



Characterisation of anucleate cells.

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## Abstract

Bacteria are beneficial in industry as they are easily genetically manipulated to express proteins, enzymes or full pathways while remaining cheap to grow in large batches with simple feed stocks. However, there are limitations associated with this as overproduction can lead to interference from complex cellular processes or metabolic burden. Metabolic burden is caused when essential cellular resources, such as energy and carbon, are diverted to the engineered pathway used for bio-production. To overcome limitations, this project aims to compartmentalise biosynthetic pathways into anucleate compartments of *Bacillus subtilis*, *Escherichia coli* and *Synechococcus elongatus* while regulating expression in nucleated cells. Division mutants were constructed in each chassis for the production of minicells or maxicells. Analysis of these anucleate producing strains was done to determine the properties of plasmid replicons with respect to gene expression and segregation into anucleate cells. Here the  $P_{lac}/\text{LacI}$  regulation system was used so expression in nucleated rod cells was repressed while expression occurred within anucleate compartments. Good repression was present in the minicell and maxicell producing strains of *B. subtilis*, however, only 50% of anucleate cells were expressing GFP. There were efforts in *B. subtilis* to alter copy number to understand the pLS20 replicon and increase the amount of anucleate cells gaining a plasmid, however, there was no single mutant which worked best in minicells. There was a dramatic increase in maxicell expression with copy number mutants but there was still a cost to the regulation system in nucleated cells. In *E. coli*, the systems regulation does not repress as efficiently in nucleated mother cells compared to *B. subtilis*. Studies from this work revealed that the RSF1010 driven plasmids were better for minicell expression where ~30-35% minicells were GFP positive. Although a minicell producing mutant was constructed for *S. elongatus*, integration of a plasmid and repression system remained problematic. However, the minicell producing phenotype was characterised in greater detail using widefield fluorescence and transmission

electron microscopy. This work contributes to the wider Portabolomics project aiming to bridge the gap between academia and industry.

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## Contents

|                                                                   |      |
|-------------------------------------------------------------------|------|
| Contents .....                                                    | i    |
| List of tables.....                                               | xi   |
| List of figures.....                                              | xi   |
| List of abbreviations .....                                       | xvii |
| Chapter 1 – Introduction .....                                    | 1    |
| 1.1 Industrial uses and biology of microbial cell factories ..... | 1    |
| 1.1.1 <i>Bacillus subtilis</i> model organism .....               | 2    |
| 1.1.2 <i>Escherichia coli</i> model organism.....                 | 5    |
| 1.1.3 <i>Synechococcus elongatus</i> model organism .....         | 8    |
| 1.1.4 Bacterial Min system for division site placement.....       | 12   |
| 1.2 Synthetic microbiology .....                                  | 18   |
| 1.2.1 Uses and advantages .....                                   | 18   |
| 1.2.2 Limitations & metabolic burden .....                        | 20   |
| 1.3 Compartmentalisation .....                                    | 21   |
| 1.3.1 Synthetic cell production .....                             | 21   |
| 1.3.2 Minimal genome cells.....                                   | 23   |
| 1.3.3 Minicell uses .....                                         | 25   |
| 1.3.4 Maxicell uses .....                                         | 27   |
| 1.4 Plasmids and transcriptional repressors .....                 | 28   |
| 1.4.1 Plasmid replication and copy number .....                   | 28   |



|                                                                        |    |
|------------------------------------------------------------------------|----|
| 1.4.2 Transcriptional repressors and promoters .....                   | 33 |
| 1.5 Aims for compartmentalisation.....                                 | 35 |
| 1.6 Portabolomics project aims and outlook.....                        | 37 |
| 1.6.1 Portabolomics compartmentalisation.....                          | 37 |
| 1.6.2 Plasmid portability .....                                        | 38 |
| 1.7 Specific aims of the project.....                                  | 39 |
| Chapter 2 - Materials and methods .....                                | 40 |
| 2.1 Growth medias and general culturing methods .....                  | 40 |
| Growth curve experiments.....                                          | 40 |
| 2.2 Bacterial strain collections.....                                  | 40 |
| 2.3 General methods of DNA amplification and plasmid construction..... | 51 |
| 2.3.1 Oligonucleotide construction .....                               | 51 |
| 2.3.2 Polymerase chain reaction for cloning.....                       | 51 |
| 2.3.3 Gel electrophoresis of DNA.....                                  | 51 |
| 2.3.4 DNA fragment clean up .....                                      | 52 |
| 2.3.5 Megaprimer PCR of Whole Plasmid (MEGAWHOP) .....                 | 52 |
| 2.3.6 NEBuilder HiFi DNA assembly .....                                | 54 |
| 2.3.7 Restriction enzyme and ligation cloning.....                     | 55 |
| 2.3.8 Colony PCR .....                                                 | 55 |
| 2.4 Media supplements .....                                            | 56 |
| 2.5 DNA extraction .....                                               | 56 |

---

|                                                                                                |    |
|------------------------------------------------------------------------------------------------|----|
| 2.5.1 Genomic DNA extraction. ....                                                             | 56 |
| 2.5.2 Plasmid extraction.....                                                                  | 57 |
| 2.6 Transformation and genetic manipulation techniques .....                                   | 57 |
| 2.6.1 <i>B. subtilis</i> transformation. ....                                                  | 57 |
| 2.6.2 <i>E. coli</i> chemical transformation. ....                                             | 58 |
| 2.6.3 <i>E. coli</i> electrocompetent transformation and recombineering. ....                  | 58 |
| 2.6.4 <i>S. elongatus</i> transformation. ....                                                 | 59 |
| 2.7 Protein analyses methods.....                                                              | 60 |
| 2.7.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses.<br>..... | 60 |
| 2.7.2 Western blotting.....                                                                    | 60 |
| 2.7.3 Coomassie staining .....                                                                 | 61 |
| 2.8 Microscopy techniques .....                                                                | 61 |
| 2.8.1 Fluorescence microscopy.....                                                             | 61 |
| 2.8.2 Cell and fluorescence counting.....                                                      | 62 |
| 2.8.3 Time-lapse microscopy.....                                                               | 62 |
| 2.8.4 Transmission electron microscopy .....                                                   | 63 |
| 2.8.5 Immunostaining .....                                                                     | 63 |
| 2.8.6 Fluorescence <i>in situ</i> hybridisation (FISH) in Cyanobacteria.....                   | 64 |
| 2.9 $\beta$ -galactosidase assay.....                                                          | 66 |
| 2.10 Minicell enrichment in <i>E. coli</i> .....                                               | 67 |
| 2.11 Flow cytometry .....                                                                      | 68 |

---

|                                                                                                      |    |
|------------------------------------------------------------------------------------------------------|----|
| 2.12 Plasmid stability assay .....                                                                   | 68 |
| 2.12.1 Spot titre and plasmid stability assays .....                                                 | 68 |
| 2.12.2 Plasmid stability assays in liquid media using CFU/ml.....                                    | 69 |
| 2.13 Quantitative PCR for plasmid copy number .....                                                  | 69 |
| 2.13.1 <i>B. subtilis</i> genomic DNA preparation .....                                              | 69 |
| 2.13.2 QIAgility robot set up .....                                                                  | 70 |
| 2.13.3 Primers for qPCR.....                                                                         | 70 |
| 2.13.4 Standards.....                                                                                | 71 |
| 2.13.5 qPCR experiments and quantification .....                                                     | 71 |
| Chapter 3 - Characterisation of minicell producing <i>B. subtilis</i> as a synthetic biology chassis | 73 |
| 3.1 Introduction.....                                                                                | 73 |
| 3.2 Results: minicell compartments of <i>B. subtilis</i> .....                                       | 76 |
| 3.2.1 Data from the project before starting .....                                                    | 76 |
| 3.2.2 Expression of $\beta$ -galactosidase and activity assays.....                                  | 79 |
| 3.2.3 LacI degradation in <i>B. subtilis</i> .....                                                   | 83 |
| 3.2.4 Plasmid copy number in minicell producing strains.....                                         | 87 |
| 3.3 Development of an anucleated system in <i>B. subtilis</i> using a DNA replication mutant .....   | 88 |
| 3.3.1 Introduction to the maxicell model .....                                                       | 88 |
| 3.3.2 Construction and characterisation of the maxicell producing mutant .....                       | 89 |
| 3.3.3 Portabolomics vector transformation and expression .....                                       | 91 |
| 3.3.4 Time lapse microscopy and monitored expression of GFP .....                                    | 97 |

---

|                                                                                                                        |     |
|------------------------------------------------------------------------------------------------------------------------|-----|
| 3.3.5 Quantification of expression using $\beta$ -galactosidase.....                                                   | 100 |
| 3.3.6 Population analyses using flow cytometry .....                                                                   | 105 |
| 3.4 DnaA is not required for pLS20 driven plasmid replication.....                                                     | 110 |
| 3.4.1 Plasmid behaviour in maxicell producing strains. ....                                                            | 110 |
| 3.4.2 pLS20 DnaA box mutagenesis.....                                                                                  | 112 |
| 3.5 Industrial uses for anucleated chassis of <i>B. subtilis</i> .....                                                 | 117 |
| 3.5.1 Using protein A constructs.....                                                                                  | 117 |
| 3.5.2 Expression and secretion of protein A .....                                                                      | 121 |
| 3.6 Discussion .....                                                                                                   | 128 |
| 3.6.1 <i>Bacillus subtilis</i> minicell chassis characterisation .....                                                 | 128 |
| 3.6.2 Maxicell chassis development and characterisation .....                                                          | 131 |
| 3.6.3 Protein A expression and secretion.....                                                                          | 134 |
| Chapter 4 – modulating plasmid copy number to increase chassis expression in <i>Bacillus subtilis</i><br>.....         | 137 |
| 4.1 Introduction.....                                                                                                  | 137 |
| 4.1.1 Plasmid replication apparatus split to modulate copy number .....                                                | 137 |
| 4.1.2 pLS20 replicon random mutagenesis.....                                                                           | 139 |
| 4.2 Results: Plasmid replication apparatus split to control copy number via expression of<br>replication protein. .... | 141 |
| 4.2.1 Design of the RepN iteron mutant and integration into the vector .....                                           | 141 |
| 4.2.2 Characterisation of transformants .....                                                                          | 144 |
| 4.2.3 $\beta$ -galactosidase assays to determine functionality .....                                                   | 146 |

---

|                                                                                                                  |     |
|------------------------------------------------------------------------------------------------------------------|-----|
| 4.3 Non-specific plasmid mutagenesis .....                                                                       | 149 |
| 4.3.1 Plasmid stability experiments .....                                                                        | 149 |
| 4.3.2 Error prone PCR mutagenesis and selection of mutants.....                                                  | 151 |
| 4.4 Characterisation of the pLS20 mutants in minicell and maxicell backgrounds of <i>Bacillus subtilis</i> ..... | 158 |
| 4.4.1 $\beta$ -galactosidase assays to quantify the difference between mutants .....                             | 158 |
| 4.4.2 Microscopy analysis of pLS20 mutants .....                                                                 | 166 |
| 4.4.3 Copy number determination for <i>oriLS20</i> mutants .....                                                 | 174 |
| 4.5 Conclusions and discussion .....                                                                             | 176 |
| 4.5.1 RepN iteron mutant approach. ....                                                                          | 176 |
| 4.5.2 pLS20 stability .....                                                                                      | 177 |
| 4.5.3 pLS20 random mutagenesis .....                                                                             | 178 |
| Chapter 5 - <i>Escherichia coli</i> minicell chassis .....                                                       | 183 |
| 5.1 Introduction.....                                                                                            | 183 |
| 5.2 Results: microscopy analyses of Portabolomics chassis.....                                                   | 184 |
| 5.2.1 Construction of a defined <i>E. coli</i> chassis .....                                                     | 188 |
| 5.2.2 Time lapse microscopy of strains with different plasmid replicons .....                                    | 192 |
| 5.2.3 Analyses of the <i>E. coli</i> $\Delta minC$ ultrastructure.....                                           | 197 |
| 5.3 Population analyses of <i>E. coli</i> Portabolomics chassis .....                                            | 199 |
| 5.3.1 Whole culture population analyses using flow cytometry .....                                               | 199 |
| 5.3.2 Minicell purification and analyses using flow cytometry .....                                              | 203 |
| 5.4 Increasing rod cell repression of the reporter .....                                                         | 207 |

|                                                                                    |     |
|------------------------------------------------------------------------------------|-----|
| 5.4.1 antisense RNA construction to improve rod repression .....                   | 207 |
| 5.5 Expression of an industrially relevant product .....                           | 213 |
| 5.5.1 Expression of protein A construct in <i>Escherichia coli</i> .....           | 213 |
| 5.5.2 Localisation of protein A .....                                              | 214 |
| 5.6 Dual replicon system in <i>B. subtilis</i> affects pLS20 replication .....     | 219 |
| 5.6.1 RSF1010 origin effects on GFP expression in minicell production strains..... | 219 |
| 5.6.2 RSF1010 effects on pLS20 driven plasmids copy number.....                    | 221 |
| 5.7 Conclusions and discussion .....                                               | 222 |
| 5.7.1 <i>E. coli</i> chassis optimal vector.....                                   | 223 |
| 5.7.2 Population analyses of minicell producing <i>E. coli</i> .....               | 226 |
| 5.7.3 Expressing an industrially relevant compound .....                           | 227 |
| 5.7.4 Integration of the dual replicon into <i>B. subtilis</i> chassis .....       | 228 |
| 5.7.5 Conclusions and future work .....                                            | 230 |
| Chapter 6 - Cyanobacterial minicell producing chassis.....                         | 231 |
| 6.1 Introduction.....                                                              | 231 |
| 6.2 Results.....                                                                   | 233 |
| 6.2.1 Construction of a minicell producing mutant .....                            | 233 |
| 6.3 Investigating ultrastructure of Cyanobacteria .....                            | 236 |
| 6.3.1 Wild type structural analyses and division .....                             | 236 |
| 6.3.2 Minicell ultrastructure.....                                                 | 243 |
| 6.4 Thylakoid curvature in division .....                                          | 246 |

---

|                                                                                          |     |
|------------------------------------------------------------------------------------------|-----|
| 6.4.1 CurT localisation.....                                                             | 246 |
| 6.4.2 Z-stack imaging of CurT thylakoid associated protein.....                          | 248 |
| 6.4.3 CurT-GFP produce a minicell producing phenotype.....                               | 250 |
| 6.5 Chromosome localisation of a minicell producing strain .....                         | 252 |
| 6.5.1 DNA distribution in Cyanobacteria .....                                            | 252 |
| 6.5.2 Chromosome origin localisation .....                                               | 254 |
| 6.6 Portabolomics chassis construction .....                                             | 256 |
| 6.6.1 Integration of a chromosome bound repressor.....                                   | 256 |
| 6.6.2 Fluorescence analyses of the minicell producing strain with plasmid expression.... | 257 |
| 6.7 Discussion and conclusions .....                                                     | 259 |
| 6.7.1 Microscopy analyses of minicell producing strains.....                             | 259 |
| 6.7.2 Transmission electron microscopy analyses .....                                    | 260 |
| 6.7.3 CurT interactions with the thylakoid membranes .....                               | 261 |
| 6.7.4 DNA & carboxysomes positioning within minicells .....                              | 261 |
| 6.7.5 Portabolomics chassis work.....                                                    | 263 |
| 6.7.6 Conclusions.....                                                                   | 264 |
| Chapter 7 – Concluding remarks and future directions .....                               | 266 |
| References.....                                                                          | 270 |
| Appendix 1 – media recipes.....                                                          | 296 |
| Complex defined media (CDM) for <i>Bacillus subtilis</i> .....                           | 296 |
| M9 minimal media for <i>Escherichia coli</i> .....                                       | 297 |

---

|                                                                                   |     |
|-----------------------------------------------------------------------------------|-----|
| BG11 media for <i>Synechococcus elongatus</i> .....                               | 297 |
| Luria-Bertani broth (LB).....                                                     | 299 |
| Penassay broth (PAB) .....                                                        | 299 |
| Appendix 2 – Buffers.....                                                         | 300 |
| Western blot/protein gel buffers .....                                            | 300 |
| Appendix 3 – oligonucleotide table .....                                          | 301 |
| Appendix 4 – $\Delta dnaA$ for pLS20 replication and immunostaining controls..... | 310 |
| $\Delta dnaA$ plasmid copy number .....                                           | 310 |
| Protein A no plasmid controls.....                                                | 314 |
| <i>Bacillus subtilis</i> no lysozyme: .....                                       | 314 |
| Appendix 5 - pGGZ32 RepN/ <i>oriN</i> region promoter scan .....                  | 317 |
| Appendix 6 – XL1-red mutagenesis .....                                            | 318 |
| Appendix 7 – qPCR standards traces.....                                           | 321 |
| Appendix 8 - RSF1010 replication in <i>B. subtilis</i> .....                      | 323 |
| Appendix 9 – 6-hour stability of plasmids in <i>E. coli</i> .....                 | 326 |
| Appendix 10 - Universal replicon integration <i>E. coli</i> .....                 | 328 |
| Supplementary 10.1 Introduction.....                                              | 328 |
| 10.2 Time course microscopy in different media .....                              | 330 |
| 10.3 Time lapse microscopy .....                                                  | 335 |
| 10.4 Addition of a degradation system .....                                       | 337 |
| 10.5 Segregation system addition .....                                            | 338 |



|                                       |     |
|---------------------------------------|-----|
| 10.6 Conclusions and discussion ..... | 343 |
|---------------------------------------|-----|

## List of tables

|                                                                                                                                                  |    |
|--------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Table 2.1. Bacterial strains used throughout this study <i>B. subtilis</i> (white), <i>E. coli</i> (blue) and <i>S. elongatus</i> (green). ..... | 41 |
| Table 2.2. Plasmid constructs used within this study including their transformation host and construction sources. ....                          | 44 |
| Table 2.3. Demonstration of antibiotic, substrate and inducer final concentrations used in liquid and solid media throughout the study. ....     | 56 |
| Table 2.4. Demonstration of oligonucleotide sequences used for qPCR experiments. ....                                                            | 70 |

## List of figures

|                                                                                                            |    |
|------------------------------------------------------------------------------------------------------------|----|
| Figure 1.1. Demonstration of wild type division in model organism <i>Bacillus subtilis</i> . .....         | 4  |
| Figure 1.2. Wild-type division in <i>Escherichia coli</i> . ....                                           | 7  |
| Figure 1.3. Demonstration of <i>Synechococcus elongatus</i> cell and carboxysome reactions. ....           | 10 |
| Figure 1.4. Chromosomal analyses and insertional sites of <i>Synechococcus elongatus</i> . ...             | 12 |
| Figure 1.5. The Min system if <i>Bacillus subtilis</i> . ....                                              | 14 |
| Figure 1.6 The Min system models in both <i>Escherichia coli</i> and <i>Synechococcus elongatus</i> . .... | 16 |
| Figure 1.7. Rolling circle replication. ....                                                               | 29 |
| Figure 1.8. Theta-type replication of plasmid DNA. ....                                                    | 30 |
| Figure 1.9. RSF1010 strand displacement replication using plasmid encoded initiation proteins. ....        | 32 |
| Figure 1.10. Image depicting the minicell expression model proposed by this study. ....                    | 35 |
| Figure 1.11. The production of ‘maxicells’ and how they may be used industrially. ....                     | 36 |
| Figure 2.1. MEGAWHOP workflow. ....                                                                        | 53 |

|                                                                                                          |            |
|----------------------------------------------------------------------------------------------------------|------------|
| <b>Figure 2.2. A demonstration of HiFi assembly reactions for cloning new vectors.....</b>               | <b>54</b>  |
| <b>Figure 3.1. Independently minicell expression of a fluorescent reporter. ....</b>                     | <b>76</b>  |
| <b>Figure 3.2. Quantification of rod and minicell expression. ....</b>                                   | <b>78</b>  |
| <b>Figure 3.3. Demonstration of plasmid encoded <i>lacZ</i> repression via chromosomal <i>lacI</i>..</b> | <b>80</b>  |
| <b>Figure 3.4. Enzyme activity in mother and minicells.. ....</b>                                        | <b>82</b>  |
| <b>Figure 3.5. PBP2B loading control for LacI degradation. ....</b>                                      | <b>84</b>  |
| <b>Figure 3.6. Western blot band quantification. ....</b>                                                | <b>86</b>  |
| <b>Figure 3.7. Plasmid copy number in sAG1333 and sAG1338 strains. ....</b>                              | <b>88</b>  |
| <b>Figure 3.8. Integrating the LacI repression system into the maxicell model.....</b>                   | <b>90</b>  |
| <b>Figure 3.9. Independent expression of GFP from maxicells. ....</b>                                    | <b>92</b>  |
| <b>Figure 3.10. Fluorescence intensity of nucleated and anucleate strains with a GFP reporter. ....</b>  | <b>94</b>  |
| <b>Figure 3.11. Analysis of populations with GFP reporter during stationary phase.....</b>               | <b>96</b>  |
| <b>Figure 3.12. Time-lapse of maxicell GFP independent expression.....</b>                               | <b>98</b>  |
| <b>Figure 3.13. Quantification of maxicell expression from time-lapse microscopy.. ....</b>              | <b>99</b>  |
| <b>Figure 3.14. Enzyme activity in maxicell producing strains whole population.....</b>                  | <b>101</b> |
| <b>Figure 3.15. Enzyme activity in maxicell producing strains whole population in rich media. ....</b>   | <b>103</b> |
| <b>Figure 3.16. Localisation of enzyme activity optimisation assay.....</b>                              | <b>104</b> |
| <b>Figure 3.17. Cell size and property analysis of wild type and maxicell producing strains. ....</b>    | <b>105</b> |
| <b>Figure 3.18. Fluorophore analysis of maxicell producing strains using flow cytometry.. ....</b>       | <b>107</b> |
| <b>Figure 3.19. Dual fluorophore detection in strains with pAG-P08 plasmid.....</b>                      | <b>109</b> |
| <b>Figure 3.20. Stability of the pLS20 replicon in maxicells.....</b>                                    | <b>110</b> |

|                                                                                                                         |     |
|-------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 3.21. Plasmid copy number in maxicell producing strains. ....                                                    | 112 |
| Figure 3.22. Demonstration of the DnaA box mutagenesis. ....                                                            | 113 |
| Figure 3.23. Quantification of plasmid per chromosome with WT <i>oriLS20</i> and <i>ΔdnaA</i> box <i>oriLS20</i> . .... | 114 |
| Figure 3.24. pLS20 DnaA box mutagenesis in maxicell producing strain. ....                                              | 116 |
| Figure 3.25. Protein A expression from wild type, mini- and maxicell strains. ....                                      | 118 |
| Figure 3.26. Attachment of a native <i>B. subtilis</i> carbohydrate binding domain. ....                                | 120 |
| Figure 3.27. Protein A expression from sAG1392 and sAG1338 with different induction conditions. ....                    | 122 |
| Figure 3.28. Protein A expression from sGG113 and sGG115 strains in different induction conditions. ....                | 124 |
| Figure 3.29. Minicell strains expression of LytC-Protein A fusions. ....                                                | 125 |
| Figure 3.30. Maxicell producing strains with LytC-Protein A fusion reporter. ....                                       | 127 |
| Figure 3.31. Demonstration of the protein A structure and carbohydrate binding domain fusion. ....                      | 136 |
| Figure 4.1. Demonstration of the proposed host restrictive region in <i>B. subtilis</i> . ....                          | 138 |
| Figure 4.2. Alp7AR operon increases the stability of low copy number plasmid. ....                                      | 140 |
| Figure 4.3. Demonstration of iteron mutations. ....                                                                     | 142 |
| Figure 4.4. Plasmid production for the host-restrictive system. ....                                                    | 143 |
| Figure 4.5. Testing colonies for colorimetric changes with X-gal and different induction conditions. ....               | 145 |
| Figure 4.6. β-galactosidase expression from minimal plasmid strains. ....                                               | 147 |
| Figure 4.7. Plasmid stability of pAG-P08 over 24 hour and 7 day periods. ....                                           | 150 |
| Figure 4.8. Error-prone PCR pLS20 origin and selection of mutants. ....                                                 | 152 |
| Figure 4.9. Mutants with sustained phenotype. ....                                                                      | 153 |

|                                                                                                                                               |     |
|-----------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 4.10. pLS20 copy number mutant intermediates.....                                                                                      | 154 |
| Figure 4.11. Colourimetric changes between pLS20 mutants. ....                                                                                | 156 |
| Figure 4.12. Computational comparison of the "cluster 1" mutants and pLS100* known<br>to be higher copy number variant of pLS20 replicon..... | 157 |
| Figure 4.13. pLS20 copy number mutants $\beta$ -galactosidase expression in sGG113.....                                                       | 159 |
| Figure 4.14. $\beta$ -galactosidase activity from expression of copy number mutants in minicell<br>producing strain sAG1338.. ....            | 161 |
| Figure 4.15. Plasmid expression from maxicell producing strain sGG115. ....                                                                   | 163 |
| Figure 4.16. Investigating the reverted maxicell mutant.....                                                                                  | 165 |
| Figure 4.17. Fluorescence analyses of pLS20 copy number mutants in sAG1392.....                                                               | 167 |
| Figure 4.18. Fluorescence of pLS20 mutants in minicell producing strains. ....                                                                | 169 |
| Figure 4.19. Maxicell parental strain analyses of pLS20 mutants in sGG113. ....                                                               | 171 |
| Figure 4.20. pLS20 mutants in the maxicell chassis fluorescence analyses. ....                                                                | 173 |
| Figure 4.21. qPCR analyses of the pLS20 mutants found by error-prone PCR.....                                                                 | 175 |
| Figure 5.1. Dual replicon system alterations for regulation.....                                                                              | 185 |
| Figure 5.2. Native LacI repression. ....                                                                                                      | 187 |
| Figure 5.3. Construction and analysis of the <i>E. coli</i> minicell chassis.....                                                             | 189 |
| Figure 5.4. Populations of GFP expression.....                                                                                                | 191 |
| Figure 5.5. Independent minicell expression of GFP reporter. ....                                                                             | 194 |
| Figure 5.6. RSF1010 driven plasmid GFP expression time lapse.....                                                                             | 196 |
| Figure 5.7. Electron microscopy imaging of <i>E. coli</i> minicells to investigate ultrastructures.<br>.....                                  | 198 |
| Figure 5.8. Analysing individual populations of wild type and minicell producing strains.<br>.....                                            | 200 |
| Figure 5.9. Fluorescence analyses of minicell producing strains.....                                                                          | 202 |

|                                                                                                                       |     |
|-----------------------------------------------------------------------------------------------------------------------|-----|
| Figure 5.10. <i>Escherichia coli</i> enrichment microscopy. ....                                                      | 204 |
| Figure 5.11. Flow cytometry analysis of <i>E. coli</i> host populations fluorescence.....                             | 206 |
| Figure 5.12. Using asRNA to aid in GFP regulation.....                                                                | 208 |
| Figure 5.13. Chromosomal expressed asRNA for reduction of GFP background.....                                         | 210 |
| Figure 5.14. Quantification of fluorescence and asRNA efficiency.....                                                 | 212 |
| Figure 5.15. Western blot analyses of protein A expression.. ....                                                     | 214 |
| Figure 5.16. Localisation of protein A in HL713.....                                                                  | 215 |
| Figure 5.17. <i>E. coli</i> minicells minicell protein A localisation. ....                                           | 217 |
| Figure 5.18. Minicells immunostaining of protein A. ....                                                              | 218 |
| Figure 5.19. RSF1010 and pLS20 dual replicon in <i>B. subtilis</i> .....                                              | 220 |
| Figure 5.20. Copy number of RSF1010/pLS20 dual replicon in a <i>B. subtilis</i> background.<br>.....                  | 222 |
| Figure 6.1. Tools for MinC knockout in <i>Synechococcus elongatus</i> . A .....                                       | 233 |
| Figure 6.2. Phenotypic and genotypic examination of transformants. ....                                               | 235 |
| Figure 6.3. <i>Synechococcus elongatus</i> wild type TEM internal structural analyses. ....                           | 237 |
| Figure 6.4. TEM of wild type division showing septum constriction. ....                                               | 239 |
| Figure 6.5. Asymmetric thylakoid folding at the site of division. ....                                                | 240 |
| Figure 6.6. Wild type asymmetric septum before invagination.....                                                      | 242 |
| Figure 6.7. Ultrastructures in a $\Delta minC$ strain producing minicells. ....                                       | 244 |
| Figure 6.8. Asymmetric thylakoid folding in a $\Delta minC$ strain. ....                                              | 245 |
| Figure 6.9. Cyanobacterial thylakoid membrane localisation using CurT-GFP fusion.<br>.....                            | 247 |
| Figure 6.10. Cyanobacteria strains were incubated in BG11 media and grown at 30°C<br>with constant illumination. .... | 250 |
| Figure 6.11. CurT-GFP fusion causes a minicell producing phenotype. ....                                              | 251 |

|                                                                                                |            |
|------------------------------------------------------------------------------------------------|------------|
| <b>Figure 6.12. Nucleoid localisation in wild type and minicell producing strains.....</b>     | <b>253</b> |
| <b>Figure 6.13. Localisation of chromosomes in minicell producing <i>S. elongatus</i>.....</b> | <b>255</b> |
| <b>Figure 6.14. Integrating the LacI repressor for the Portabolomics model.....</b>            | <b>256</b> |
| <b>Figure 6.15. Flow cytometry analyses of Cyanobacteria fluorescence.....</b>                 | <b>258</b> |

## List of abbreviations

**µl** – microlitre(s)

**µm** – micrometre(s)

**µM** – micromolar

**Amp** – ampicillin

**asRNA** – antisense ribonucleic acid

**ATP** – Adenosine triphosphate

**bp** – base pair(s)

**BSA** – bovine serum albumin

**CAA** – casamino acids

**Cam** – chloramphenicol

**CDM** – complex defined media

**Dam** - DNA Adenine Methylase

**DAPI** - 4,6-diamidino-2-phenylindole

**DMSO** - Dimethyl sulfoxide

**DNA** – deoxyribonucleic acid

**dsDNA** – double stranded DNA

**EDTA** - Ethylenediaminetetraacetic acid

**Erm** – erythromycin

**FtsZ** – filamentous temperature-sensitive mutant Z

**g** – grams

**gDNA** – genomic DNA

**GFP** – green fluorescent protein

**GM** – Genetically modified

**HEPES** - 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

**IPTG** - Isopropyl β-D-1-thiogalactopyranoside

**Kan** – kanamycin

**Kb** – kilobase(s)

**KDa** – kilodaltons

**L** – litre

**LB** - Luria-Bertani broth

**M** – molar

**mg** – milligrams



**mg/ml** - milligrams per millilitre

**mM** – millimolar

**MW** – molecular weight

**NA** – nutrient agar

**°C** – degrees centigrade

**OD600** – optical density at a wavelength of 600 nm

**ONPG** - 2-Nitrophenyl  $\beta$ -D-galactopyranoside

**ori** – origin of replication

**PBP** – penicillin binding protein

**PBS** – phosphate buffered saline

**PCR** – polymerase chain reaction

**PG** – peptidoglycan

**RFP** – red fluorescent protein

**RNA** - Ribonucleic acid

**rpm** - Revolutions per minute

**SDS-PAGE** - Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

**SMM** – Spizizens minimal media

**Spec** – spectinomycin

**SSC** - saline sodium citrate

**ssDNA** – single stranded DNA

**TAE** - Tris-Acetate-EDTA

**TEM** – transmission electron microscopy

**ter** – terminus of chromosome replication

**X-Gal** - 5-bromo-4-chloro-3-indoly1-  $\beta$ -D-galactopyranoside

## Chapter 1 – Introduction

### 1.1 Industrial uses and biology of microbial cell factories

Bacterial cells have made major contributions to industrial production, “cell factories” have been utilised in many ways for the bioproduction of vaccines, enzymes, pesticides and many more primary and secondary metabolites (Demain, 2000). The use of bacteria can be an environmentally friendly solution to fuel production, where it has been shown that bacterial species can produce hydrocarbons, acetone, butanol, ethanol and biodiesel (Rojo, 2008; Demain, 2000). Whereas it has also been shown that microbial bioreactors can produce medically relevant products such as antibiotics, vitamins, anti-cancer and anti-inflammatory agents (Pham *et al.*, 2019). Although yeast and mammalian cells are increasingly popular for expression, it is estimated that prokaryotic cells are still used for production of ~30% of biopharmaceutical products (Sanchez-Garcia *et al.*, 2016). The addition of genetic engineering has aided in reprogramming host cells to optimise production, enhance protein folding, as well as increasing secretion (Davy *et al.*, 2017). However, selecting the right microbial host is significant when designing a microbial bioreactor as host protein synthesis properties, product toxicity and culturing conditions all need to be considered. The host strain is often selected in accordance to where the protein originated and its ability to synthesise the required by-products while preserving stability and enzyme activity (Glick, 1995). Using a native producer may be beneficial as the hosts ability to phosphorylate, acetylate or glycosylate will be unhindered, however, where this was not possible a host with similar attributes should be chosen (Glick, 1995).

Model organisms are widely characterised and easily cultured or maintained in a laboratory setting which are utilised to understand biological processes. They are representative of a small

portion of prokaryotic species and often represent Gram positive or Gram negative organisms which are often compared to species with greater complexity (Leonelli and Ankeny, 2013).

### **1.1.1 *Bacillus subtilis* model organism**

*Bacillus subtilis* is a Gram positive rod-shaped bacterium which offers an advantage in industrial microbiology because of the ability to secrete products directly into the media, making retrieval of the product significantly easier (Chen *et al.*, 2015). *B. subtilis* are known for easy genetic engineering and manipulation, the species has a long history as a cell factory for the production of enzymes, vitamins and sugars (Liu *et al.*, 2017; van Dijl and Hecker, 2013). Moreover, it has been shown that fast growth rates and high yields (~20-25 g/L) are possible, both characteristics making them attractive for large scale fermentation used in industry (Schallmeyer *et al.*, 2004; van Dijl and Hecker, 2013).

In addition to the industrial applications, *B. subtilis* have aspects of their biology that have attracted academics. Gene regulation has been of interest, particularly in respect to sporulation which is an example of cellular differentiation. Furthermore, *Bacillus* spp. have developed responses to low nutrient conditions such as motility, hydrolytic enzyme upregulation and competence (Harwood, 1992). If cells are in unfavourable nutrient limited conditions, they will form endospores which are extremely hardy and can resist many physical and chemical treatments (Russell, 1990). Endospores are not active and can remain latent for long periods of time before they are able to germinate in favourable conditions (Errington and Wu, 2017). The endospore of *B. subtilis* are not industrially favourable because of their dormancy, however, competence induced via starvation can be extremely useful for uptake of DNA. Uptake of DNA can either be whole plasmids or fragments with the ability to induce homologous recombination into the chromosome. *B. subtilis* recombination machinery is beneficial at the onset of stationary phase as extracellular DNA can be acquired and gain new genetic information, driving genetic diversity. The DNA will undergo random cleavage events to cause nicks and

strand breaks, the recombination machinery will then associate any homology with single stranded DNA (ssDNA) which will invade double stranded DNA (dsDNA) where DNA synthesis will aid in insertion into the chromosome (Fernández *et al.*, 2000).

*B. subtilis* is known to have a thick cell wall of ~30 layers of peptidoglycan (PG) which is used to determine cellular shape and withstand the high turgor pressure from within the cell (fig.1.1A). The PG is composed of an intricate polymer structure of alternating N-acetylglucosamine and N-acetylmuramic acid units to form glycan strands. Glycan strands are crosslinked by flexible peptide chains protruding from N-acetylmuramic acid units to form a net-like structure (Archibald *et al.*, 1993). To protect the cell against osmotic pressure, PG is constantly maintained by a number of synthases and hydrolases throughout the lifecycle (Hayhurst *et al.*, 2008). When the cell has reached a critical stage in growth, according to the ‘adder’ model when the cell has increased length by ~3-5 micrometres (µm) depending on growth conditions, division will be initiated where the division complex assembles at the mid-cell (Taheri-Araghi *et al.*, 2015). *B. subtilis* firstly elongates the cell before starting division, demonstrated in figure 1.1C. A dynamic division protein complex (divisome) assembles in 2 steps of early and late division proteins, the first stage is marked by the assembly of the most concurrent cell division protein in bacteria, FtsZ (Gamba *et al.*, 2009). FtsZ is a structural protein which polymerises to form the Z-ring at the mid cell, the Z-ring drives PG synthesis and cross wall construction which eventually leads to the formation of 2 daughter cells (Fig.1.1D; Gamba *et al.*, 2009). Chromosome replication initiation must be coordinated with the cell cycle in order to produce two daughter cells which both possess a single chromosome. The *oriC* sequence contains multiple short repeats known as DnaA boxes, where the DnaA protein binds to the dsDNA to initiate bidirectional replication (Fuller *et al.*, 1984). Firstly, the DNA is melted to expose single strands where the helicase unwinds the DNA for the rest of the replisome to assemble (Bramhill and Kornberg, 1988). DnaA and the DnaA boxes are

highly conserved within different species at the origin of replication (*oriC*) (Mott and Berger, 2007). The FtsZ ring can only assemble within the chromosome free gap at the mid cell positioning because of the Nucleoid occlusion system (Noc) which prevents division machinery establishing a functional complex where chromosomes are present (Wu and Errington, 2004).

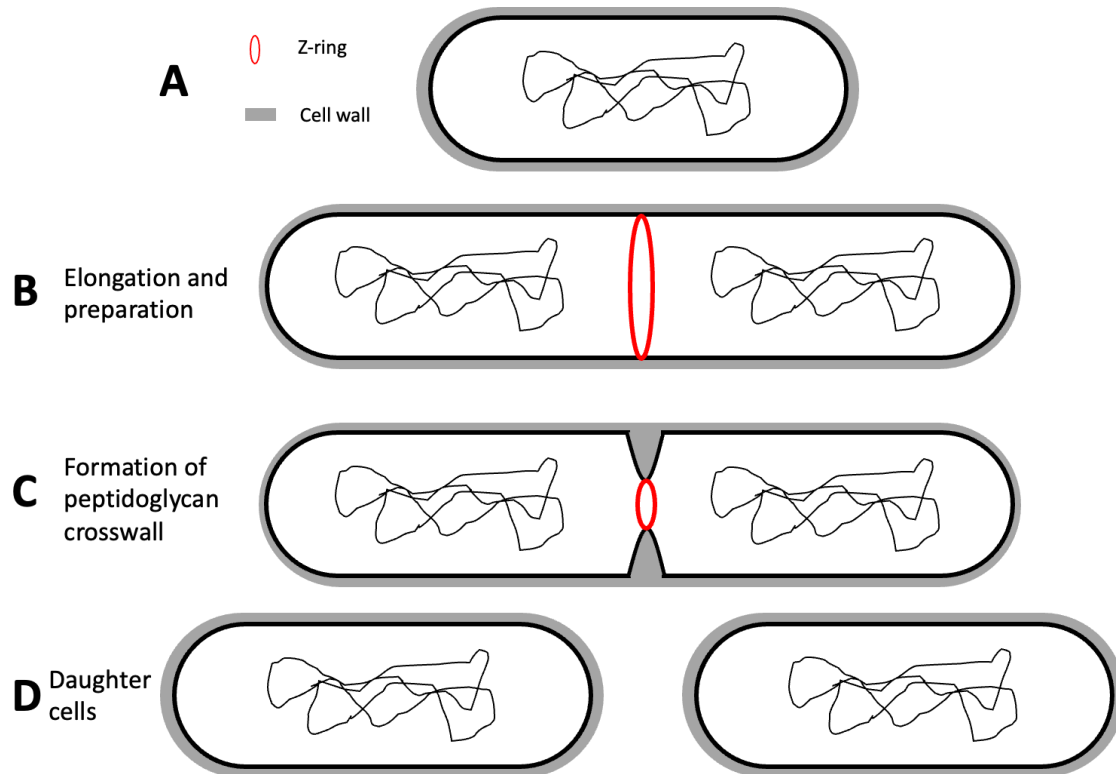


**Figure 1.1. Demonstration of wild type division in model organism *Bacillus subtilis*.** (A + B) Once cells reach a critical mass, the peptidoglycan cell wall is synthesised to elongate the cell ready for division. The FtsZ ring will only assemble where there is no chromosome, therefore the ring is positioned at the mid-cell. (C) Division drives PG synthesis, forming a cross wall between the segregated chromosomes. (D) The crosswall forms the two new poles of the daughter cells.

### 1.1.2 *Escherichia coli* model organism

*Escherichia coli* is the workhorse of modern bioproduction used widely within industry and academia for expression of heterologous proteins as well as ease of genetic manipulation by plasmids. A review by Selas-Castiñeiras *et al.*, described *E. coli* as one of the most popular hosts for expression of therapeutic biological products because of their rapid growth, scalability and the ability to culture in high densities (Selas-Castiñeiras *et al.*, 2018). *E. coli* is estimated to account for expression of ~30% therapeutic proteins from prokaryotes used by biopharmaceutical companies because they are known to produce high yields of functional recombinant proteins/peptides (Rodriguez *et al.*, 2014; Baeshen *et al.*, 2015). As mentioned previously, it has become common for eukaryotes or yeast to be used, however, the authors report that the use of *E. coli* has made a resurgence in industry for applications including gene therapy and DNA vaccination by producing plasmid DNA (Selas-Castiñeiras *et al.*, 2018). *E. coli* species used in industry are very well characterised, however, there can be limitations to the modifications some strains can perform which is why a range of strains have been developed for specific functionality (Marisch *et al.*, 2013). Major work has also been investigated for growth conditions of *E. coli* strains to produce good quality protein at high yields, these investigations looked at the growth rates, soluble protein yields and metabolic loads to identify strengths and weaknesses (Marisch *et al.*, 2013; Kadisch *et al.*, 2017).

As *E. coli* is a Gram negative organism, the structure of the cell is different to that described in *B. subtilis*. Where Gram positive organisms have thick multi-layered PG, Gram negative bacteria only contain a single layer. The PG structure has the same purpose in both organisms to protect the cell against osmotic pressure and retain cell shape, however, the single layer of PG in *E. coli* is encased between the cytoplasmic and an outer membrane (fig.1.2A; Vollmer *et al.*, 2008). In Gram negative organisms, the PG layer is firmly anchored to the outer membrane via outer membrane bound proteins (Typas *et al.*, 2010). The cell envelope is

believed to serve several functions from resisting internal turgor pressure, determining cellular shape, division and protection from hostile environments/secondary metabolites where specific transporters are the only means of passing nutrients and waste (Silhavy *et al.*, 2010).

Similar to what was described in *B. subtilis*, FtsZ polymerises at the mid-cell to form a ring like structure where the division complexes are assembled. The *E. coli* cell elongates while dividing to create daughter cells, the divisome adds new PG material to the existing cell wall to make new poles for daughter cells between the chromosomes (Typas *et al.*, 2012). The Z-ring constricts and drives invaginations to occur simultaneously for inner and outer membranes (fig.1.2C & D). It was hypothesised that the Z-ring was able to facilitate invaginations of the PG and inner membrane, however, it was found the outer membrane was pulled in via the linkage of the outer membrane protein to the PG and the inner membrane (Gerding *et al.*, 2007). Z-ring placement at the mid cell is an accurately controlled mechanism requiring two systems to precisely localise the divisome. In *E. coli*, the nucleoid occlusion system that prevents Z-ring polymerisation over the chromosome is known as SlmA. SlmA is associated with the chromosome and is a FtsZ ring inhibitor which, in turn, prevents division rings forming over the chromosome by direct interactions with FtsZ (Bernhardt and de Boer, 2005). When the protein was knocked out there was obvious divisome assembly at sites where chromosomes were present (Bernhardt and de Boer, 2005). The second system for division site placement is known as the Min system, which is described in detail in section 1.1.4.

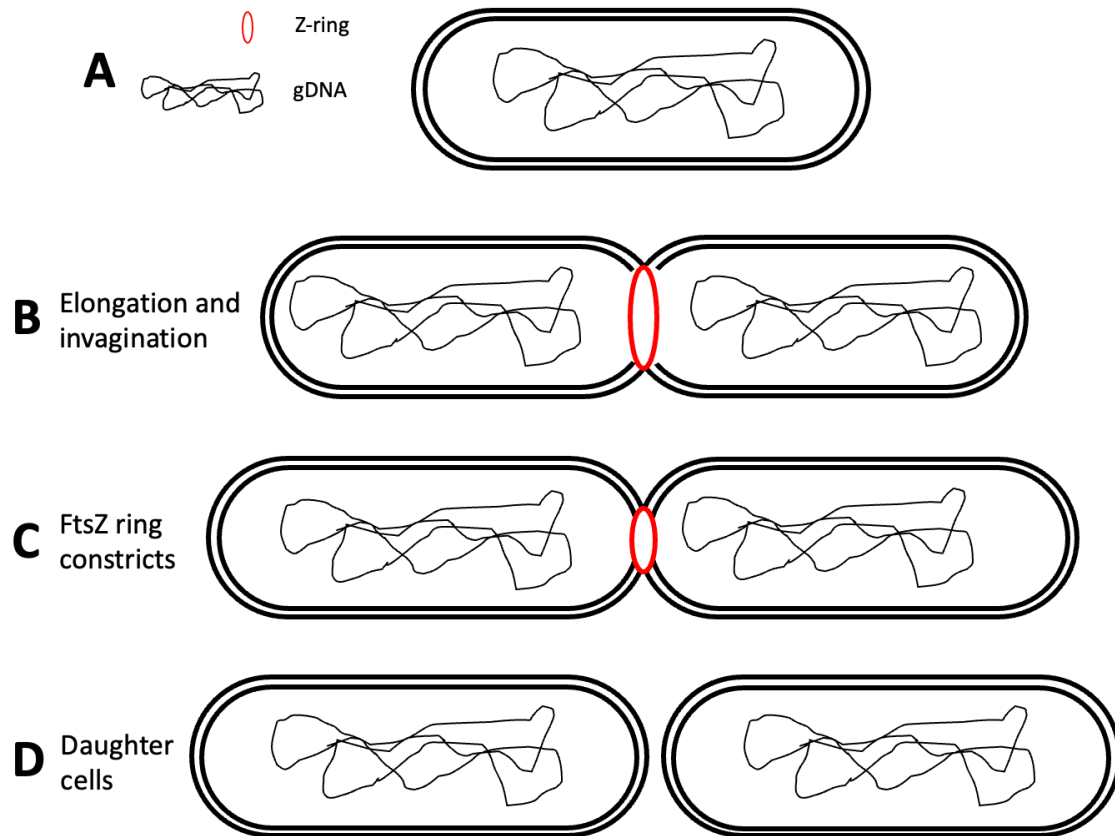


**Figure 1.2. Wild-type division in *Escherichia coli*.** (A) Gram negative organisms contain 2 membranes with a single layer of peptidoglycan in between. (B) For division, the FtsZ ring polymerises at the mid-cell where there is a chromosome free gap. (C) New peptidoglycan is synthesised to form the new daughter cell poles and the Z-ring constricts inwards to form an invagination. (D) Both cell membranes are pulled inwards to form 2 new daughter cells.



### 1.1.3 *Synechococcus elongatus* model organism

*Synechococcus elongatus* PCC 7942 is also a Gram negative rod-shaped organism, however, it is a member of the cyanobacteria family which are known for photosynthetic energy production as opposed to using a carbon source. Cyanobacteria are often optimal models for the study of nitrogen fixation, carbon fixation and circadian rhythms in laboratory settings. Since molecular tools were developed in the 1980-90's there has been a growing interest in using these strains as industrial models (Al-Haj *et al.*, 2016). There have been many examples of cyanobacteria being used to tackle climate change by utilising atmospheric carbon to yield fuels or chemicals using the unique photosynthetic system (Atsumi *et al.*, 2009). Examples are published where the carbon uptake system of Cyanobacterial species were exploited for production of isobutyraldehyde/isobutanol, isoprene, hydrogen, and other chemicals reviewed by Nozzi *et al.*, 2013 (Atsumi *et al.*, 2009; Lindberg *et al.*, 2010; Dutta *et al.*, 2005). Although having minimal nutritional requirements, only needing CO<sub>2</sub>, Na<sub>2</sub>CO<sub>3</sub>, K<sub>2</sub>HPO<sub>4</sub> and trace metals, there are draw backs to the strains as they are known to be incredibly slow growing and certain experiments may take weeks or months to complete; where *E. coli* hosts could perform a similar task within days (Yu *et al.*, 2015). Ongoing studies have found and characterised a relative of *Synechococcus* PCC 7942, known as *Synechococcus elongatus* UTEX 2973, which has a 2-fold significantly lower doubling time (Yu *et al.*, 2015). This would be desirable for industrial application as increased growth rates would mean the densities needed to extract certain products would be achieved quicker, therefore increasing efficacy of the chassis.

The circadian clock is a commonly studied component of cyanobacterial species, although the species requires light for energy production, it is able to oscillate activities in accordance with light availability (night/day 12-hour cycles). The circadian clock is able to regulate many cellular processes such as cell division, metabolism and competence where data even suggests that natural competence is at its greatest efficiency when darkness occurs at dusk (Taton *et al.*,

2020). There have been growing interests in transplantation of the circadian clock in non-circadian organisms, such as *E. coli*, to rhythmically control expression. In studies by Chen *et al.*, 2015, it was shown that the circadian clock could be used to induce and repress expression of a fluorescent reporter in 24-hour rhythms. This method of gene expression regulation is of interest because of its predictable oscillations in relation to time.

Cyanobacterial species have different intracellular structures compared to the 2 model bacteria described earlier, this organism is polyploid and contains organelle like structures known as thylakoid membranes and carboxysomes demonstrated in figure 1.3. Photosynthesis is carried out within the thylakoid membranes which are present just beneath the cytoplasmic membrane and essentially surround the cytoplasm of the cell, demonstrated in fig.1.3. Thylakoid membranes concentric stacked structures, first reported by Nierzwicki-Bauer *et al.* in 1983, have been hypothesised to increase surface area within small cellular compartments to maximise electron transport and provide an abundance of energy (Nevo *et al.*, 2007). The stacked layers of thylakoid membranes have been shown to have openings which are hypothesised to allow the passage of proteins to cytoplasmic membranes, however, it has also been suggested that these membranes may be permeable and allow passage of proteins through them (Nevo *et al.*, 2007; MacCready *et al.*, 2016). The photosynthetic machinery is composed of photosystem II, photosystem I, cytochrome and ATP synthases aided by electron chaperones (Liu, 2016). The photosystems are activated by light energy (visible light – 500-670 nm), photosystem II is connected to a reaction where H<sub>2</sub>O molecules are split and electrons flow through the transport chain (Rochaix, 2011; Liu, 2016). Thereafter, proton pumping into the thylakoid membrane is used to power synthases for production of ATP (Rochaix, 2011). In photosystem I, after light energy is provided, electron carriers are charged which leads to the reduction of ferredoxin, this in turn is processed through transfer reactions that creates NADPH

(Liu, 2016). NADPH and ATP are utilised for cellular processes such as  $\text{CO}_2$  fixation and metabolism.

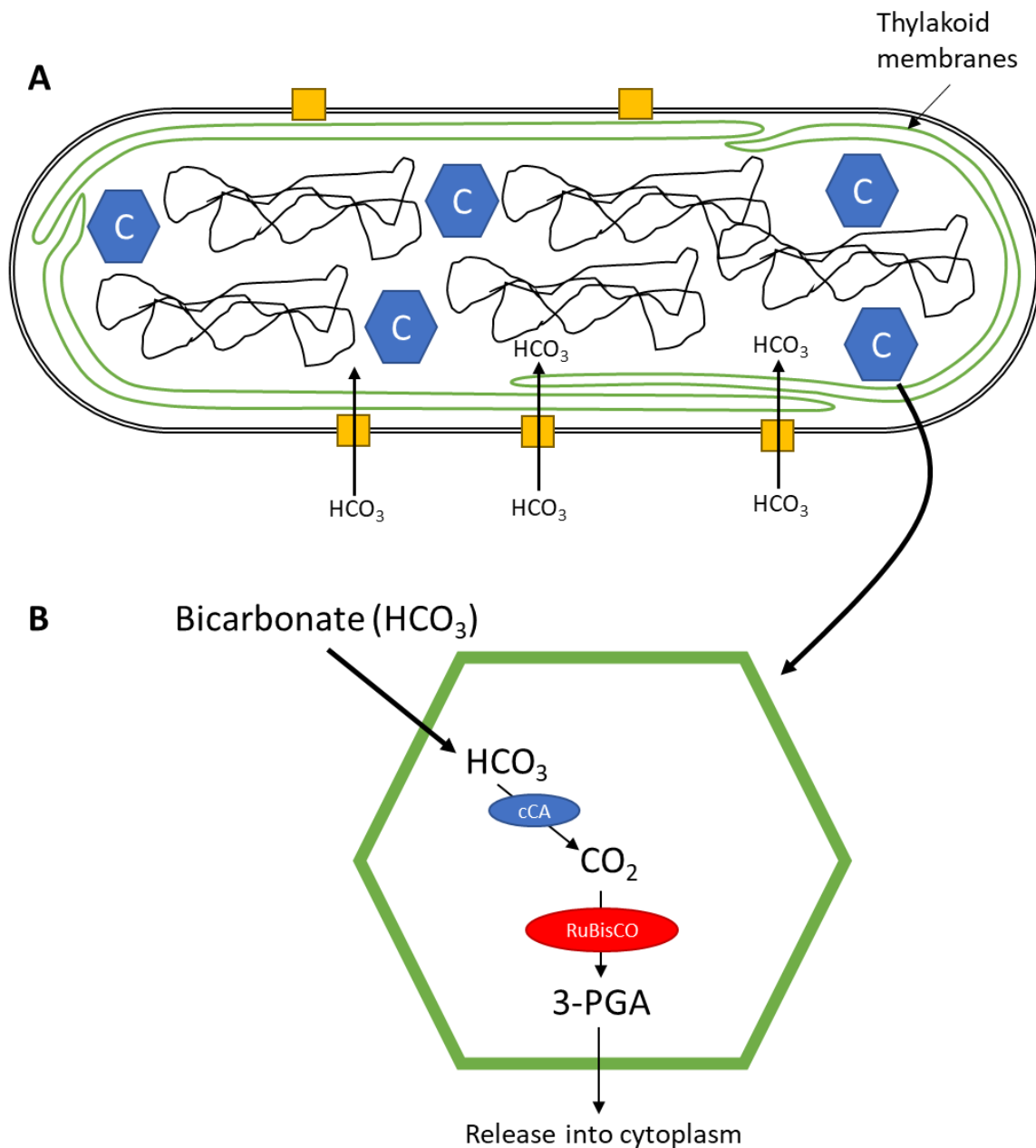


**Figure 1.3. Demonstration of *Synechococcus elongatus* cell and carboxysome reactions.** **A)** *S. elongatus* is a polyploid organism with 2-8 chromosomes, the thylakoid membranes are outlined with green lines encasing the cytoplasm. The cytoplasm contains carboxysome organelles and yellow boxes indicate transporters which draw bicarbonate into the cell. **B)** function of carboxysome demonstration housing the Carboxysomal Carbonic Anhydrases (cCA) and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) enzymes to fix  $\text{CO}_2$  into 3 carbon sugar (3-PGA) which is released into the cytoplasm. Image adapted from Rae et al., 2013.

Carboxysomes, shown in figure 1.3B, are a bacterial microcompartment used to house the CO<sub>2</sub> fixation protein ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). Uptake of bicarbonate (HCO<sub>3</sub>) from the cytoplasm is firstly processed by carbonic anhydrases to create CO<sub>2</sub>, then RuBisCO performs carbon fixation to produce a sugar known as 3-phosphoglycerate (3-PGA) (Rae *et al.*, 2013). RuBisCO is immensely sensitive to oxygenation, which is why these reactions must be housed within microcompartments as reactions with oxygen result in phosphoglycolate which is toxic and an inefficient use of the enzyme (Cleland *et al.*, 1998).

The chromosome of *S. elongatus* is quite small at 2695903 base pairs (bp) but there are 2-8 chromosomes per cell, increasing the amount of DNA present and duplicating genes many times. As there were no nucleoid occlusion homologues, it was assumed that having many chromosomes meant they could be dispensable and septa may form over a chromosome (Miyahishima *et al.*, 2005). However, more recent data has shown that there does seem to be a chromosome segregation pathway which aligns chromosomes before cytokinesis occurs and a non-essential ParA-like protein has also been identified which affects this arrangement when knocked out (Chen *et al.*, 2012; Watanabe *et al.*, 2018). There are common sites identified within the chromosome favoured for cloning labelled as neutral site I, II and III. Neutral sites I and II are both close to the origin of replication (*oriC*) at ~80-100 (Kb) either side (fig.1.4). Neutral site III is further away from the origin at ~730 Kb upstream, each site has been recorded in the literature for uses including inducible or constitutive gene expression (Golden *et al.*, 1987). The cyanobacterial genome consists of 55.96% GC content with many overlapping genes indicated by the inner and outer rings in fig.1.4.

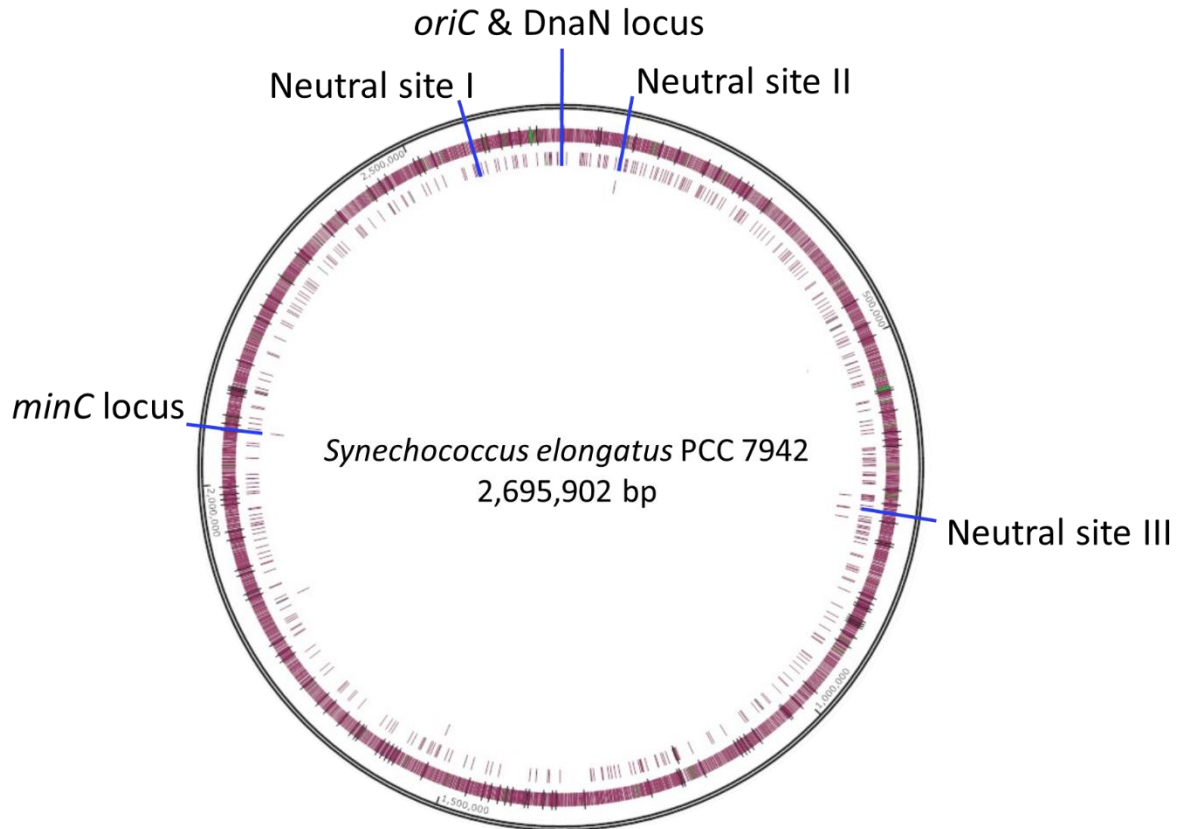


**Figure 1.4. Chromosomal analyses and insertional sites of *Synechococcus elongatus*.** Chromosome map was generated using SnapGene, thick blue lines show the position of genes commonly used for chromosomal insertion. Neutral sites were identified by Golden *et al.* (1987). The two inner and outer rings indicate genes, the two rings show genes that are overlapping the coding sequence of one another. The genome is 2.69 megabases (Mb) in size.

### 1.1.4 Bacterial Min system for division site placement

It was mentioned in sections 1.1.1 and 1.1.2 that there were Noc and SlmA systems in both Gram positive and Gram negative organisms respectively, these systems both aid in division site placement to prevent Z-ring constriction over chromosomes. However, how bacteria avoid dividing at the pole of the cell, where there is no nucleoid, requires a separate complex known as the Min system which prevents Z-ring formation at the cell poles. Min systems are present in *B. subtilis*, *E. coli* and *S. elongatus*, however, the mechanism of the complexes are different between Gram positive and Gram negative organisms. Primarily, in both Gram positive and negative organisms, the main inhibitor of the Z-ring is a dimer known as MinC, it has an N-terminal domain which interacts with FtsZ and a C-terminal domain that interacts with MinD

(Hu and Lutkenhaus, 2000). The role of MinD is to sequester MinC at the poles of the cell as it interacts with negatively curved membranes on its own in *E. coli* or through protein-protein interactions in *B. subtilis* (Rowlett and Margolin, 2013; van Baarle and Bramkamp, 2010).

In *B. subtilis*, MinJ is present to aid in localisation of MinCD complexes through interaction with DivIVA, a protein which remains stationary at the negatively curved membranes of the cell poles (fig.1.5A – blue ovals) (van Baarle and Bramkamp, 2010). MinCDJ complexes stationed at the poles of the cell allow MinC to exert Z-ring inhibition, and therefore prevent septum formation at the poles. After chromosome replication and segregation, the Z-ring can fully assemble in the chromosome free gap at the mid-cell through regulation via Min and Noc systems. When the septum is fully formed and starting to constrict, DivIVA moves to the newly formed cell pole as it binds to the curved membrane of the growing septum (fig.1.5B). MinCDJ follows DivIVA and aids in disassembly of the divisome at the new pole when division is completed, while some complexes remain at the old pole of the cell to prevent Z-rings forming (van Baarle and Bramkamp, 2010; fig.1.5C). Recent modelling work from Feddersen *et al.*, 2021 demonstrated that the Min complex of *B. subtilis* was in fact a dynamic system moving towards active division sites in protein complex clusters. This dynamic movement to new cell poles was important to ensure the division site does not reinitiate at newly formed cell poles.

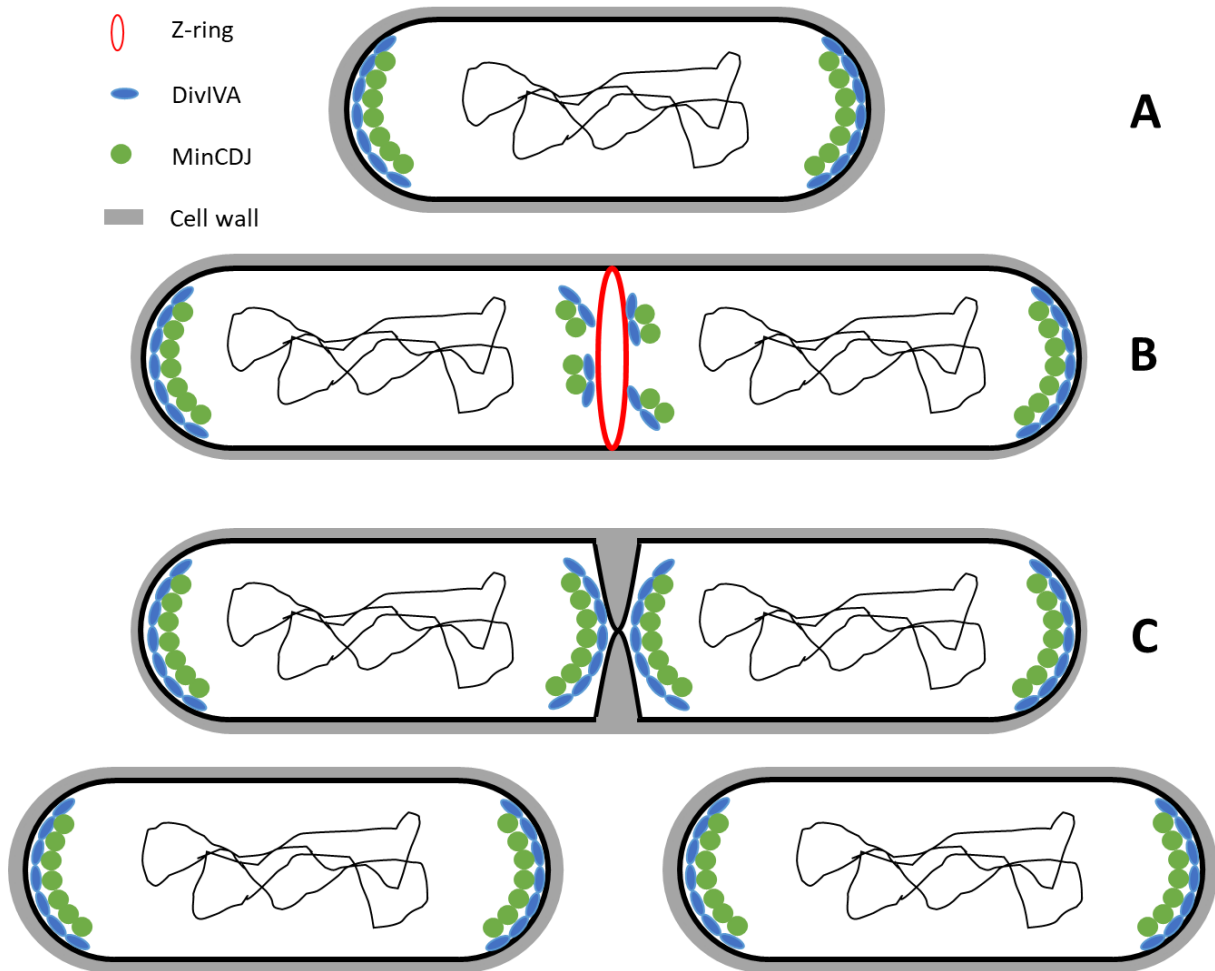


**Figure 1.5. The Min system in *Bacillus subtilis*.** The *B. subtilis* Min system is stationary at the poles of the cells. **A)** The protein complexes consist of DivIVA bound to the negatively curved polar membranes, MinJ bridging DivIVA and MinCD to station at the poles. MinC is the main inhibitor of FtsZ polymerisation to prevent septal rings forming at the poles of the cell. **B)** During division, the complex moves to the division site to decorate the new poles of the daughter cells formed to ensure there is no division sites formed inappropriately. **C)** when division completes, two new daughter cells are formed. Image adapted from Rowlett and Margolin, 2013.

In contrast, the Min system in *E. coli* and *S. elongatus* is very mobile as it oscillates between the two cell poles and does not have MinJ or DivIVA (Raskin and de Boer, 1999a; MacCready *et al.*, 2016). The MinCDE machinery prevents septum formation at the poles, but also regulates mid-cell placement of the Z-ring. MinC works directly on inhibition of FtsZ polymerisation but requires stimulation by MinD interactions, MinD has a role in driving oscillations and carrying MinC to opposing poles (Raskin and de Boer, 1999b), a knockout in either of these genes will result in formation of septa at the cell poles (Raskin and de Boer, 1999a). To prevent MinC disassembling mid-cell Z-rings, a regulatory component, MinE, is present to maintain oscillations from pole to pole (Raskin and de Boer, 1999b). Furthermore, knockout of *minE* produces long filamentous cells which are unable to divide (de Boer *et al.*, 1989). For oscillation to occur, MinCD complexes form at one pole of the cell to inhibit FtsZ polymerisation, then MinE will move towards the pole and disassemble the complex (fig.1.6 – blue ring; Rowlett and Margolin, 2013). After disassembly, MinCD complexes move rapidly towards the next pole through the cytoplasm, thus, MinE drives oscillation of MinD and subsequently MinC (Rowlett and Margolin, 2013). In support of this model, MinE has been shown to interact directly with MinD but cannot directly inhibit MinC activity, therefore, this implies that MinE prevents MinD from residing at the mid-cell membrane to successively prevent MinC inhibition (Zhao *et al.*, 1995; Raskin and de Boer, 1999b). Although the Min and SlmA systems are both utilised to position the chromosome at the mid-cell, studies in *E. coli* have demonstrated that these systems are mainly required for precision of the division ring placement. When both Min and SlmA systems were deleted in *E. coli*, Z-rings were able to form over chromosomes and at the cell poles but there was still evidence that mid-cell placement was favoured. When the authors grew the mutants slowly, it was evident that mid-cell placement was more prominent, but positioning was not as precise (Bailey *et al.*, 2014).



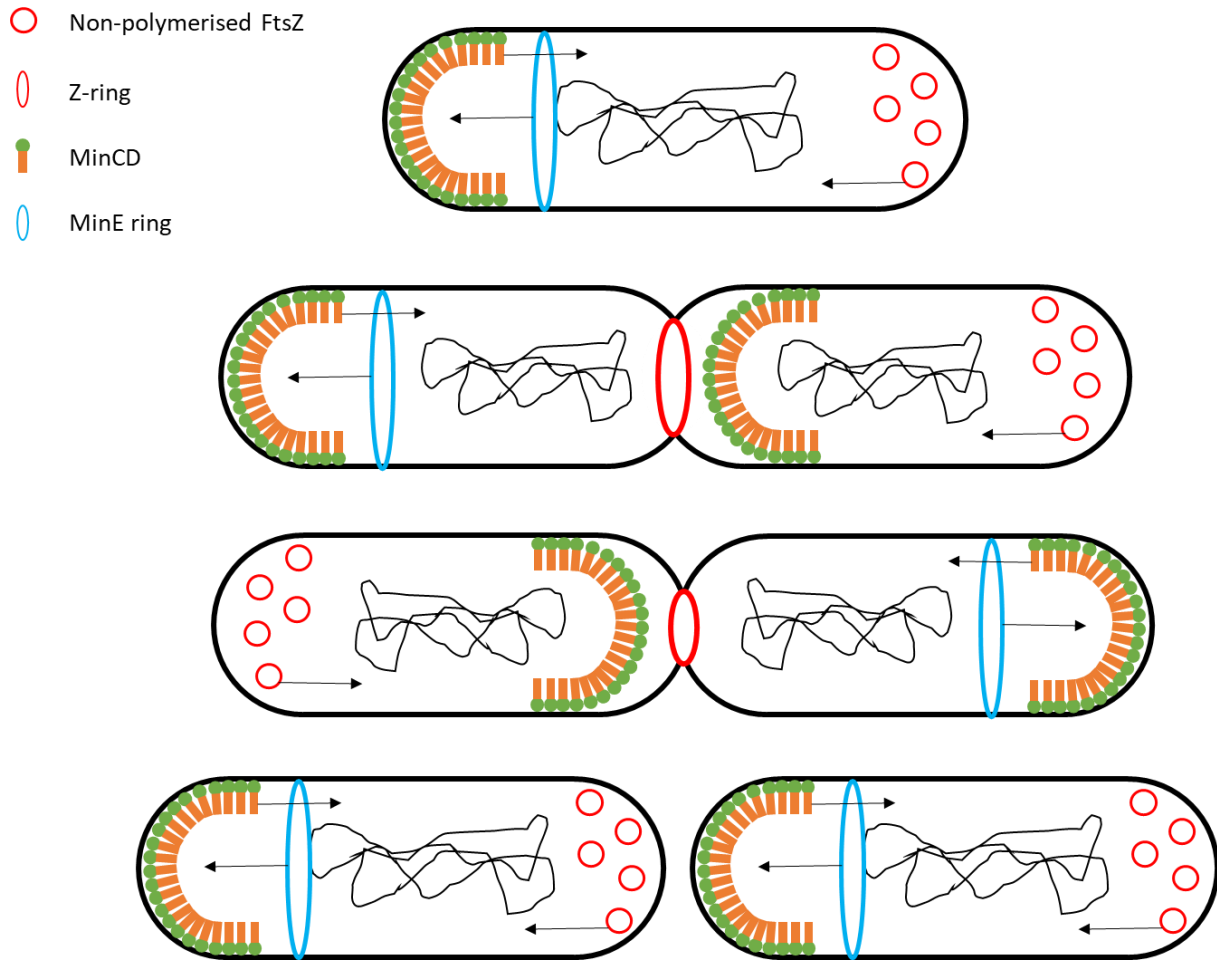


**Figure 1.6 The Min system models in both *Escherichia coli* and *Synechococcus elongatus*.** *E. coli* and *S. elongatus* has an oscillatory Min system which moves between the poles to prevent FtsZ proteins from polymerising. MinE moves to disassemble the MinCD complex, drive oscillation and promote midcell FtsZ polymerisation. Image adapted from (Rowlett and Margolin, 2013).

Min systems in both models are not essential but do cause phenotypic changes when knocked out, the most significant being the appearance of anucleated minicells where the FtsZ ring is allowed to polymerise at the poles. Minicells are produced by various deletions causing the septum to develop in any chromosome free zones. As Min and nucleoid occlusion systems work independently of one another, septum formation at the mid-cell is still regulated to prevent division over chromosomes. However, Min mutations do allow FtsZ ring formation at the cell poles which subsequently forms minicells. An exception being in deletions of *minE* in *E. coli* and *minJ* or *divIVA* in *B. subtilis* as these mutations cause rod cells to become filamentous as MinCD complexes are no longer stationed at the cell poles and allow for Z-ring inhibition throughout the cell (Bramkamp *et al.*, 2008).

Minicells were first described in *E. coli* by Adler *et al.*, when a mutagenesis screening found a phenotypic change where small, rounded anucleated cells were identified. These minicells were found to be present throughout all growth phases and were described as ‘normal division’ at the poles of the cells, which did not affect normal mid-cell division (Adler *et al.*, 1967). In *B. subtilis*, the minicell phenotype was described by Reeve *et al.* in 1973 after nitrosoguanidine mutagenesis found DivIV-A1/B1 mutations produced minicells, with greatest yields being recovered in a rich broth during exponential growth. In both studies, separation of minicells was achievable as they were much smaller than rod cells and sucrose gradient methods were beneficial for purification of small cells (Adler *et al.*, 1967; Reeve *et al.*, 1973). The discovery of asymmetric septa in *S. elongatus* has been a much more recent discovery, first published by MacCready *et al.* in 2016, where  $\Delta minD$  and  $\Delta minC$  mutants showed FtsZ rings formed at the poles of the cell. This was not surprising as the presence of *minCDE* genes suggested an oscillatory pathway.

## 1.2 Synthetic microbiology

### 1.2.1 Uses and advantages

Since the emergence of synthetic microbiology, there has been many innovations made within the field from optimising protein expression to studies of metabolism. Synthetic biology is a broad subject which involves applying engineering principles to biology to permit the repurposing of biological systems, therefore constructing strains and circuits which can sustainably produce a range of products (Clarke and Kitney, 2016; Engstrom and Pfleger, 2017). With the growing uses of synthetic biology, the accompaniment of computer modelling has also become imperative to success. Computer modelling gives laboratory scientists confidence in engineering by using existing data to predict the outcome of genetic engineering, this can even be done on large genome wide metabolic models to find candidates which could increase efficacy of the host strain (Medema *et al.*, 2012). Advances in genetic manipulation has increased usage of microorganisms, where genetic circuits are used to give cells new functionality such as expression of industrially relevant products from fine chemicals to biofuels (Zhang and Nielsen, 2014; Macdonald and Deans, 2016).

In industry, there are numerous enzymes made using microbial systems with approximately half being from fungi and one third from bacteria, other sources include plant and animal cells (Illanes *et al.*, 2012). Synthetic biology developments in microbiology can be advantageous as a wider range of sustainable products can be created and optimised (Singh *et al.*, 2016). Microbial bioreactor products are highly diverse, in that they are able to produce medicinal enzymes as well as enzymes in household commodities and chemicals. For example, sacrosidases produced from *Saccharomyces cerevisiae* can be used to treat genetic disorders where patients cannot hydrolyse sucrose, allowing patients to normalise diets (Vellard, 2003). Industrial applications for microbial bioreactors can also be used to make common products, for example, proteases manufactured in *Bacillus* and *Aspergillus* sp. may be used for dead skin

removal in cosmetic products (Singh *et al.*, 2016). Phage therapies are also of growing interest as a therapy to treat infections where last resort medications are not effective because of antimicrobial resistance. Synthetic biology has been influential in the study of phage treatment as viral genome engineering can be employed to design a specific phage with a greater host range and efficiency (Barbu *et al.*, 2016). Where bacteriophage resistance mechanisms have been found to evolve rapidly, the use of synthetic biology has the potential to evade resistance mechanisms which adds interesting possibilities (Labrie *et al.*, 2010; Barbu *et al.*, 2016). The genes and pathways introduced into the cell are often referred to as “genetic circuits” that are engineered specifically for the model organisms and the function required.

Microbial bioreactors are used in enzyme production as they are sustainable, can be manipulated with ease, and produce high quality products without the labour intensive recovery involved in conventional methods for processing animal tissue to purify enzymes (Boland *et al.*, 1989; Singh *et al.*, 2016). Synthetic biology does not only have a scope for the production of materials, there has also been evidence for medical application where it has been shown that genetically altered microbes could be used to alter the human gut microbiota. This would allow responses to microflora changes to occur by using biosensing, this is hypothesised to be an effective way to preserve the microbiota during antibiotic treatment (de la Fuente-Nunez *et al.*, 2017). There is also possibility that synthetic biology could be utilised for the upkeep of biodiversity within the natural world, however, this requires ethical consideration as containment of genetically modified (GM) organisms and their potential impact on the natural micro and macroflora would need to be accounted for (Redford *et al.*, 2014).

### 1.2.2 Limitations & metabolic burden

Engineering biological cells to perform a specific function is often an unpredictable approach as living cells possess normal processes which are complex and largely misunderstood, therefore, often interfere with expression of genes or pathways from introduced genetic circuits (Kwok, 2010). Studies have shown that when cells are in unfavourable conditions, such as metabolic load or heterologous toxic products, the cell has a reduced the ability to fold proteins correctly which can be detrimental for industrial applications (Baneyx and Mujacic, 2004). Another limitation to overexpression of proteins are inclusion bodies which are formed from misfolded, insoluble protein aggregates, these aggregates require considerable processing in order to extract, solubilise, fold and purify the protein (Carrió *et al.*, 2000; Singh *et al.*, 2015). An example of this was shown with the expression of Onconase, an anti-cancer agent, in *E. coli* that could be expressed at high yields but often required lengthy purification and refolding steps because of inclusion body formation (Notomista *et al.*, 1999). Therefore, to reduce laborious processing, a cell-free system was constructed using the lysates of *E. coli*. This method permitted bypassing of complex cellular processes interfering with enzyme expression while allowing usage of cytoplasmic contents. Overall, it was found that cell-free protein synthesis using *E. coli* lysates produced active enzymes that could be applied to downstream assays without complex purification (Salehi *et al.*, 2016). However, there are limitations associated with cell free systems as essential components are turned over and not replaced such as those involved in transcription and translation. Subsequently, this limits the quantity of product these systems can produce.

Expression of a heterologous gene(s) can lead to a metabolic load, especially with overexpression, when the hosts resources are significantly exploiting energy (ATP or GTP), amino acids, polymerases and ribosomes (Glick, 1995). Metabolic load has major implications which affects product yields, growth, mutation occurrence and energy reductions which may

broadly affect cellular functions and fitness (Wu *et al.*, 2016). Evidence of metabolic burden in a bacterial system was demonstrated by the introduction of a plasmid encoding genes for chloramphenicol-acetyl-transferase (*cat*) (Bentley *et al.*, 1990). Here it was found that increased expression consistently reduced growth rates regardless of media conditions (Bentley *et al.*, 1990). Burden has also been associated with plasmid replication and copy number, when the copy number increases, energy to maintain the plasmid and express its encoding gene incurs a greater metabolic load on the host (Bentley *et al.*, 1990; Birnbaum and Bailey, 1991). Stress induced discrepancies in growth rate can then cause plasmid segregation instability as divisions are irregular causing fewer daughter cells to retain the plasmid, leaving the bioreactor unproductive as it has no “circuit” to express (Imanaka and Aiba, 1981). Efforts have been made to try and use plasmid free systems such as those described by Striedner *et al.*, where expression of the GFP or human superoxide dismutase from the genome showed reduced leaky expression and theoretically greater expression in an *E. coli* host strain (Striedner *et al.*, 2010). However, plasmid-based systems are often favoured by academia and industry because of quick cloning techniques in a wide range of model organisms, combined with fast transformation and lower costs (Marish *et al.*, 2013).

## **1.3 Compartmentalisation**

### **1.3.1 Synthetic cell production**

New approaches in the synthetic biology field to overcome limitations, such as metabolic load and cellular interventions, are to build synthetic cells using a range of techniques. Cell free systems have been created to evade unwanted cellular responses, these have developed from simple DNA free lysates transferred into artificial cell liposomes. This enables introduction of DNA integration/segregation, transcription/translation initiation and cell free regulation (Olivi *et al.*, 2021). Interestingly, within these synthetic cell systems, there is an interest to reconstitute

cellular division systems such as Min, FtsZ and even using DNA origami to curve lipid membranes (Litschel *et al.*, 2018; Godino *et al.*, 2020; Franquelim *et al.*, 2018). Some artificial cells are designed to aid in understanding of the basic requirements for life as well as understanding evolution and how life may have emerged (Olivi *et al.*, 2021). Alternatively, liposomes have also been shown as synthetic biological compartments to produce industrially relevant proteins.

As mentioned, there are studies to reconstitute cellular systems such as transcription/translations in liposomes, however, there are other studies which use cell extracts from HeLa cell lines with hemin to increase protein synthesis initiation (Weber *et al.*, 1975). This allows researchers to utilise the cytoplasmic contents with little interference from normal cellular processes mediated by the chromosome. An example of this model is “Synells”, these synthetic cells are produced from lipid vesicles with HeLa cell extracts which can make channel forming proteins for the influx of inducers such as IPTG and doxycycline (Adamala *et al.*, 2017). HeLa cell extracts are not the only examples of providing synthetic cells with the ability to express proteins as Murtas *et al.*, also demonstrated this was possible with *E. coli* lysates (Murtas *et al.*, 2007). Synells can contain one or more genetic circuits which encode the appropriate gene(s) for protein synthesis, Synell liposomes demonstrated the ability to fuse together, thus, it is probable to compile multiple genetic circuits into one large vesicle (Adamala *et al.*, 2017).

Cell free systems are currently very popular within academia with research showing that these systems can express functional proteins such as variations of GFP, membrane proteins, *Pseudomonas* exotoxin A (PE), and polypeptides (Murtas *et al.*, 2007; Yu *et al.*, 2001; Krinsky *et al.*, 2016; Oberholzer *et al.*, 1999). Although there are many examples of cell free protein expression for purification and industrial purposes, there has been potential for medical application shown in recent studies where Krinsky *et al.* demonstrated the potential to use these

cells for tumour treatment. The authors describe how synthetic cells are able to express proteins, even for up to 50 minutes after injection into mouse host tumours (Krinsky *et al.*, 2018). The study showed that these cells could be used as a therapeutic approach, liposomes would be ideal for treatment as they are non-pathogenic.

Synthetic cells show great promise for future engineering projects to overcome metabolic burden and avoid interference which may occur from normal cellular processes. However, thus far there are limitations associated with these artificial cell systems compared to biological cells. For example, cell extracts mean cellular resources are not renewed, synthetic cells cannot interact/react within populations, cannot divide independently and it is predicted that synthetic cells will be unlikely to surpass biological cell expression (Elani, 2021).

### **1.3.2 Minimal genome cells**

In an effort to reduce metabolic load, remove complex cellular interferences and increase yields, some research had been directed toward the construction of cells with minimal genomes. The rationale being that if redundant cell processes can be removed, this liberates a greater proportion of cellular resources for the cell to express heterologous proteins/pathways. Minimal genomes have been presented in multiple bacterial species such as *Mycoplasma mycoides*, *E. coli* and *B. subtilis* (Gibson *et al.*, 2010; Manabe *et al.*, 2011; Morimoto *et al.*, 2008). There are various applications for minimal genome cells such as synthetic biology application, understanding genes for antimicrobial resistance or to understand the minimum requirements for life (Zhang *et al.*, 2010).

Cells with minimal genomes can be produced by 2 targeted approaches, the first is the ‘bottom up’ approach in which a reduced genome is designed specifically and transplanted into the host cell. This approach has been described by Gibson *et al.*, where a 1.08 megabase chemically synthesised genome of *M. mycoides* was successfully transplanted and proved to be fit enough



to replicate and used for further synthetic applications (Gibson *et al.*, 2010; Zhang *et al.*, 2010). The second approach is known as the ‘top down’ approach where genes are systematically deleted from existing genomes (Zhang *et al.*, 2010). The challenge is knowing which genes to delete, therefore computational tools have been developed to help researchers make sensible deletions in stretches ranging in size (Wang and Maranas, 2018). Examples within *E. coli* and *B. subtilis* are prevalent in the literature and some have even demonstrated better expression. In earlier examples of minimal *Bacillus* genomes, the deletion of 20.7% of the genome exhibited greater promoter activity and recombinant protein expression (Manabe *et al.*, 2011; Morimoto *et al.*, 2008). This was also demonstrated in *B. subtilis* by van Tilburg *et al.*, where the genome was reduced by 36% and showed better expression of lantibiotic as the toxic precursors no longer affected the strain. Additionally, the minimal genome strain was described as providing a better background for secondary metabolite production and therefore a desirable host strain for biotech applications in antibiotic expression (van Tilburg *et al.*, 2020). Minimal genome strains of *B. subtilis* are described in the literature as having a greater ability to express ‘difficult’ heterologous proteins such as *Staphylococcus* spp. Antigens, again making minimal genome hosts a prime candidate for industrial expression applications (Aguilar Suárez *et al.*, 2019). Conversely, *E. coli* has been published with an engineered genome reduction of 29.7% using a top-down approach (Kato and Hashimoto, 2007). However, further studies with this model demonstrated that deletions had a significant effect on phenotype, growth rate and nucleoid organisation (Hashimoto *et al.*, 2005).

Limitations associated with this are that there is an emergence of ‘redundant’ genes which have been found to function when essential genes were inactive. Functional redundancy could also be of concern where singular genes can be deleted as other proteins may be able to perform the same activity. However, a knockout in both genes produces non-viable cells. Examples include: inactivated *pbp2B* rescued by *pbpC*, *pbp2A/pbpH* and *mreB/mreBH* in *B. subtilis* (Sassine *et*

*al.*, 2017; Wei *et al.*, 2003; Kawai *et al.*, 2009). Using these examples, it could be postulated that many ‘redundant’ genes do fulfil a role and mass deletions of these genes could have effects on regulation, survival, and basal resistance. Therefore, a greater understanding of functionally redundant genes and pairs would be required to make targeted genome reductions in bacterial species.

### **1.3.3 Minicell uses**

As minimal genome strains and cell free systems have shown promising results for increasing expression from genetic circuits and reduced toxicity for secondary metabolite production, it was clear that avoiding responses driven by genome expression changes were necessary for a good microbial bioreactor. As described in section 1.1.4, minicells are produced when the bacterial Min system fails to fulfil the task of preventing Z-ring polymerisation at the poles of the cell. The last two sections have described the use for synthetic cells and minimal genome cells, however, minicells can be of great use because of their anucleated nature while retaining cytoplasmic contents and native secretion systems. The minicell contains a snapshot of the mother cell cytoplasm at the time of septum constriction, meaning they contain normal cell wall structures including peptidoglycan and lipid bilayers, alongside ribosomes, RNA, proteins and plasmids (Frazer and Curtiss, 1975). Although minicells are able to express from a plasmid, the contents, protein half-life and time frame they express for are unknown.

There are many examples of minicells being used for various fields of research because of their non-viable nature while retaining virulent properties. For example, in the medical field, minicells of *Salmonella typhimurium*, *E. coli*, *Shigella flexneri*, *Pseudomonas aeruginosa* and *Listeria monocytogenes* were purified for the use of therapeutic treatment (MacDiarmid *et al.*, 2007). In order to treat diseases, MacDiarmid *et al.* incubated purified minicells with chemotherapeutic drugs before using bispecific antibodies to attach to minicells to cancer cell targets for engulfment and therefore, drug delivery. While MacDiarmid used minicells for

treating diseases, Carleton *et al.* provided a proof of principle that *S. typhimurium* minicells were able to express, assemble and insert type III secretion needles into the membrane. Thus, the authors hypothesised that such minicells could be used for antigen delivery and therefore vaccination (Carleton *et al.*, 2013). Minicells are also an ideal candidate for vaccination as they are not able to replicate and therefore non-pathogenic, while remaining biologically active, which would be beneficial for immunocompromised patients.

Minicells do not only have applications for medical uses, as they retain ribosomes, RNA, proteins and plasmids they are also a prime candidate for biosensors and there are numerous examples in the literature demonstrating this. Biosensors only become active when they encounter the specific material engineered to induce expression of a reporter, drug or antigen. Rampley *et al.*, presented that *E. coli* minicells carrying genetic circuits could induce GFP reporter expression in the presence of small molecules such as glucarate, acrylate and arabinose. This showed that minicells do have potential to react to molecules in their surrounding environment and display reporter expression. However, the authors showed the importance of ATP as SimCells incubated at 37°C significantly decreased ATP quantities which reflected on expression levels after induction. As a result of this, SimCell preparations for increased expression and storage were optimised by being maintained at 4°C temperatures for preparation and storage (Rampley *et al.*, 2017). This was significant for any potential industrial uses of minicells as it is imperative that these cells work to their highest potential for these systems to be adopted. Future studies with SimCells showed that the system was able to be adapted as an aspirin biosensor, the authors described this as a promising step towards using SimCells to sense the environment and express from controllable genetic circuits for diagnostic and therapeutic uses in humans (Chen *et al.*, 2019). A recent example of minicells potential for use within the medical field as biosensors for drug delivery was demonstrated by Ni *et al.*, where minicells of *E. coli* were shown to be chemotactic and motile. The authors showed that

*E. coli* minicells could express flagella systems from inducible genetic circuits and, at higher induction, minicells were very motile with the ability to move towards chemoattractants (Ni *et al.*, 2021). Although swimming abilities were not as rapid or directionally accurate as wild type/normal cells, the minicells were still able to move efficiently towards stimulants which demonstrated great potential for movement within animal tissues for treatment (Ni *et al.*, 2021).

### 1.3.4 Maxicell uses

Larger anucleate compartments were first identified in relation to chromosome replication mutants when a 103-bp segment of *oriC* was deleted (fig.1.8A). *oriC* is essential meaning a knockout is lethal but this study used a secondary self-replicating mini-replicon integrated into the chromosome derived from pLS32, a plasmid native to *Bacillus natto* (Hassan *et al.*, 1997). The study by Hassan *et al.* found the pLS32 replicon rescued the strain but reduced chromosome replication. There was a common phenotype in the *oriC<sup>-</sup> repN/oriN<sup>+</sup>* mutants where a population composed of ~40% anucleate cells became prominent (Hassan *et al.*, 1997). The anucleate production phenotype has also been identified when DnaA regulators were mutated (Scholefield *et al.*, 2012).

In academia, one of the first examples in *B. subtilis* of maxicells was described by East & Errington in 1989, where *B. subtilis* cultures were irradiated to remove all genetic material and consequently stop host protein expression. Therefore, the resulting maxicells could be used to look at bacteriophage protein synthesis (East and Errington, 1989). SimCells have been described in the last section as a derivative from minicells, however, the same laboratory group have also used a different method to make larger anucleate compartments in *E. coli*, *Pseudomonas putida* and *Ralstonia eutropha* (Fan *et al.*, 2020). Fan *et al.* utilised endogenous enzymatic degradation of the chromosome to cause irreparable double strand breaks and therefore remove the chromosome while retaining plasmids for expression. The cytoplasmic contents remained intact and functional. This publication highlighted the importance of having

a secondary plasmid for maintaining ATP/NADH levels for compartment efficiency, in fact it was shown that ATP/NADH expression increased yields of the reporter significantly within the first 3 days of expression (Fan *et al.*, 2020).

## **1.4 Plasmids and transcriptional repressors**

### **1.4.1 Plasmid replication and copy number**

Plasmids are mobile genetic elements which are small in size, easy to manipulate and can be dispensable to the host strain. As these plasmids can be dispensable, it is important to have good segregation mechanisms to ensure the genetic information is passed to each daughter cell, especially when plasmids are low copy number. Plasmid copy number refers to the number of plasmids which are within the host cell, these can be described as low, medium or high copy number. Although high copy number can be good for protein expression, it was found that an extra metabolic burden was imposed on *E. coli* cells with these plasmids (Ceroni *et al.*, 2015). Low copy number plasmids can be good for regulation control and reducing metabolic load, however, it can also come with issues related to segregation into daughter cells. As there are not as many plasmids within the cytoplasm, random diffusion does not work efficiently with low copies, therefore, segregation systems are usually utilised to direct movement into daughter cells (Tomalsky, 2013).

There are 3 types of plasmid replication mechanisms known: Theta-type, strand displacement and rolling circle (del Solar, 1998). Strand displacement models are most commonly found within broad-host range plasmids, which can carry the risk of cross contamination to other bacterial species. Rolling circle mechanisms facilitate unidirectional replication (fig.1.7) and historically rolling circle replication was commonly associated with small vectors isolated from Gram positive organisms but there are examples from Gram negative bacteria (Meijer *et al.*, 1998; del Solar, 1998). Rolling circle replication starts when the origin DNA is nicked

(fig.1.7A), the 3' end of the origin is extended by DNA polymerase while the 5' strand is displaced (fig.1.7B; Kahn, 1997). DNA synthesis is ongoing until the whole plasmid is replicated (fig.1.7D & A). Rolling circle replication produces single stranded intermediates which are synthesised into dsDNA plasmids, made clear from the diagram in figure 1.7E.

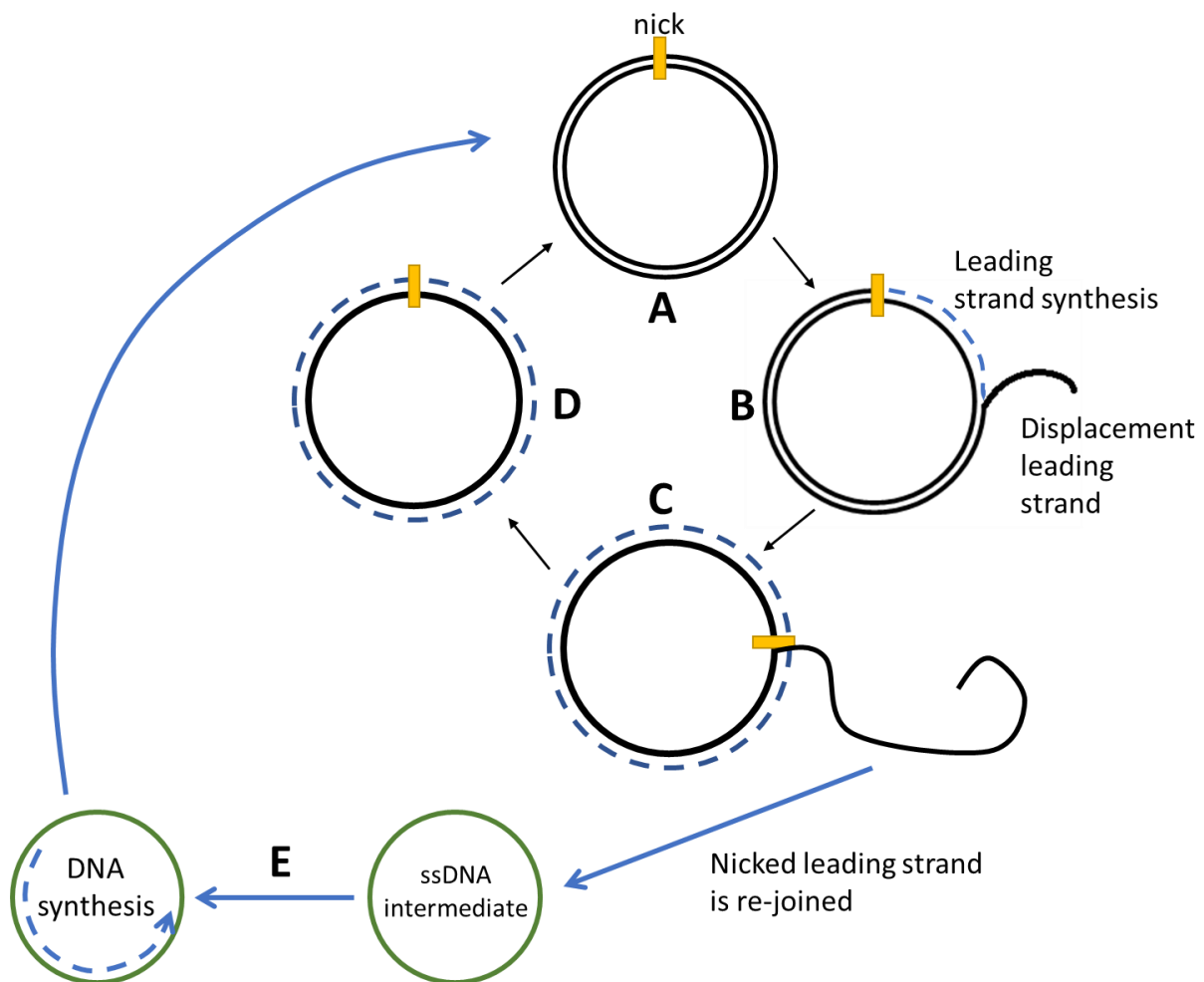


**Figure 1.7. Rolling circle replication.** The rolling circle plasmid replicates via nicking dsDNA, the leading strand is displacing to allow for synthesis. The single stranded intermediates also have DNA synthesis to produce 2 new daughter plasmids. **A)** origin DNA is nicked (origin yellow triangle) **B)** leading strand displacement and DNA synthesis of new leading strand **C)** nicked leading strand is re-joined **D)** Plasmid with newly synthesised leading strand **E)** single stranded intermediate has a new strand synthesis to make a double stranded plasmid. Image adapted from Wawrzyniak *et al.*, 2017.

Theta-type replication was named because the replicating plasmid bears similarities to the Greek letter  $\theta$  (Brewer and Fangman, 1987). Theta-type replication does not require DNA strand breaks, nor does it produce single stranded intermediates which makes the replicon much more stable (Meijer *et al.*, 1995). For expression within a host, it is common to use native plasmid replicon for expression as the plasmid is already optimised for the host (Meijer *et al.*, 1995). Theta-type replication starts at the origin of replication where the DNA is melted to dissociate the double stranded structure, primer RNA's are annealed to DNA which allows DNA polymerases to attach new nucleotides to the existing strand (Kornberg and Baker, 1992). DNA synthesis is preceded bidirectionally by leading strand synthesis and discontinuous lagging strand synthesis in each replication fork, seen as grey circles in fig.1.8A. DNA replication leads to Y-shaped forks (fig.1.8B) from leading strand and lagging strand synthesis, the leading strand is replicated continuously in the same direction the fork moves. Whereas the lagging strand is synthesised as small Okazaki fragments as it is synthesised in the reverse direction of the replication fork (Maslowska *et al.*, 2018; fig.1.8B).

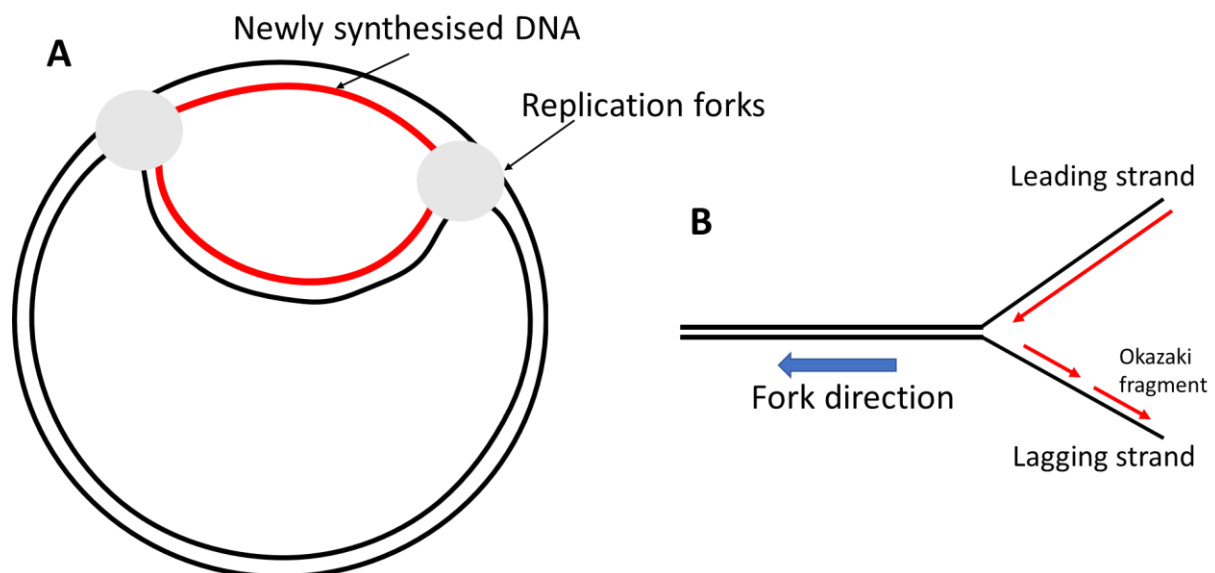


**Figure 1.8. Theta-type replication of plasmid DNA.** **A)** The double helix structure of the DNA is opened at the origin of replication, this in turn allows the replisome to form and replication forks work bidirectionally. **B)** in each replication fork there is DNA synthesis of the leading and lagging strand, the leading strand is made

continuously, whereas the lagging strand is made in discontinuous Okazaki fragments which are linked together by DNA ligase to form a leading strand. Image adapted by Maslowska *et al.*, 2018.

As mentioned in section 1.1.1, DnaA is the main initiator for DNA replication and binds to specific small sequences at the origin to open the DNA duplex (Bramhill and Kornberg, 1988). Some plasmids do not rely on the host's machinery for replication initiation by DnaA but do still require some host factors such as DNA polymerase, DnaA dependent replication of theta-type plasmids are similar to chromosome replication (Lilly and Camps, 2015). Other plasmids encode their own replication initiation proteins, often called 'self-replicating', which can either work alone or in tandem with DnaA. For example, pSC101 RepA protein was described to bind to its cognate sites and strengthen DnaA binding to distantly spaced DnaA boxes (Stenzel *et al.*, 1991).

Broad host range plasmids such as RSF1010, which replicate via strand displacement, encode all the proteins required for replication initiation and only utilise some host replication machinery such as polymerases for DNA synthesis. The dedicated replication initiation protein (RepC – fig.1.9 orange oval) binds to specific AT rich iteron sequences in order to open the DNA duplex, a plasmid encoded helicase (RepA – fig.1.9 black circle) is then recruited to aid in melting the duplex and DNA unwinding in a 5'-3' direction which subsequently exposes *ssi* sites (Lilly and Camps, 2015; del Solar *et al.*, 1998). After exposing the *ssi* sites, the plasmid encoded primase (RepB – fig.1.9 blue boxes) specifically binds to and initiates DNA synthesis using the hosts polymerase (red arrows fig.1.9). Replication from *ssi* sites is continuous in each direction with RepA aiding in displacement of the parental DNA which forms a theta-type structure with two D-loops (del Solar *et al.*, 1998; Honda *et al.*, 1991). However, in contrast to theta-type replication, only one strand is replicated in each direction resulting in a ssDNA intermediate stage in the replication process.



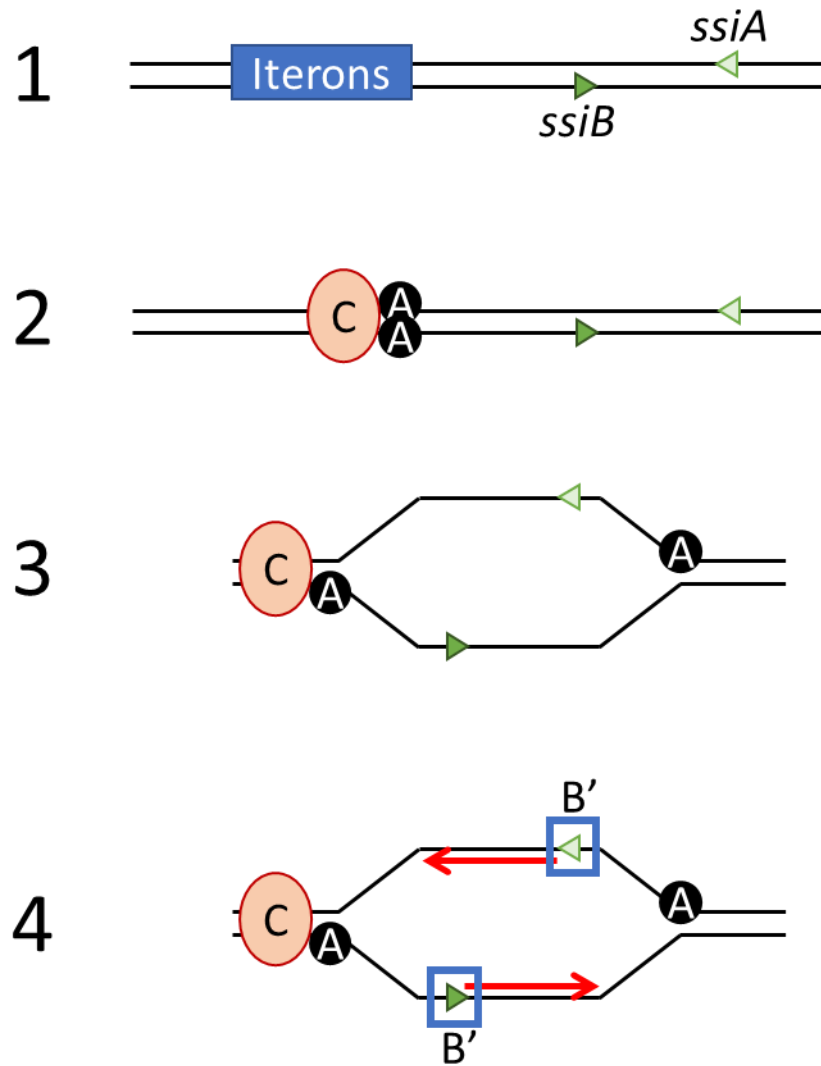


**Figure 1.9. RSF1010 strand displacement replication using plasmid encoded initiation proteins.** 1) the iteron sequences of the origin sequence is positioned close to *ssi* sites (green triangles) 2) the initiator protein RepC (orange oval) binds to AT rich region and recruits RepA helicases (black circles) 3) the helicases help melt the DNA duplex and opens to expose *ssi* sites 4) RepB (blue squares) recognises the *ssi* sites and primes the DNA for the synthesis of new DNA (red arrows) by the hosts own polymerase. Image adapted from del Solar *et al.*, 1998 and Honda *et al.*, 1991.

### 1.4.2 Transcriptional repressors and promoters

A promoter is a site within a DNA sequence where RNA polymerase can start transcription of the gene(s) which the promoter controls. Transcription from promoter sequences is regulated in accordance with cell cycle, stress and external stimuli that can influence cellular responses to aid in survival (Yoon *et al.*, 2003; Jacob and Monod, 1961). Choice of promoter can make a significant change in the expression of products from bacterial cells. For high levels of expression in *E. coli* the promoter must be strong with the ability to induce expression, however, it is known that high protein expression can burden metabolic pathways, leading to growth impairment and even cell death (Makrides, 1996; Marisch *et al.*, 2013). In addition to promoter strength, translational efficiency must also be considered. The spacing between the ribosome binding sites (RBS) and start codons are important for efficient translation where 7-9 nucleotides was found optimal for both *E. coli* and *B. subtilis*. Shorter spacing sequences were shown to be translated less efficiently, especially in *B. subtilis* (Vellanoweth and Rabinowitz, 1992).

In both *B. subtilis* and *E. coli*, LacI is a transcriptional repressor used in order to prevent expression within rod cells – a system native to *E. coli* first described by Jacob and Monod (Jacob and Monod, 1961). In *E. coli*, the *lac* operon is composed of *lacZ*, *Y* and *A*, this operon is upregulated in the presence of lactose to allow expression of  $\beta$ -galactosidase which metabolises lactose into glucose and galactose (Marbach and Bettenbrock, 2012). *lacZ* is also responsible for producing allolactose, the natural induction molecule of the *lac* operon (Marbach and Bettenbrock, 2012). The lactose repressor is known as LacI, when present it will bind to the promoter to prevent transcription via blocking RNA polymerase (Lewis, 2013). However, this repression can be regulated with the use of a non-metabolisable inducer, Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG), which mimics natural allolactose to bind and reduce affinity of the repressor for its DNA-binding site (Glacock and Weickert, 1998). The

*E. coli* model utilises the transcriptional repressor mutant *lacI<sup>Q</sup>*, the mutation is comprised of two base pair changes in the -35 promoter of *lacI* which increases the amount of repressor 10-fold in comparison to the native *lac* repressor (Müller-Hill *et al.*, 1968; Calos and Miller, 1981). This well characterised and easily inducible system was highly desirable in other organisms. Therefore, Yansura and Henner worked on transferring the system into *B. subtilis* for the ability to regulate gene expression. The Lac repressor (*lacI*) and operator (*lacO*) systems of *E. coli* were used to repress and induce expression of penicillinase and leukocyte interferon with success using IPTG (Yansura and Henner, 1984). The first IPTG inducible promoter in *B. subtilis*, *P<sub>spac</sub>*, was a hybrid between the *lacO* operator sequence and a *B. subtilis* phage promoter (SPO-1) (Yansura and Henner, 1984). Yansura and Henner placed the LacI repressor under the control of a penicillinase promoter, known as *P<sub>pcn</sub>*, native to *Bacillus licheniformis* as it was constitutive to ensure LacI was abundant. The *P<sub>spac</sub>* promoter and LacI have been used widely since the system was constructed for many uses such as determining the function of gene products by “turning off” to permit controlled expression of recombinant products (Vagner *et al.*, 1998).

An alternative mechanism for expression control is provided by antisense-RNA (asRNA) which are of growing interest in prokaryotic cells, they are utilised for post-transcriptional gene repression found to occur natively in bacteria (Copeland *et al.*, 2014). Native uses were often related to stress conditions where the cell prevented expression of certain proteins in order to maintain resources for essential pathways, asRNA's are complementary to the target mRNA which primes RNA for degradation by Rnase E or simply blocked ribosome binding sites (Copeland *et al.*, 2014). Repression by asRNA's required pairing with Hfq, which forms ring-like complexes with asRNA, aiding in annealing and stabilising asRNA to mRNA targets (De Lay *et al.*, 2013).

## 1.5 Aims for compartmentalisation

In section 1.3.3, all minicell models described required purification steps, soaking with drugs, and/or further induction steps. Whereas section 1.3.4 demonstrated most methods of producing maxicells required destruction of the genome by varying methods. The minicell model within this study is unique as it aims to prevent plasmid encoded protein expression in rod cells by utilising a transcriptional repressor, minicells can then independently express once they are segregated from nucleated cells, as demonstrated in figure 1.10. The transcriptional repressor was expressed only from the chromosome, whereas the plasmid bound reporter was controlled by the cognate inducible promoter. Therefore, when the plasmid was within nucleated cells, where the repressor was expressed, there was no expression. Once the plasmid was compartmentalised, the transcriptional repressor was depleted, and expression induced in minicells. This system was designed to continually make minicells so there was constant production of minicells expressing proteins or antigens. This would mean that nucleated cells did not bear a metabolic burden but did recurrently produce minicells which were expressing from engineered genetic circuits utilising cytoplasmic contents.

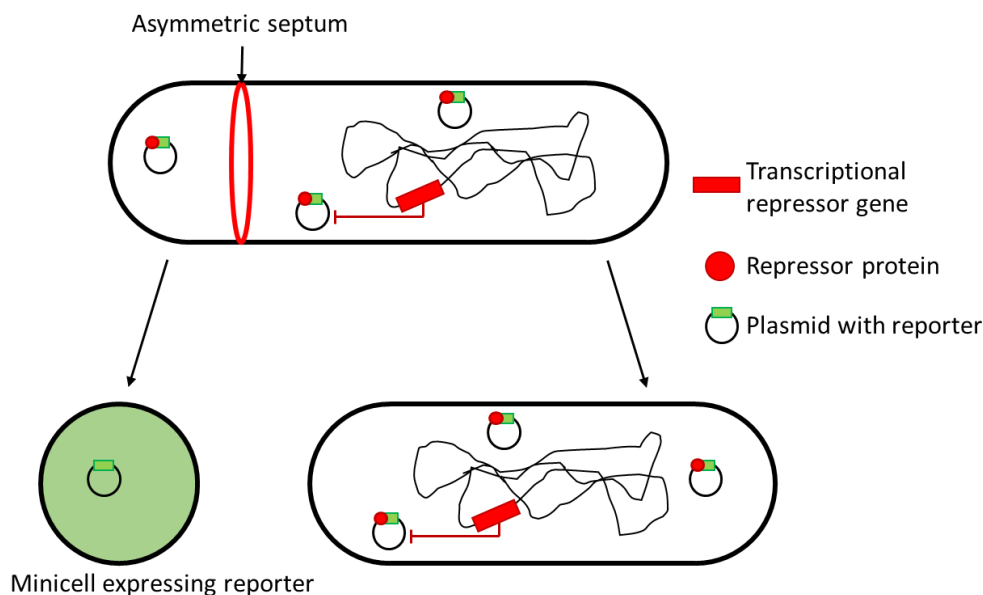


**Figure 1.10. Image depicting the minicell expression model proposed by this study.** Transcriptional repressor (red triangle and dot) is expressed from the chromosome of the mother cell and actively repress expression from

the plasmid. Once the asymmetric septum constricts, minicells which gain a plasmid can express freely once the repressor is depleted. The rod cells of the culture continually make minicells which are able to express from the plasmid (Image adapted from Farley *et al.*, 2016).

This study proposes another model for expression in larger anucleated compartments, known as maxicells. To make maxicells for this project chromosome replication was planned to be exploited. Using the knowledge of chromosome replication initiation (see section 1.1.1), a larger anucleate compartment could be formed by slowing DNA replication initiation while the divisome works appropriately to produce a daughter cell negated of chromosomal DNA. The maxicell model was similar to that what was presented for the minicell model (fig.1.10). The transcriptional repressor was constitutively expressed from the chromosome, while chromosome replication was perturbed and the divisome worked to form anucleated cells. The anucleated cells would then be able to independently express from the plasmid when the repressor was diluted out. It was hypothesised that, as maxicells are larger in size, they would contain much greater cytoplasmic contents than a minicell and therefore be able to express for longer periods of time. This would be optimal for an industrial chassis as more product could be made over time without the issues of burden affecting nucleated cell populations.

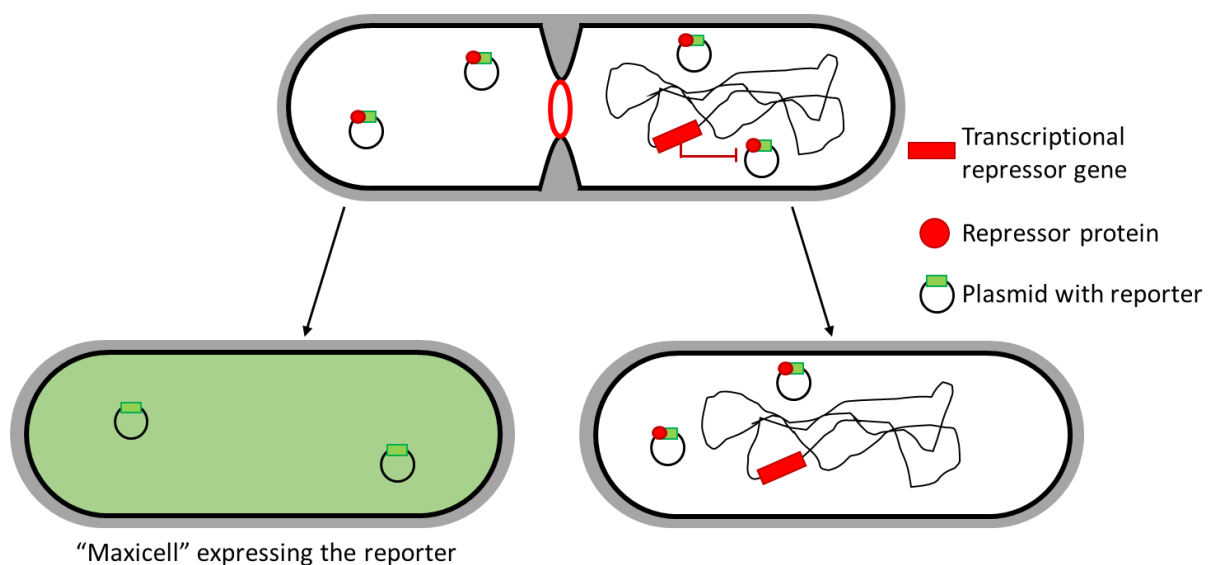


**Figure 1.11. The production of ‘maxicells’ and how they may be used industrially.** A model of how the proposed maxicell system will work, chromosome replication initiation will be reduced enough so daughter cells

will not have a chromosome as cellular division works temporally the same while the cell cannot produce a chromosome rapidly enough to provide for the new daughter cell. A transcriptional repressor expressed from the chromosome will stop expression in nucleated mother cells but expression will be allowed in maxicells.

## **1.6 Portabolomics project aims and outlook**

This research contributes to a larger project, known as Portabolomics, the aim of which is to make genetic circuits and host organism systems universally compatible between hosts. Compartmentalising gene expression or enzymes to enhance efficiency. This contributes to standardising the field and removing the time-consuming burden of re-engineering circuits for each new organism. The project combines the use of computer sciences for modelling and predicting cellular behaviours coupled with laboratory work to test models and increase efficacy for industrial applications.

### **1.6.1 Portabolomics compartmentalisation**

The use of bacterial compartments has been described throughout section 1.3 for the ability to aid in increasing expression yields, evade stress responses/cellular interference and produce non-reproductive biologically active cells. Compartmentalisation using minicell or maxicells is relevant to the project to redirect gene expression into anucleate compartments, subsequently removing metabolic burden imposed on nucleated cells and allowing control of expression using cytoplasmic contents. This way, nucleated cells remained fit as they do not express from the genetic circuit but also provide a continuous quantity of anucleated cells which do express. Like the cell free system, compartmentalisation benefits from the lack of cellular interference from chromosomal induced responses, although cytoplasmic turnover limitations are still present in anucleate compartments. However, this limitation does not affect the Portabolomics compartmentalisation system as significantly because chromosomal cells within the culture can continuously produce new compartments.

To understand the host strains better and for quick diagnosis of metabolic burden, the project has a strand investigating stress landscapes of the cell. This improves efficacy of the host strain as identified stress biomarkers could lead to rapid actions taken to modulate gene expression where appropriate. Using transcriptome data, gene expression was analysed, and 10 biomarker genes were identified with further analyses showing the biomarkers were effective at sensing cellular growth states (Huang *et al.*, 2021).

### **1.6.2 Plasmid portability**

As one of the aims was to standardise plasmids for portability across different host species, the universal replicon strand was involved in construction of this system. The system developed a plasmid replicon which could be moved between Gram positive and Gram negative bacteria such as *B. subtilis* and *E. coli* with scope to move to *Corynebacterium* and yeast. To ensure the broad host system was safe and to prevent unwanted transfer to environmental bacteria, therefore spreading antibiotic resistance cassettes, the system was designed to be split where replication initiation proteins were only expressed from the chromosome (A. Johns, unpublished). Therefore, if the plasmid did move to an unwanted organism, the bacteria would not have the ability to replicate the plasmid (More information in Appendix 10). This is comparative to the Standard European Vector Architecture (SEVA) system designed within Victor de Lorenzo's laboratory group as both Portabolomics and pSEVA systems aim to produce a standardised plasmid system where functional modules, such as terminators, reporter genes, or antibiotic selection markers can be swapped interchangeably (Silva-Rocha *et al.*, 2013). However, the Portabolomics universal replicon system contrasts to the SEVA system as the aim was to construct a single origin of replication which only replicated in specifically engineered bacterial strains.

The overall aim between the compartmentalisation and plasmid portability strands was to integrate compartmentalisation with the universal replicon. Therefore, the plasmid split-

replicon system would be implemented in a mini- or maxicell producing strain for compartmentalised gene expression.

## 1.7 Specific aims of the project

The purpose of this study was to produce a range of characterised microbial reactors able to divert metabolic burden from nucleated cells for continuous manufacture of products. Genetic circuits were also considered as they were vital to ensure efficient production and stability within all hosts. Using different host strains was important for determining portability within the Portabolomics project, species diversity demonstrated how anucleate production could be interchangeable.

The aims investigated within this work were:

1. Construct anucleated cell producing strains and characterise industrial practicality.
  - a. Minicell production.
  - b. Maxicell production.
2. Investigate regulation systems in host stains to maintain nucleated cell fitness.
3. Optimise genetic circuits for use across host ranges.
4. Enhance genetic circuits specifically in *B. subtilis* hosts to increase yields of compartments with a plasmid.



## Chapter 2 - Materials and methods

### 2.1 Growth media and general culturing methods

Media recipes are attached to appendices 1, growth in rich media used Luria-Bertani broth (LB) for both *B. subtilis* and *E. coli* species, but minimal medias differed between species. For *B. subtilis* a complex defined media (CDM) was used, whereas, for *E. coli* M9 minimal media was used (both recipes in appendices 1). *E. coli* or *B. subtilis* overnight cultures were grown in 5 millilitre (ml) volumes in tubes, whereas cultures used for experiments were grown in 125 ml Erlenmeyer flasks with culture volumes at approximately 15-20 % of flask size. *S. elongatus* was exclusively grown in BG11 media, described in appendices 1, at 50-100 ml volumes with constant light. Growth in all experiments was carried out with agitation, either shaking horizontally or orbital shaking, at 30°C unless otherwise indicated in figure legends.

**Growth curve experiments.** Growth curves were created using cultures grown in 125 ml Erlenmeyer flasks grown at 30°C with shaking. To monitor optical density at 600 nm (OD600) a spectrophotometer (Thermofisher Genesys 20) was used, and time points were measured across 6-8-hour periods. Sterile media was used to set the spectrophotometer to zero. Cuvettes were filled to a 1 ml volume either with whole culture or diluted 10-fold in sterile media. Graphs were plotted to identify stages of growth from lag phase, exponential growth and stationary phase, most experiments thereafter were performed within exponential phase when populations were simultaneously growing.

### 2.2 Bacterial strain collections

Bacterial strains from all species used throughout this study are listed in table 2.1. Where a gene has been knocked out/in or mutated a selection marker was integrated which is reflected within the genotype. Genes and the resistance they convey are as follows: *bla* (Ampicillin), *cat*

(Chloramphenicol), *kan/Neo* (Kanamycin), *erm* (Erythromycin), *spec/aadA* (Spectinomycin), *apr* (Apramycin) and *zeo* (Zeocin). Antibiotic selection concentrations are in section 2.4.

Table 2.1. Bacterial strains used throughout this study *B. subtilis* (white), *E. coli* (blue) and *S. elongatus* (green).

| Strain name | Genotype                                                                                                        | Homologous recombination matrix <sup>1</sup> | Construction, reference/source            |
|-------------|-----------------------------------------------------------------------------------------------------------------|----------------------------------------------|-------------------------------------------|
| 168 CA      | <i>trpC2</i>                                                                                                    | -                                            | Laboratory strain                         |
| sAG1331     | <i>trpC2 minCD::(ΔminC minD+ Ωkan)</i>                                                                          | -                                            | A Guyet                                   |
| sAG1333     | <i>trpC2 aprE::P<sub>pcn</sub>-lacI-cat</i>                                                                     | -                                            | A Guyet                                   |
| sAG1338     | <i>trpC2 aprE:: P<sub>pcn</sub> -lacI-cat<br/>minCD::(ΔminC minD+ Ωkan)<br/>amyE::WALP-Spec</i>                 | -                                            | A Guyet                                   |
| sAG1392     | <i>trpC2 aprE:: P<sub>pcn</sub> -lacI-cat<br/>amyE::WALP-Spec</i>                                               | -                                            | A Guyet                                   |
| sGG104      | <i>trpC2 aprE:: P<sub>pcn</sub> -lacI-cat<br/>minCD::(ΔminC minD+ Ωkan)<br/>amyE::RepN-iteron mutant</i>        | pGGNIM                                       | This work                                 |
| sGG106      | <i>trpC2 aprE::lacI-Km</i>                                                                                      | pAPNC-Km                                     | This work                                 |
| HM1099      | <i>dnaAG152S::cat</i>                                                                                           | -                                            | H. Murray                                 |
| HM1337      | <i>dnaAG152S::cat amyE::neo(P<sub>hbs</sub>-hbs-gfp)</i>                                                        | -                                            | H. Murray                                 |
| sGG107      | <i>trpC2 aprE::lacI-Km dnaA::G152S-cat</i>                                                                      | pAPNC-Km;<br>gDNA HM1099                     | This work                                 |
| sGG121      | <i>trpC2 aprE:: P<sub>pcn</sub> -lacI-cat<br/>minCD::(ΔminC minD+ Ωkan)<br/>amyE::RepN-iteron mutant pGGP01</i> | pGGNIM                                       | This work                                 |
| sGG122      | <i>trpC2 aprE:: P<sub>pcn</sub> -lacI-cat<br/>minCD::(ΔminC minD+ Ωkan)<br/>amyE::RepN-iteron mutant pGGP02</i> | pGGNIM                                       | This work                                 |
| HM722       | <i>trpC2 amyE::cat(P<sub>hu</sub>-hu-mCherry)</i>                                                               | -                                            | H. Murray                                 |
| HM1376      | <i>trpC2 dnaAG152S::zeo</i>                                                                                     | -                                            | H. Murray                                 |
| sGG108      | <i>trpC2 amyE::cat(P<sub>hu</sub>-hu-mCherry)<br/>aprE::lacI-Kan</i>                                            | pAPNC-kan,<br>HM722 gDNA                     | This work                                 |
| sGG109      | <i>trpC2 aprE::lacI-Kan dnaAG152S::zeo</i>                                                                      | pAPNC-kan,<br>HM1376 gDNA                    | This work                                 |
| sGG110      | <i>trpC2 amyE::cat(P<sub>hu</sub>-hu-mCherry)<br/>aprE::lacI-Kan dnaAG152S::zeo</i>                             | pAPNC-kan,<br>HM722 gDNA,<br>HM1376          | This work                                 |
| sGG111      | <i>trpC2 amyE::P<sub>pcn</sub>-lacI-spec</i>                                                                    | pAPNC213                                     | This work                                 |
| sGG112      | <i>trpC2 pGGR02</i>                                                                                             | -                                            | This work                                 |
| 4743        | <i>168ca ΩamyE::(Ωcat::neo) hbsU-mCherry</i>                                                                    | -                                            | Dr Ling Juan Wu (Wu <i>et al.</i> , 2020) |
| sGG113      | <i>trpC2 aprE::P<sub>pcn</sub>-lacI-spec, amyE::neo-hbsU-mCherry integration</i>                                | pAPNC-spec;<br>gDNA 187C<br>(Gifted L. Wu)   | This work                                 |

|                      |                                                                                                                  |                                                                               |                                         |
|----------------------|------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------|-----------------------------------------|
| sGG114               | <i>trpC2 aprE::Ppcn-lacI-spec, dnaAG152S-cat</i>                                                                 | pAPNC-spec;<br>gDNA HM1099                                                    | This work                               |
| sGG115               | <i>trpC2 aprE::Ppcn-lacI-spec, amyE::neo-hbsU-mCherry, integration dnaAG152S::cat</i>                            | pAPNC-spec;<br>gDNA HM1099,<br>187C (Gifted L. Wu)                            | This work                               |
| sGG137               | <i>trpC2 aprE::Ppcn-lacI-spec, amyE::neo-hbsU-mCherry, integration dnaAG152S::cat dnaAPhe265Leu pGG013</i>       | -                                                                             | This work                               |
|                      |                                                                                                                  |                                                                               |                                         |
| <b>Strain name</b>   | <b>Genotype</b>                                                                                                  | <b>Homologous recombination matrix</b>                                        | <b>Construction or reference source</b> |
| JM109                | <i>endA1, recA1, gyrA96, thi, hsdR17 (rk-, mk+), relA1, supE44, Δ(lac-proAB), [F' traD36, proAB, lacIqZΔM15]</i> | -                                                                             | New England Biosciences                 |
| DH5α                 | <i>fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>                   | -                                                                             | New England Biosciences                 |
| MG1655               | <i>F-, lambda-, rph-1</i>                                                                                        | -                                                                             | Laboratory stock strain                 |
| XL1-blue             | <i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIqZΔM15 Tn10 (Tetr)]</i>                        | -                                                                             | Agilent                                 |
| TB28                 | <i>MG1655 ΔlacZYA &lt; &gt;frt</i>                                                                               | -                                                                             | Laboratory stock strain                 |
| BW2511               | <i>F- DE(araD-araB)567 lacZ4787(del)::rrnB-3 LAM- rph-1 DE(rhaD-rhaB)568 hsdR514</i>                             | -                                                                             | Baba <i>et al.</i> , 2006               |
| BW2511 Δ <i>minC</i> | <i>F- DE(araD-araB)567 lacZ4787(del)::rrnB-3 LAM- rph-1 DE(rhaD-rhaB)568 hsdR514 ΔminC</i>                       | -                                                                             | Baba <i>et al.</i> , 2006               |
| HL713                | <i>MG1655 ΔintS::lacIQ-Km</i>                                                                                    | -                                                                             | Han Lim (Hussein , 2011)                |
| sGG204               | <i>MG1655 ΔlacZYA &lt; &gt;frt ΔminC-km</i>                                                                      | BW2511 Δ <i>minC</i> locus PCR product                                        | This work                               |
| sGG205               | <i>MG1655 ΔlacZYA &lt; &gt;frt ΔminC-&lt; &gt;frt</i>                                                            | BW2511 Δ <i>minC</i> locus PCR product, markerless via FLT recombinase        | This work                               |
| sGG206               | <i>MG1655 ΔlacZYA &lt; &gt;frt ΔminC-&lt; &gt;frt ΔintS::lacIQ-Kan</i>                                           | BW2511 Δ <i>minC</i> locus PCR product, markerless via FLT recombinase, HL713 | This work                               |

|                     |                                                                                                                                                                               |                                                    |                                         |
|---------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------|-----------------------------------------|
|                     |                                                                                                                                                                               | $\Delta$ intS::lacIq<br>PCR product                |                                         |
| sGG209              | <i>MG1655 <math>\Delta</math>lacZYA &lt; &gt;frt <math>\Delta</math>minC-&lt; &gt;frt <math>\Delta</math>intS::lacIq-Kan pGGS01</i>                                           | -                                                  | This work                               |
| sGG210              | <i>MG1655 <math>\Delta</math>lacZYA &lt; &gt;frt <math>\Delta</math>minC-&lt; &gt;frt <math>\Delta</math>intS::lacIq-Kan pGGR01</i>                                           | -                                                  | This work                               |
| sAJ015              | <i>MG1655 <math>\Delta</math>lacZYA &lt; &gt;frt <math>\Delta</math>pspG-qorA::lacIq-repA-cat</i>                                                                             | -                                                  | A. Johns                                |
| sAJ016              | <i>MG1655 <math>\Delta</math>lacZYA &lt; &gt;frt <math>\Delta</math>pspG-qorA::lacIq-repA-cat <math>\Delta</math>minC-km</i>                                                  | BW2511 $\Delta$ minC<br>locus PCR<br>product       | A. Johns                                |
| sAJ024              | <i>MG1655 <math>\Delta</math>lacZYA &lt; &gt;frt <math>\Delta</math>pspG-qorA::lacIq-repA-cat pAJ053</i>                                                                      | -                                                  | A. Johns                                |
| sAJ025              | <i>MG1655 <math>\Delta</math>lacZYA &lt; &gt;frt <math>\Delta</math>pspG-qorA::lacIq-repA-cat <math>\Delta</math>minC-km pAJ053</i>                                           | BW2511 $\Delta$ minC<br>locus PCR<br>product       | A. Johns                                |
| sAG1562             | <i>MG1655 <math>\Delta</math>lacZYA &lt; &gt;frt <math>\Delta</math>pspG-qorA::lacIq-repA-cat pAG-P35</i>                                                                     | -                                                  | A. Guyet                                |
| sAG1563             | <i>MG1655 <math>\Delta</math>lacZYA &lt; &gt;frt <math>\Delta</math>pspG-qorA::lacIq-repA-cat pAG-P36</i>                                                                     | -                                                  | A. Guyet                                |
| sGG217              | <i>MG1655 <math>\Delta</math>lacZYA &lt; &gt;frt <math>\Delta</math>pspG-qorA::lacIq-repA-cat <math>\Delta</math>minC-km pAG-P35</i>                                          | BW2511 $\Delta$ minC<br>locus PCR<br>product       | This work                               |
| sGG218              | <i>MG1655 <math>\Delta</math>lacZYA &lt; &gt;frt <math>\Delta</math>pspG-qorA::lacIq-repA-cat <math>\Delta</math>minC-km pAG-P36</i>                                          | BW2511 $\Delta$ minC<br>locus PCR<br>product       | This work                               |
| sGG219              | <i>MG1655 <math>\Delta</math>lacZYA &lt; &gt;frt <math>\Delta</math>pspG-qorA::lacIq-repA-cat pGG017</i>                                                                      |                                                    | This work                               |
| sGG220              | <i>MG1655 <math>\Delta</math>lacZYA &lt; &gt;frt <math>\Delta</math>pspG-qorA::lacIq-repA-cat <math>\Delta</math>minC-km pGG017</i>                                           | -                                                  | This work                               |
| sGG221              | <i>MG1655 <math>\Delta</math>intS::lacIQ-Km pGGR04</i>                                                                                                                        |                                                    | This work                               |
| sGG222              | <i>MG1655 <math>\Delta</math>lacZYA &lt; &gt;frt <math>\Delta</math>minC-&lt; &gt;frt <math>\Delta</math>intS::lacIq-Kan pGGR04</i>                                           | -                                                  | This work                               |
| sGG223              | <i>MG1655 <math>\Delta</math>lacZYA &lt; &gt;frt <math>\Delta</math>minC-&lt; &gt;frt <math>\Delta</math>intS::lacIq-Kan <math>\Delta</math>arsB::PtetO-asRNA-spec</i>        | pGG011 PCR<br>product                              | This work                               |
| sGG226              | <i>MG1655 <math>\Delta</math>lacZYA &lt; &gt;frt <math>\Delta</math>minC-&lt; &gt;frt <math>\Delta</math>intS::lacIq-Kan <math>\Delta</math>arsB::PtetO-asRNA-spec pGGS01</i> |                                                    | This work                               |
| sGG227              | <i>MG1655 <math>\Delta</math>lacZYA &lt; &gt;frt <math>\Delta</math>minC-&lt; &gt;frt <math>\Delta</math>intS::lacIq-Kan <math>\Delta</math>arsB::PtetO-asRNA-spec pGGR01</i> |                                                    | This work                               |
|                     |                                                                                                                                                                               |                                                    |                                         |
| <b>Strain name</b>  | <b>Genotype</b>                                                                                                                                                               | <b>Homologous recombination matrix<sup>1</sup></b> | <b>Construction or reference source</b> |
| <i>S. elongatus</i> | Wild type                                                                                                                                                                     | -                                                  | Gifted from Luning Liu                  |

<sup>1</sup> Homologous recombination matrix is the plasmid/PCR product used to homologously recombine genes into the chromosome.

|                              |                                          |        |                                              |
|------------------------------|------------------------------------------|--------|----------------------------------------------|
| PCC 7942                     |                                          |        | laboratory collection                        |
| <i>S. elongatus</i> CurT-GFP | Wild type native <i>curT-gfp</i>         | -      | Gifted from Luning Liu laboratory collection |
| sGG301                       | $\Delta$ <i>minC-aadA</i> Transformant 2 | pGGCA1 | This work                                    |
| sGG302                       | $\Delta$ <i>minC-aadA</i> Transformant 3 | pGGCA1 | This work                                    |
| sGG303                       | $\Delta$ <i>minC-aadA</i> Transformant 4 | pGGCA1 | This work                                    |

Plasmids used within this study are shown in table 2.2 with colour coding to reference which bacterial host the plasmid was intended to be transformed within. The plasmid genotype is shown which indicates origin of replication(s), promoters, reporters and antibiotic resistance cassettes.

Table 2.2. Plasmid constructs used within this study including their transformation host and construction sources.

| Plasmid                   | Genotype                                                                 | Transformation host                              | Construction or reference source  | Comment                                                                                                    |
|---------------------------|--------------------------------------------------------------------------|--------------------------------------------------|-----------------------------------|------------------------------------------------------------------------------------------------------------|
| <b><i>B. subtilis</i></b> |                                                                          |                                                  |                                   |                                                                                                            |
| pAG-P08                   | <i>oriLS20 oriBR322 P<sub>spac</sub>-gfp alp7AR erm bla</i>              | sAG1333, sAG1338, sGG106, sGG107, sGG113, sGG115 | A. Guyet (unpublished)            | pLS20 minimal replicon with <i>gfp</i> reporter (fig.3.3A).                                                |
| pGG08Z                    | <i>oriLS20 oriBR322 P<sub>spac</sub>-gfp'(15 bp)-lacZ alp7AR erm bla</i> | sAG1333, sAG1338, sGG106, sGG107, sGG113, sGG115 | This work                         | pLS20 minimal replicon with <i>lacZ</i> reporter (fig3.3B).                                                |
| pDR111                    | <i>oriBR322 bla amyE::P<sub>hyspank</sub>-MCS spec</i>                   | <i>B. subtilis</i>                               | Ben-Yehuda <i>et al.</i> , 2003   | Vector for genomic integration at the <i>amyE</i> locus in <i>B. subtilis</i> and IPTG inducible promoter. |
| pSHP1                     | <i>oriBR322 bla amyE::P<sub>xyl</sub>-mNeonGreen spec</i>                | <i>B. subtilis</i>                               | Stijn Henrik Peters (unpublished) | Vector for genomic integration at the <i>amyE</i> locus, <i>P<sub>xyl</sub></i> and                        |

|           |                                                                                               |                    |           |                                                                                                                                                                   |
|-----------|-----------------------------------------------------------------------------------------------|--------------------|-----------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|           |                                                                                               |                    |           | mNeonGreen protein.                                                                                                                                               |
| pGGNIM    | <i>oriBR322 bla amyE::P<sub>xyI</sub>-repN-iteron mutant spec</i>                             | sAG1338            | This work | pSHP1 backbone with mNeonGreen replaced with designed RepN iteron mutant (chapter 4).                                                                             |
| pGG08Z011 | <i>oriLS20 oriBR322 P<sub>spac</sub>-gfp'(15 bp)-lacZ alp7AR erm bla - no mutations found</i> | XL1-red, sAG1338   | This work | Plasmids found from XL1 red cloning. pGG08Z backbone and random mutagenesis attempted. However, no mutations were found when sequenced (described in appendix 6). |
| pGG08Z012 | <i>oriLS20 oriBR322 P<sub>spac</sub>-gfp'(15 bp)-lacZ alp7AR erm bla - no mutations found</i> | XL1-red, sAG1338   | This work |                                                                                                                                                                   |
| pGG08Z013 | <i>oriLS20 oriBR322 P<sub>spac</sub>-gfp'(15 bp)-lacZ alp7AR erm bla - no mutations found</i> | XL1-red, sAG1338   | This work |                                                                                                                                                                   |
| pGG08Z014 | <i>oriLS20 oriBR322 P<sub>spac</sub>-gfp'(15 bp)-lacZ alp7AR erm bla - no mutations found</i> | XL1-red, sAG1338   | This work |                                                                                                                                                                   |
| pGG08Z02  | <i>oriLS20 oriBR322 P<sub>spac</sub>-gfp'(15 bp)-lacZ alp7AR erm bla - no mutations found</i> | XL1-red, sAG1338   | This work |                                                                                                                                                                   |
| pGG08Z021 | <i>oriLS20 oriBR322 P<sub>spac</sub>-gfp'(15 bp)-lacZ alp7AR erm bla - no mutations found</i> | XL1-red, sAG1338   | This work |                                                                                                                                                                   |
| pGG08Z022 | <i>oriLS20 oriBR322 P<sub>spac</sub>-gfp'(15 bp)-lacZ alp7AR erm bla - no mutations found</i> | XL1-red, sAG1338   | This work |                                                                                                                                                                   |
| pGG08Z023 | <i>oriLS20 oriBR322 P<sub>spac</sub>-gfp'(15 bp)-lacZ alp7AR erm bla - no mutations found</i> | XL1-red, sAG1338   | This work |                                                                                                                                                                   |
| pGGZ32    | pGG08Z backbone with <i>oriLS20</i> swapped with <i>pLS32 repN/oriN</i> mini replicon.        | <i>B. subtilis</i> | This work | pLS20 <i>ori</i> replaced with the <i>repN/oriN</i> replicon native to pLS32 (fig.4.4C).                                                                          |
| pGGP01    | <i>pGGZ32 Δ683 bp upstream of iteron sequence 'oriLS32</i>                                    | sGG104             | This work | pGGZ32 backbone with <i>repN/oriN</i> truncation to remove the promoter and protein                                                                               |

|                          |                                                                                                                    |                    |                                  |                                                                                                                                                                                                 |
|--------------------------|--------------------------------------------------------------------------------------------------------------------|--------------------|----------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                          |                                                                                                                    |                    |                                  | sequence upstream of identified iteron sequences (fig.4.4C).                                                                                                                                    |
| pGGP02                   | <i>pGGP02</i> $\Delta$ 683 bp upstream of iteron sequence, $\Delta$ 147 bp downstream of iteron sequence 'oriLS32' | sGG104             | This work                        | pGGP01 backbone with protein sequence downstream of iteron sequences truncated (fig.4.4C).                                                                                                      |
| pAPNC213                 | <i>oriBR322 bla aprE::P<sub>pcn</sub>-lacI spec</i>                                                                | <i>B. subtilis</i> | Gifted from Errington Laboratory | Vectors for genomic integration at the <i>aprE</i> locus in <i>B. subtilis</i> with varying antibiotic resistance cassettes for selection. Used to integrate the LacI gene into the chromosome. |
| pAPNC-Cat                | <i>oriBR322 bla aprE::P<sub>pcn</sub>-lacI cat</i>                                                                 | <i>B. subtilis</i> | Gifted from Errington Laboratory |                                                                                                                                                                                                 |
| pAPNC-Km                 | <i>oriBR322 bla aprE::P<sub>pcn</sub>-lacI kan</i>                                                                 | <i>B. subtilis</i> | Gifted from Errington Laboratory |                                                                                                                                                                                                 |
| pAPNC-Erm                | <i>oriBR322 bla aprE::P<sub>pcn</sub>-lacI erm</i>                                                                 | <i>B. subtilis</i> | Gifted from Errington Laboratory |                                                                                                                                                                                                 |
| pMUTIN4                  | <i>oriColE1 bla amyE::P<sub>spac</sub>-MCS lacZ P<sub>pcn</sub>-lacI erm</i>                                       | <i>B. subtilis</i> | Vagner <i>et al.</i> , 1998      | Plasmid used to amplify <i>lacZ</i> .                                                                                                                                                           |
| pGGN32                   | <i>oriLS32 oriBR322 P<sub>spac</sub>-gfp alp7AR erm bla</i>                                                        | sAG1333, sAG1338   | This work                        | pGGZ32 backbone with <i>gfp</i> reporter replacing <i>lacZ</i> .                                                                                                                                |
| pGGAZ1                   | <i>oriLS20 oriBR322 P<sub>spac</sub>-lacZ alp7AR erm bla</i>                                                       | sAG1333, sAG1338   | This work                        | pGG08Z backbone with <i>lacZ</i> truncation to test both start atg start codon sites.                                                                                                           |
| pAG-P08 $\Delta$ oriLS20 | <i>oriBR322 P<sub>spac</sub>-gfp alp7AR erm bla</i>                                                                | <i>B. subtilis</i> | This work                        | pAG-P08 backbone with pLS20 <i>ori</i> deleted and plasmid religated, used in appendix 8.                                                                                                       |
| pGGR01                   | <i>oriLS20 oriRSF1010 P<sub>spac</sub>-gfp alp7AR erm bla</i>                                                      | <i>B. subtilis</i> | This work                        | Plasmid map demonstrated in fig.5.1A. Used in appendix 8.                                                                                                                                       |

|         |                                                                                                                           |                                           |           |                                                                                                                                                |
|---------|---------------------------------------------------------------------------------------------------------------------------|-------------------------------------------|-----------|------------------------------------------------------------------------------------------------------------------------------------------------|
| pGGR02  | <i>oriRSF1010 P<sub>spac</sub>-gfp<br/>alp7AR erm bla</i>                                                                 | <i>B. subtilis</i>                        | This work | pGGR01<br>backbone with<br>pLS20 <i>ori</i><br>deletion, used<br>in appendix 8.                                                                |
| pGG013Z | <i>oriLS20:cluster1<br/>oriBR322 P<sub>spac</sub>-<br/>gfp'(15 bp)-lacZ<br/>alp7AR erm bla</i>                            | sAG1333,<br>sAG1338,<br>sGG113,<br>sGG115 | This work | pGG08Z<br>backbone with<br>pLS20 origin<br>random<br>mutations.<br>Sequenced and<br>identified<br>mutations<br>demonstrated<br>in fig.4.11B-E. |
| pGG014Z | <i>oriLS20:cluster1(last<br/>base) oriBR322 P<sub>spac</sub>-<br/>gfp'(15 bp)-lacZ<br/>alp7AR erm bla</i>                 | sAG1333,<br>sAG1338,<br>sGG113,<br>sGG115 | This work |                                                                                                                                                |
| pGG015Z | <i>oriLS20:cluster1(firs<br/>t base) oriBR322<br/>P<sub>spac</sub>-gfp'(15 bp)-lacZ<br/>alp7AR erm bla</i>                | sAG1333,<br>sAG1338,<br>sGG113,<br>sGG115 | This work |                                                                                                                                                |
| pGG016Z | <i>oriLS20:cluster1(firs<br/>t and last base)<br/>oriBR322 P<sub>spac</sub>-<br/>gfp'(15 bp)-lacZ<br/>alp7AR erm bla</i>  | sAG1333,<br>sAG1338,<br>sGG113,<br>sGG115 | This work |                                                                                                                                                |
| pGGC21  | <i>oriLS20:cluster2(3<br/>bases) oriBR33 P<sub>spac</sub>-<br/>gfp'(15 bp)-lacZ<br/>alp7AR erm bla</i>                    | sAG1338                                   | This work | pAG-P08<br>backbone with<br>pLS20 origin<br>random<br>mutations.<br>Sequenced and<br>identified<br>mutations<br>demonstrated<br>in fig.4.10.   |
| pGGC22  | <i>oriLS20:cluster2(firs<br/>t and last bases)<br/>oriBR322 P<sub>spac</sub>-<br/>gfp'(15 bp)-lacZ<br/>alp7AR erm bla</i> | sAG1338                                   | This work |                                                                                                                                                |
| pGG013  | <i>oriLS20:cluster1<br/>oriBR322 P<sub>spac</sub>-gfp<br/>alp7AR erm bla</i>                                              | sAG1333,<br>sAG1338,<br>sGG113,<br>sGG115 | This work |                                                                                                                                                |
| pGG014  | <i>oriLS20:cluster1(last<br/>base) oriBR322 P<sub>spac</sub>-<br/>gfp alp7AR erm bla</i>                                  | sAG1333,<br>sAG1338,<br>sGG113,<br>sGG115 | This work |                                                                                                                                                |
| pGG015  | <i>oriLS20:cluster1(firs<br/>t base) oriBR322<br/>P<sub>spac</sub>-gfp alp7AR erm<br/>bla</i>                             | sAG1333,<br>sAG1338,<br>sGG113,<br>sGG115 | This work |                                                                                                                                                |
| pGG016  | <i>oriLS20:cluster1(firs<br/>t and last base)<br/>oriBR322 P<sub>spac</sub>-gfp<br/>alp7AR erm bla</i>                    | sAG1333,<br>sAG1338,<br>sGG113,<br>sGG115 | This work |                                                                                                                                                |
| pAM011  | <i>oriLS20 oriBR322<br/>P<sub>spac</sub>-spa alp7AR erm<br/>bla</i>                                                       | sAG1333,<br>sAG1338,<br>sGG113,<br>sGG115 | This work | pAG-P08<br>backbone with<br><i>gfp</i> reporter<br>replaced with<br>protein A<br>coding<br>sequence ( <i>spa</i> )                             |



|                       |                                                                             |                                           |                                                                |                                                                                       |
|-----------------------|-----------------------------------------------------------------------------|-------------------------------------------|----------------------------------------------------------------|---------------------------------------------------------------------------------------|
|                       |                                                                             |                                           |                                                                | from <i>Staphylococcus aureus</i> (plasmid map, fig.3.25).                            |
| pGG018                | <i>oriLS20ΔdnaAbox<br/>oriBR322 P<sub>spac</sub>-gfp<br/>alp7AR erm bla</i> | sAG1333,<br>sAG1338,<br>sGG113,<br>sGG115 | This work                                                      | pAG-P08 backbone, with <i>dnaA</i> box mutation demonstrated in fig.3.22B.            |
|                       |                                                                             |                                           |                                                                |                                                                                       |
| <b><i>E. coli</i></b> | <b>Genotype</b>                                                             | <b>Transformation host</b>                | <b>Construction or reference source</b>                        | <b>Comments</b>                                                                       |
| pSIM5                 | <i>oriSC101 gam-exo-bet cat</i>                                             | DH5α                                      | Datta <i>et al.</i> , 2006                                     | Plasmid used to clone SC101 origin.                                                   |
| pRL1383a              | <i>oriRSF1010 spec</i>                                                      | DH5α                                      | Peter Wolk (Addgene plasmid # 70692) Wolk <i>et al.</i> , 2007 | Plasmid used to clone RSF1010 origin.                                                 |
| pKD46                 | <i>oriR101 repA101ts<br/>araBp-gam-bet-exo bla</i>                          | MG1655/TB28                               | Datsenko and Wanner, 2000                                      | Lambda red recombinase expression plasmid, used for recombineering (chapter 5).       |
| pGGS01                | <i>oriLS20 oriSC101<br/>P<sub>spac</sub>-gfp alp7AR erm bla</i>             | sGG206                                    | This work                                                      | Plasmid map demonstrated in fig.5.1A.                                                 |
| pGGR01                | <i>oriLS20 oriRSF1010<br/>P<sub>spac</sub>-gfp alp7AR erm bla</i>           | sGG206                                    | This work                                                      | Plasmid map demonstrated in fig.5.1A.                                                 |
| pCP20                 | <i>ori repA101<br/>repA101ts lambdats<br/>FLP bla cat</i>                   | BW25113                                   | Baba <i>et al.</i> , 2006                                      | Plasmid with FLP recombinase to allow antibiotic cassette excision between FRT sites. |
| pUC19                 | <i>oriColE1 MCS bla</i>                                                     | DH5α                                      | Norrandar <i>et al.</i> , 1983 (acquired from NEB)             | <i>E. coli</i> cloning vector.                                                        |
| pUC19::spec           | <i>oriColE1 MCS spec</i>                                                    | sGG206                                    | This work                                                      | pUC19 backbone with ampicillin cassette replaced with spectinomycin                   |

|         |                                                               |              |                                                           |                                                                                                                               |
|---------|---------------------------------------------------------------|--------------|-----------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------|
|         |                                                               |              |                                                           | resistance cassette.                                                                                                          |
| pGGAS1  | <i>pUC19 P<sub>tet</sub>-asRNA</i>                            | sGG206       | This work                                                 | pUC19 vector with designed asRNA and <i>P<sub>tet</sub></i> promoter cloned (illustrated in fig.5.12).                        |
| pGGAS2  | <i>pUC19::spc P<sub>tet</sub>-asRNA</i>                       | sGG206       | This work                                                 | pUC19::spec vector with designed asRNA and <i>P<sub>tet</sub></i> promoter cloned (illustrated in fig.5.12).                  |
| pAJ045  | <i>oriP Kan</i>                                               | sAJ015/016   | A Johns (unpublished)                                     | Minimal universal replicon, demonstrated in appendix 10.                                                                      |
| pAJ053  | <i>oriP P<sub>spac</sub>-gfp erm</i>                          | sAJ015/016   | A Johns (unpublished)                                     |                                                                                                                               |
| pAG-P30 | <i>oriP P<sub>spac</sub>-gfp-laa (ssrA tagged) erm</i>        | sAJ015/016   | A Johns/A Guyet (unpublished)                             |                                                                                                                               |
| pAG-P35 | <i>oriP P<sub>spac</sub>-gfp alp7AR erm</i>                   | sAJ015/016   | A Johns/A Guyet (unpublished)                             |                                                                                                                               |
| pAG-P36 | <i>oriP P<sub>xyI</sub>-gfp alp7AR erm</i>                    | sAJ015/016   | A Johns/ A Guyet (unpublished)                            |                                                                                                                               |
| pGG007  | <i>pUC19::'arsR-arsB-arsC'</i>                                | sGG206/HL713 | This work                                                 | Integration vectors constructed to allow asRNA integration into the chromosome using Lambda red recombineering (chapter 5.4). |
| pGG011  | <i>pUC19 'arsR-ΔarsB::P<sub>tet</sub>-asRNA-spec-arsC'</i>    | sGG206/HL713 | This work                                                 |                                                                                                                               |
| pAJ060  | <i>oriP P<sub>spac</sub>-gfp ywjL::105bp ram sites erm</i>    | sAJ015/016   | A Johns (unpublished)                                     | Minimal universal replicon, demonstrated in appendix 10.                                                                      |
| pGG017  | <i>pBAD322::P<sub>bad</sub>-DivIVA/RacA</i>                   | sAJ015/016   | Derived from pBAD322-DivIVA-msfGFP gifted by H Strahl lab |                                                                                                                               |
| pGGR04  | <i>oriLS20 oriRSF1010 P<sub>spac</sub>-spa alp7AR erm bla</i> | sGG206/HL713 | This work                                                 | pGGR01 backbone with <i>gfp</i> reporter replaced with protein A                                                              |

|                            |                                   |                                |                                                                       |                                                                                |
|----------------------------|-----------------------------------|--------------------------------|-----------------------------------------------------------------------|--------------------------------------------------------------------------------|
|                            |                                   |                                |                                                                       | coding sequence ( <i>spa</i> ). Used in chapter 5.5.                           |
|                            |                                   |                                |                                                                       |                                                                                |
| <b><i>S. elongatus</i></b> | <b>Genotype</b>                   | <b>Transformation host</b>     | <b>Construction or reference source</b>                               | <b>Comments</b>                                                                |
| pGGCA1                     | <i>pUC19 minC::aadA</i>           | <i>S. elongatus</i> WT         | This work                                                             | Integration vector designed to disrupt <i>minC</i> gene, plasmid map fig.6.1A. |
| pAM1573                    | <i>oriBR322 bla NSII::MCS cat</i> | <i>S. elongatus</i> WT, sGG301 | Susan Golden (Addgene plasmids #40239) Andersson <i>et al.</i> , 2000 | Neutral site II integration vector.                                            |
| pGG005                     | <i>pAM1573 NSII::lacI</i>         | <i>S. elongatus</i> WT, sGG301 | This work                                                             | pAM1573 backbone with <i>lacI</i> integration. Illustrated in fig.6.14.        |

## **2.3 General methods of DNA amplification and plasmid construction**

### **2.3.1 Oligonucleotide construction**

Oligonucleotides used to amplify DNA were designed using Snapgene software to produce complimentary primers for the sequence to be amplified (Insightful Science; @ snapgene.com). Custom designed primers were synthesised by Eurogentec (Kaneka Corporation). Stocks were diluted to a working concentration of 10 micromolar ( $\mu\text{M}$ ) prepared in MiliQ water which were stored at  $-20^{\circ}\text{C}$  and used at a final concentration of 0.3-1  $\mu\text{M}$  (per primer) for amplification (section 2.3.2). All oligonucleotides used in this study are listed in appendices 3.

### **2.3.2 Polymerase chain reaction for cloning**

For the amplification of DNA, polymerase chain reactions (PCR) were carried out, mainly using NEB Q5 DNA polymerase unless otherwise stated following the manufacturers instruction. The outlined thermal cycling conditions were used: initial denaturation 30 seconds  $98^{\circ}\text{C}$ , 30 cycles of - 30 second denaturation at  $98^{\circ}\text{C}$ , 30 second annealing at  $55-62^{\circ}\text{C}$  and 30 seconds per kilobase(s) (kb) extension at  $72^{\circ}\text{C}$  ending with a  $72^{\circ}\text{C}$  final extension of 2 minutes.

### **2.3.3 Gel electrophoresis of DNA**

A 0.8 % agarose gel was made by dissolving agarose powder (Melford) with 1x Tris-Acetate-EDTA (TAE - 2 Molar (M) Tris/HCl pH 8.3, 5.7% acetate, 50 millimolar (mM) sodium acetate) buffer. After heating to dissolve and cooling, any dyes were added to appropriate volumes before a comb was inserted into the tray to make wells and the molten agarose was poured. After cooling to a gel, the tray was submerged into 1x TAE buffer before applying DNA mixed with loading buffer (Phenol red in 50% glycerol) to wells.

To stain the DNA for imaging using illumination in the Biorad Gel Doc there were 2 methods used. The first was SYBR Gold Nucleic Acid gel stain applied directly to the DNA in loading buffer before loading into the gel. The second, and most commonly used method was to add Nancy-520 at a final concentration of 0.12 µg/ml (Sigma) directly into molten 0.8% agarose before casting in the gel tray (agarose + 1x TAE). To migrate the DNA an electric current was run at 100 Volts (V) for between 45 minutes – 1 hour before detection using the Biorad Gel doc to image migration patterns using blue light.

#### **2.3.4 DNA fragment clean up**

To clean small fragments of DNA, Qiagen clean up kits were used. Following the manufacturers instructions, buffers from the Qiagen PCR purification kit were used to clean DNA after PCR and restriction digests with QIAquick column centrifugal filters.

Cleaning of specific bands was carried out using the Qiagen Gel extraction kit with specific buffers and protocols provided. DNA products were run on an agarose gel dissolved in TAE before specific band(s) were excised. Following the manufacturer protocols, excised gels were weighed and dissolved in buffer QG, incubating at 50°C until dissolved. Isopropanol was added before applying to QIAquick column centrifugal filters, washing in the supplied buffers to purify DNA before elution in nuclease free water.

#### **2.3.5 Megaprimer PCR of Whole Plasmid (MEGAWHOP)**

For insertion of new genetic material into plasmids, MEGAWHOP was used by means of PCR to make a megaprimer and integrate into the plasmid described by Miyazaki (2011). Oligonucleotides were designed *in silico*, with 5' homology to the target vector for annealing, using SnapGene software (Insightful Science) (Oligonucleotide sequences in appendices 3). The oligonucleotides were used at 1 µM in a 50 µl PCR using Q5 High-Fidelity DNA polymerase (NEB) to produce the insert with regions of homology. The PCR product then

served as a megaprimer to target the recipient vector (fig.2.1). For this PCR products were excised from an agarose gel (1x TAE, 0.8% agarose, loading dye, and 1 µl SYBR gold) and purified using the QIAquick Gel Extraction Kit, eluting at 30 µl for greater DNA concentrations. A 250-fold molar excess of megaprimer to target plasmid was used for the secondary PCR reaction where the megaprimer annealed to the target vector. PrimeStar DNA polymerase (Takara) for long templates was used for MEGAWHOP assembly following the manufactures instructions. A subsequent DpnI digest was used to degrade the DNA adenine methylated (Dam) template DNA, using 10 units and a 37°C incubation for 1 hour. A control was performed without DpnI digestion to measure parental plasmid background (fig.2.1). 5 µl of the resulting digests were used to transform DH5α and spread onto selective media.

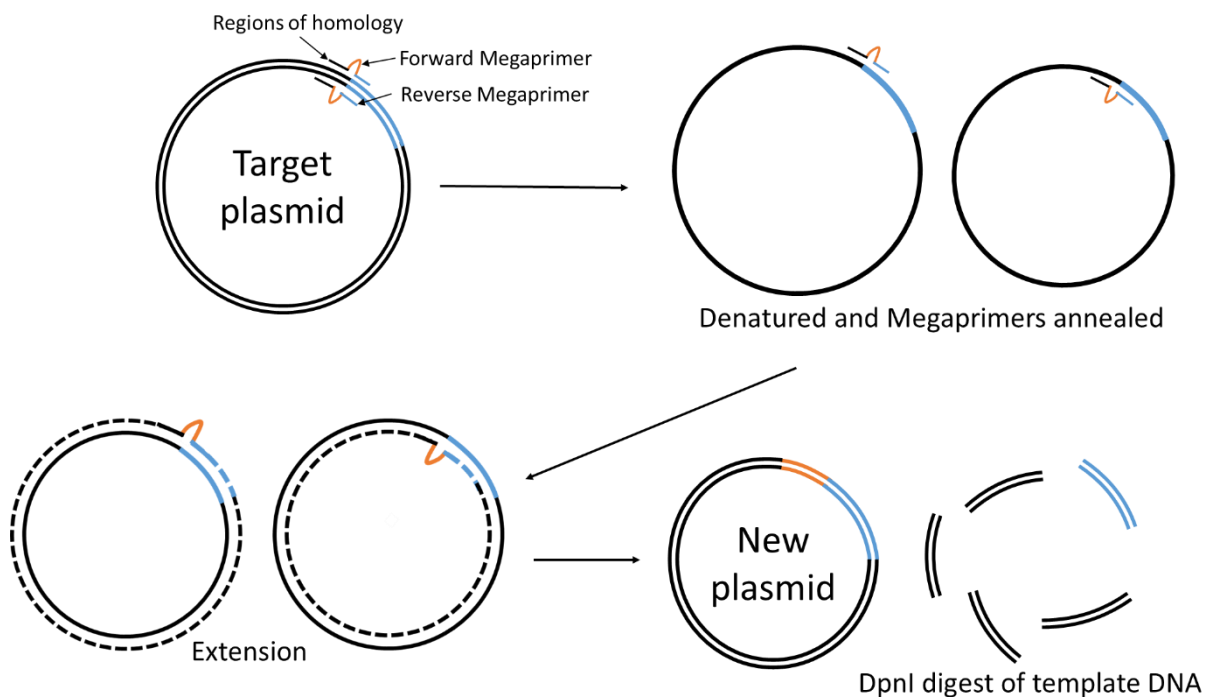


**Figure 2.1. MEGAWHOP workflow.** Megaprimers are amplified with homologous arms to anneal to the target vector. During the PCR reaction, the plasmid is synthesised throughout extension including the new insert region (yellow). The parental DNA is degraded via DpnI digestion to prevent transformation contamination. (Miyazaki, 2011).

### 2.3.6 NEBuilder HiFi DNA assembly

Vector and inserts with homologous ends were amplified using PCR described in section 2.3.2, depicted in fig.2.2A & B. If there were more than one fragment for insertion, then multiple PCR's would be performed with homologous arms demonstrated in fig.2.2B. DNA amplified from a plasmid template were treated with 10-units DpnI (NEB) at 37°C to degrade methylated DNA and prevent parental plasmid contamination. The NEBuilder HiFi master mix (2x) was diluted by half in a 20 µl reaction with the cleaned vector and inserts to a ratio of 1:2 respectively, adding nuclease free water to volume. Mixtures were incubated at 50°C for 60 minutes before transformation into *E. coli* using 10-20 µl of mixture in 50 µl of chemically competent cells described in section 2.6.2.

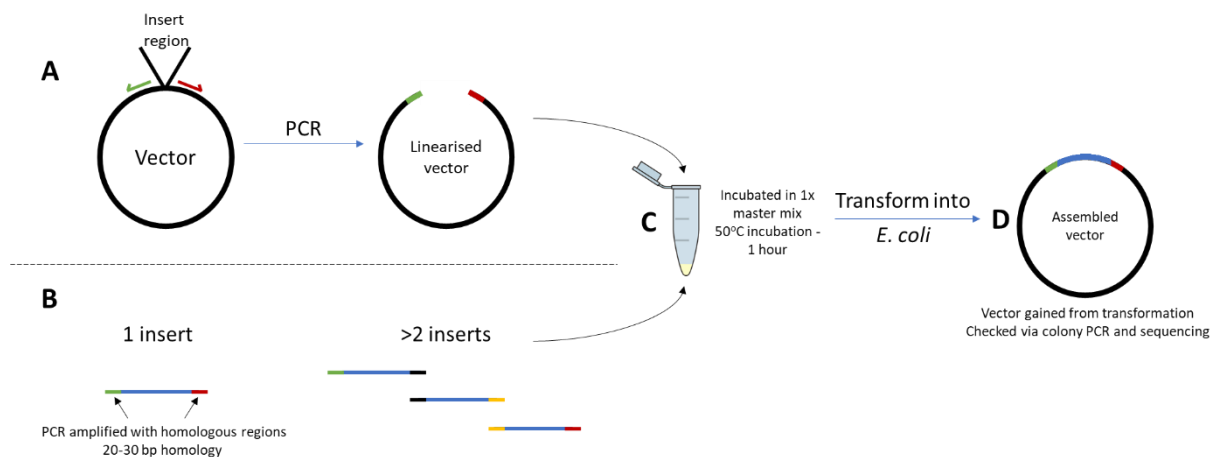


**Figure 2.2. A demonstration of HiFi assembly reactions for cloning new vectors.** **A)** PCR amplification of the vector around the site where the insert will be integrated. **B)** Insert amplification with homologous arms (red and green) to the vector and a depiction of when more than 1 insert was amplified with homology to each fragment (yellow and black). **C)** depiction of the master mix and primers being mixed before 50°C incubation for 1 hour. **D)** The resulting assembled vector with the insert integrated into the site with the homology (red and green arms).

Clker.com – Eppendorf image

### 2.3.7 Restriction enzyme and ligation cloning

The gene of interest to be cloned would be amplified where oligonucleotides were designed to make PCR fragments containing restriction enzyme cut sites complementary to the vector in which to be cloned. Restriction sites are palindromic sequences recognised and cut by restriction enzymes to reveal either blunt or sticky ends for ligation. Digestion of DNA was attained following the manufacturer's instructions using 10 units of enzyme per reaction in the preferred buffer (1x), digests were incubated at 37°C (unless otherwise stated) for 1-3 hours. Digested vectors were dephosphorylated using thermosensitive shrimp alkaline phosphatase (TSAP – Promega) added directly to the digested DNA and incubated for 1 hour, while incubating at 37°C. Reactions were stopped via heat inactivation at 65°C for 20 minutes.

For assembly of cleaned fragments, T4 DNA ligase (NEB) was used in 20 µl reaction overnight at 16°C, insert to vector ratio was always 2:1. Ligations were held at 16°C for ~16 hours and 5-10 µl was transformed into 50 µl chemically competent *E. coli* (section 2.6.2).

### 2.3.8 Colony PCR

For colony PCR, Taq polymerase (Promega) was used according to manufacturer's instruction using the following thermal cycling conditions: initial denaturation 2 minutes 95°C, 25-30 cycles of - 30 second denaturation at 95°C, 30 second annealing at 42-65°C and 1 minutes per kb extension at 72°C, a 72°C final extension was held for 2 minutes. After reaction mixtures were made, a single colony was resuspended direct into the PCR buffer for *E. coli* and *S. elongatus* were suspended directly into the reaction mix from 1 µl of liquid culture. For *B. subtilis* a single colony was suspended in 300 µl 50% Dimethyl sulfoxide (DMSO) and heated at 65°C for 20 minutes before applying 1 µl to the reaction mix.



## 2.4 Media supplements

To select for transformants or induce expression supplements were added to the media (solid or liquid). Concentrations are described in table 2.3 and show individual applications for each species.

Table 2.3. Demonstration of antibiotic, substrate and inducer final concentrations used in liquid and solid media throughout the study.

| Antibiotics                                                     | <i>B. subtilis</i><br>concentrations | <i>E. coli</i><br>concentrations | <i>S. elongatus</i><br>concentrations |
|-----------------------------------------------------------------|--------------------------------------|----------------------------------|---------------------------------------|
| <b>Ampicillin</b>                                               | -                                    | 100 µg/ml                        | -                                     |
| <b>Chloramphenicol</b>                                          | 5 µg/ml                              | 10 µg/ml                         | 5 µg/ml                               |
| <b>Spectinomycin</b>                                            | 60 µg/ml                             | 50 µg/ml                         | 50-80 µg/ml                           |
| <b>Erythromycin</b>                                             | 0.5-1 µg/ml                          | 100 µg/ml                        | -                                     |
| <b>Kanamycin</b>                                                | 5 µg/ml                              | 25 µg/ml                         | -                                     |
| <b>Zeocin</b>                                                   | 10 µg/ml                             | -                                | -                                     |
| Substrates &<br>inducers                                        |                                      |                                  |                                       |
| <b>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)</b> | 40 µg/ml                             | 80 µg/ml                         | -                                     |
| <b>Isopropylthio-β-galactoside (IPTG)</b>                       | 1 mM                                 | 1 mM                             | 1 mM                                  |
| <b>Xylose</b>                                                   | 0.5%                                 | 0.5%                             | 0.5%                                  |

## 2.5 DNA extraction

### 2.5.1 Genomic DNA extraction.

Crude preparations of genomic DNA (gDNA) were performed for uses in transformation of *B. subtilis* from other *Bacillus* spp. Firstly, the strain was cultured in LB at 37°C until turbid before harvesting using centrifugation and removing supernatant. The pellet was resuspended in 1 ml 1x saline sodium citrate (SSC) buffer and 200 µg/ml lysozyme (Sigma) followed by 20-minute incubation at 37°C. After incubation 500 µl 4 Molar NaCl was added before filter sterilisation

using a 0.45  $\mu\text{m}$  syringe filter. These preparations were stored at  $-20^{\circ}\text{C}$  and only used for transformation purposes.

For clean gDNA preparations used in PCR and transformations, cultures were grown overnight in LB at  $30^{\circ}\text{C}$ . The Sigma GenElute kit was used following protocols provided. Cells were lysed in buffers specifically stated for Gram positive or Gram negative bacteria. Lysates were treated with ethanol before loading onto GenElute Miniprep Binding Columns, the DNA bound to the column was washed and finally eluted in nuclease free water.

### **2.5.2 Plasmid extraction.**

Plasmid extractions from *E. coli* and *B. subtilis* were performed using the Qiagen Miniprep kit according to the manufacturer's instructions using the specified buffers and centrifugal columns. Cultures were grown overnight in rich media (LB) before harvesting the next day via centrifugation (13,000  $\times g$  3 minutes) and disposing the supernatant. The *B. subtilis* pellets required the addition of lysozyme (Sigma) at 1 milligrams per millilitre (mg/ml) final concentration into buffer P1 with a 10-minute incubation step at  $37^{\circ}\text{C}$ .

## **2.6 Transformation and genetic manipulation techniques**

### **2.6.1 *B. subtilis* transformation.**

Methods by Anagnostopoulos and Spizizen were used for transformation which were updated by Hamoen *et al.* where *B. subtilis* were grown in media with limited nutrition. After starvation DNA (plasmid or chromosomal) was added to competent cells and incubated for 1 hour before spreading onto selective media and plates were incubated overnight at  $37^{\circ}\text{C}$  (Anagnostopoulos and Spizizen 1961; Hamoen *et al.*, 2002)

### **2.6.2 *E. coli* chemical transformation.**

Chemically competent *E. coli* (DH5 $\alpha$ , JM109) were made using methods described by Hanahan *et al.* with rubidium chloride and heat shock transformations were performed using methods described by Bergmans *et al.* (Bergmans *et al.*, 1981; Hanahan *et al.*, 1991). Transformations were selected for by growth on selective nutrient agar overnight at 37°C.

Where unique strains were to be transformed, *E. coli* made competent via calcium chloride preparation were grown to an OD600 of 0.6 in LB media. Cultures were divided and incubated on ice for 15 minutes before centrifugation in benchtop centrifuge at 9000 rpm for 5 minutes. Supernatant was discarded and pellets resuspended in ice-cold 0.1 M CaCl<sub>2</sub> followed by incubation on ice for 15 minutes. Tubes were spun again at 9000 rpm for 8 minutes and pellets resuspended 0.1 M CaCl<sub>2</sub> 15% glycerol solution. These cultures were then used for heat shock transformation with ~100 ng plasmid.

### **2.6.3 *E. coli* electrocompetent transformation and recombineering.**

Recombineering of the *E. coli* chromosome was done according to published work by Datsenko and Wanner (2000). To make electrocompetent cells of the recipient *E. coli* strain with pKD46 plasmid, an overnight culture was firstly made in LB and grown overnight at 30°C. Next day, the cultures were diluted into fresh sterile LB to an optical density of 0.06 and returned to 30°C. After 1 hour of growth, to induce the  $\lambda$  Red recombinase system phage from pKD46, 20% sterile arabinose was added during early exponential growth at a final concentration of 0.02%. Cultures were grown to an OD600 of 0.7 and harvested via centrifugation at 4°C for 10 minutes. Supernatant was removed and pellets resuspended in chilled sterile distilled water with 2 more centrifugation and wash steps before resuspending the final pellet in 300  $\mu$ l chilled sterile water. To electroporate, the electrocompetent cells were mixed with ~1  $\mu$ g PCR amplified DNA in a 2 mm Biorad MicroPulser Cuvette which was inserted into the Biorad GenePulse II using 2.5

kV, 200 mA, 25  $\mu$ F. The cuvette was returned to ice and 900  $\mu$ l LB added before transferring to 1.5 ml tube and incubating at 30°C for 1 hour 30 minutes outgrowth. Transformants were then spread onto selective media incubated at 30°C overnight and single colonies picked would be screened for integration and grown at 42°C to lose the temperature sensitive pKD46.

The knockout strain for *ΔminC* deletion was obtained from the Keio collection (Baba *et al.*, 2006) and the locus was PCR amplified using oligonucleotides oGG022/023 (Q5 NEB) and cleaned before mixing with MG1655 electrocompetent cells. To knock in the *lacI<sup>q</sup>* gene, the *intS::lacI<sup>q</sup>-kan* locus was PCR amplified using oligonucleotides oGG068/069 from *E. coli* strain HL713 (provided by Han Lim (Addgene plasmid # 52931)). Both PCR products included ~300 bp homology either side of the gene to be disrupted and were used for  $\lambda$  red recombineering of sGG205 electrocompetent cells.

#### 2.6.4 *S. elongatus* transformation.

To construct the *minC* gene interruption in *S. elongatus*, the pGGCA1 plasmid was constructed in accordance to instructions by Clerico *et al.* (2007). Three PCR fragments were assembled into the pUC19 backbone using the NEBuilder HiFi DNA assembly kit (see section 2.3.6). Two ~350 bp sized homologous arms were incorporated from upstream and downstream of *minC*, amplified from *S. elongatus* gDNA. The gene disrupting *minC* was the *aadA* spectinomycin cassette, fragments were assembled into the plasmid by methods shown in fig.2.2B.

*S. elongatus* was transformed via growth in BG11 media, cells were harvested at an OD750 of 0.7 using centrifugation (6000 xg 10 minutes) and discarding supernatant. Pellets were washed in 10 mM sterile NaCl and centrifuged again. Pellets were resuspended in 300  $\mu$ l of fresh BG11 media and 100 ng plasmid was incubated overnight at 30°C with shaking, tubes were wrapped in aluminium foil to shield from light (Clerico *et al.*, 2007). After overnight incubation

transformation mixtures were spread onto selective solid BG11 (0.4% agarose) where half were incubated with natural daylight and half were incubated with constant light. Single colonies were picked, and antibiotic passage was performed by back diluting cultures in increasing concentrations of Spectinomycin until a full knockout was identified using colony PCR.

## **2.7 Protein analyses methods**

### **2.7.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses.**

To analyse proteins from total lysates, cultures grown from mid- to late-exponential phase were harvested via centrifugation (13,000 xg, 3 minutes) before processing cell pellets in SDS loading buffer (Laemmli SDS loading dye – recipe in appendices 2). After resuspending the pellet, the cells were lysed via sonication for 1 minute before storing on ice. The lysates were heated to 85°C for 10 minutes before running 20 µl in NuPAGE 4-12% Bis-Tris midi protein gel (Invitrogen) using a benchmark prestained ladder (Abcam). The gels were run in a XCell SureLock midi gel tank using 1x SDS MES running buffer (Invitrogen), 150 V currents were run until the loading buffer had run off the end of the gel.

### **2.7.2 Western blotting**

After running the SDS PAGE the proteins were transferred to 0.45 µm PVDF membrane (Amersham) activated via 100% methanol soaking for 1 minute. Using the Biorad Trans-Blot Turbo transfer system, stacks were made with tissue paper containing the PVDF membrane and SDS-PAGE. ~200 ml transfer buffer was used to soak the stack components (300 mM Tris, 300 mM Glycine, 140 mM Tricine, 0.05% SDS, 2.5 mM EDTA). The Biorad Trans-Blot was run with 5V for 30 minutes to transfer proteins to the membrane.

After transfer according to the manufacturer's guidance, the membrane was blocked in blocking buffer (5% skimmed milk, phosphate buffered saline (PBS) solution) for 1 hour at room temperature with agitation, the blocking buffer was removed before adding blocking buffer + primary antibody ( $\alpha$ -PBP2B 1:5000,  $\alpha$ -LacI 1:2000,  $\alpha$ -Protein A 1:2000). Incubation was done at 4°C overnight with gentle rocking. Next day, the membrane was washed 3 times with PBS with rocking for 10 minutes between each disposal and addition of PBS. The membrane was blocked again in blocking buffer for 30 minutes at room temperature with agitation. The secondary antibody (HRP) was then applied (1:5000  $\alpha$ -rabbit and  $\alpha$ -mouse) for 1 hour at room temperature with rocking, as described previous. Before detection the membrane was washed in PBS 3 times before applying Western blotting substrate by Pierce ECL Plus in accordance to manufacturers instructions. The membrane was developed using GE Healthcare ImageQuant LAS 4000 mini, taking images at increments of 1-minute intervals for 20 minutes.

### **2.7.3 Coomassie staining**

To stain the gel directly and see total protein contents of lysates, after running the SDS-PAGE the gel was removed from the cassette and incubated with Expedeon Instant blue dye overnight with agitation. Images were taken via Epson perfection V800 scanner.

## **2.8 Microscopy techniques**

### **2.8.1 Fluorescence microscopy**

Microscopy images were acquired using 1  $\mu$ l cultures applied onto 1.2% agarose pads (agarose + water) and drying before applying cover slip and immersion oil. Images were acquired using Nikon Ti eclipse and fluorescence were excited using either the Lambda LS xenon-arc light source or CoolLED illumination light source with adjustable intensity. Microscopes were connected with Sony Cool-Snap HQ2 CCD camera (Roper scientific) using Metamorph 6

imaging software (Universal imaging) to control image acquisition. Brightfield images were taken with 150 ms exposure. Staining was applied in the following concentrations: DAPI 1  $\mu\text{g/ml}$  and FM5-95 0.2  $\mu\text{g/ml}$  added directly to the culture or agarose pad respectively. All images were analysed using ImageJ (Schneider *et al.*, 2012).

For Z-stack imaging at different focal planes of cells, cultures were mounted to 1.2% agarose pads in GeneFrames (Thermofisher). Cell spots were allowed to dry before applying the cover slip. The cells were focussed on the centre of the cell before using the Nikon Ti to image 2.11  $\mu\text{m}$  through cells by taking 22 images at 0.1  $\mu\text{m}$  steps. Images were acquired by Metamorph 6 imaging software and processed using ImageJ.

### **2.8.2 Cell and fluorescence counting**

When analysing fluorescence of rod-shaped cells, the line width was set to 7 (pixels width) and a line drawn from pole to pole, sometimes using membrane staining to show the septum. The ‘measure’ function was then used to analyse the fluorescence signal. This data was then transferred to GraphPad prism 9 to allow graphs to be plotted. Minicells were counted using the analyse particle function, selecting for minicells specifically by setting ‘roundness’ to 0.89-1.00 and using the “circled” minicells to assess fluorescence.

### **2.8.3 Time-lapse microscopy**

Time-lapse microscopy slides were prepared using a GeneFrame (Thermofisher) where 200  $\mu\text{l}$  of agarose with nutrients were applied (recipes in appendices 1). 2  $\mu\text{l}$  of cell cultures were applied to the agarose pad and allowed to dry before applying to top cover slip. Immersion oil was added in excess and imaged using Nikon Ti with CoolLED illumination light source. Images were taken every 6 minutes at 5-8 different stage positions of the slide. Images were acquired using Metamorph 6 imaging software (Universal imaging).

### 2.8.4 Transmission electron microscopy

Transmission electron microscopy imaging was done using the Electron Microscopy Research Services (EMRS) in all species. The strains were grown and fixed in glutaraldehyde in house but epoxy resin embedding was performed by the electron microscopy facility based in Newcastle university by either Tracey Davey or Ross Laws.

1 ml of cultures were harvested during mid-exponential grown via centrifugation, cells were fixed in 1 ml of 2% glutaraldehyde, 0.1 M sodium cacodylate buffer and stored overnight at 4°C. The electron microscopy facility staff would process the cells firstly via washing in 0.1 M sodium cacodylate. A secondary fixation step was performed in 1% osmium tetroxide for 1 hour before dehydration in varying concentrations of acetone (25, 50, 75 then 100%). Cells were saturated with increasing resin concentrations before the final incubation at 60°C, a microtome was used to cut ultrathin sections of 70 nm. These ultrathin sections were stained within pioloform-filmed copper grids using 2% aqueous uranyl acetate and 3% lead citrate using a Leica EM AC20. Imaging took place on a Hitachi HT7800 120kV TEM with an EMSIS CMOS Xarosa camera system.

### 2.8.5 Immunostaining

To localise protein A in rod cells and minicells, *B. subtilis* strains were grown in CDM and *E. coli* strains were grown in M9 media. Overnight cultures were grown at 37°C. The cultures were diluted back into fresh competence media with 1 µg/ml erythromycin to the same optical density (OD<sub>600</sub> ~0.05) and incubated at 30°C for up to 4 hours. Cells were fixed during mid-exponential growth by mixing equal volumes of culture and 1% paraformaldehyde. The culture and paraformaldehyde mixtures were held on ice for 30 minutes. Cells were washed in PBS 3 times via centrifugation and pellet resuspension. Finally, fixed pellets were resuspended in GTE buffer (50 mM glucose, 25 mM Tris, 10 mM EDTA – pH 8.0) using the optical density



to determine the volume of buffer to resuspend pellets, therefore obtaining the same density of cells in each sample.

On a Multi-Spot Shandon 12 well microscope slide the cells suspended in GTE buffer were spotted in 20 µl volumes and allowed to stand for 5 minutes, excess solution was aspirated, and the slide allowed to dry. 0.01% poly-L-lysine was then applied and left for 2 minutes, excess liquid was pipetted off then wells allowed to dry. For *B. subtilis* treatment with lysozyme was not required, however, for *E. coli* staining the culture spots were treated with 10 mg/ml lysozyme (Sigma) dissolved in GTE for 1 minute before washing wells in PBS then allowing wells to dry. Culture spots were rehydrated in PBS before blocking in PBS with 2% Bovine serum albumin (BSA). The primary antibodies ( $\alpha$ -Protein A (Sigma)) were diluted 1:250 ratio in PBS with 2% BSA, applied to cell spots and incubated overnight at 4°C. Next day, cell spots were washed 10 times with PBS and the secondary antibody (anti-rabbit FITC (sigma)) was applied in PBS with 2% BSA with a 1:250 dilution. Cell spots were incubated with the secondary antibody for 1 hour in the dark, a 10x PBS washing step followed. 2 µl of VectaShield antifade mounting medium was added to each well before applying the coverslip for imaging.

### 2.8.6 Fluorescence *in situ* hybridisation (FISH) in Cyanobacteria

Methods were carried out as described by Watanabe *et al.*, 2018.

**Probe preparation.** Probe templates were made via NEB Q5 polymerase amplification of the origin and terminus regions. To span large areas, three sets of primers were used for each *ori* and *ter* to amplify ~13 kb for each area. Primers used for amplification of the origin and terminus segments for fluorescence *in situ* hybridisation. Primers were designed and documented by Watanabe *et al.*, 2018. Probes were made fluorescent via random primed DNA

labelling kit (Roche) with Fluoresceine-12-uridine 5'-triphosphate (UTP) (Sigma) both in accordance with the manufacturer's instructions.

Ethanol precipitation was performed to remove non-incorporated substrate, first adding sodium acetate to the DNA to a concentration of 1/10<sup>th</sup> of the sample volume. Pre-chilled ethanol (100%) was added to 2.5 times the volume of DNA solution and the sample was kept at -20°C overnight. Next day, the samples were spun at 14,000 xg for 15 minutes at 4°C, supernatant was removed and 200 µl of cold 70% ethanol added. After resuspending the DNA, the tubes were centrifuged again at 14,000xg for 10 minutes (4°C). Supernatant was removed, and ethanol was allowed to evaporate off at 37°C. Pellets were resuspended in hybridisation buffer (50% formamide, 2x SSC and 100 µg ml<sup>-1</sup> salmon sperm DNA). Probes were firstly sonicated for 2 minutes at 4°C before denaturing by boiling at 100°C for 10 minutes. Probes were then used for hybridisation.

To crudely determine the degree of labelling, the labelled DNA probes were separated via electrophoresis on an agarose TAE gel. The gel was directly screened using a GE Typhoon laser scanning Phosphorimager at: Laser – 488 (blue), Emission – 520 nm, with a resolution of 100 microns.

**FISH.** *S. elongatus* wild-type and  $\Delta minC$  strains were grown in liquid BG11 media from glycerol stocks for 1 week. After this, the cultures were diluted back to an OD<sub>750</sub> of 0.1 in fresh BG11 media and illuminated with constant light for 24 hours. The cells were harvested via centrifugation, and the pellets resuspended in chilled fixation buffer (methanol containing 1% paraformaldehyde and 10% DMSO). Pellets were incubated in the fixation buffer for 5 minutes at -80°C and washed twice in 1x PBS. Before permeabilization, the cells were treated with PBS mixed with 0.05% Triton X-100. For permeabilization the cells were incubated with lysis buffer (0.2 mg/ml lysozyme, 25 mM Tris-HCL (pH7.5), 10 mM EDTA) at 37°C for 30

minutes. To remove any remaining enzyme, the cells were centrifuged and washed twice with 1x PBS.

Poly-L-lysine coated slides were made via 0.01% poly-L-lysine solution (Sigma) which was pipetted directly onto slide wells (Thermo Shandon). The solution was left on slides for 5 minutes before pipetting off excess solution and allowing to dry at room temperature. 10  $\mu$ l of fixed Cyanobacterial cells were applied to slides, spread onto the wells and cell spots dried at room temperature. Wells were washed with sterile deionised water before incubation with pre-chilled 70% ethanol for 5 minutes, the wells were washed with chilled ethanol solutions of increasing concentration for 5 minutes each before drying at room temperature (90% then 100%, respectively).

**Hybridisation and imaging.** To hybridise the probe to the chromosome, cells adhered to glass slides were incubated overnight with the denatured probe described in the probe preparation section. Slides were placed into a hybridisation chamber and incubated overnight at 42°C in a moist box. Post hybridisation washes were firstly done twice with 50% formamide then, to ensure non-specifically binding probes were removed, 1x SSC for 10 minutes each time at 37°C. Subsequent washes in 2x and 4x SSC solutions were carried out at room temperature before the slides were dried. Mounting medium was applied with 0.2  $\mu$ g/ml DAPI (antifade – Vectashield) to each well before the cover slip was applied for imaging. Imaging was acquired using a Nikon Eclipse Ti equipped with cool LED PE-300 white light source and Photometrics prime camera. Images were edited and annotated using ImageJ software.

## 2.9 $\beta$ -galactosidase assay

Parental and minicell/maxicell producing strains with or without plasmids were grown overnight with appropriate antibiotic (to select for plasmid or chromosome integrated cassette). Next morning, cultures were diluted back 1/40 to an OD600 of 0.05 in CDM and incubated for

8-hour at 30°C. The OD600 was read every 30 minutes. At every time point 200 µl was aliquoted for β-galactosidase assay, frozen in liquid nitrogen and stored at -80°C.

For β-galactosidase assays, the 200 µl samples were defrosted in a 30°C water bath. 400 µl lysis solution was added (200 µg/ml lysozyme, 100 µg/ml DNase, 1.25% Triton-X100 (all Sigma)) and incubated for 10 minutes. β-galactosidase activity was determined by adding 200 µl 4 mg/ml 2-Nitrophenyl β-D-galactopyranoside (ONPG- Sigma) and measuring the time started and time finished after solutions turn yellow. After colouration was seen 400 µl stop solution was added (1 M Na<sub>2</sub>CO<sub>3</sub>). Reactions not exhibiting activity were left for 1 hour until stopping. Tubes were centrifuged for 5 minutes (13,000 rpm) and transferred to cuvettes for spectrophotometer readings at an optical density of 420 nm. β-galactosidase activity was calculated in miller units using the following formula: Miller Units = (A<sub>420</sub> x 6 x 1000) / (t<sub>ONPG</sub> reaction time x OD600).

## **2.10 Minicell enrichment in *E. coli***

To enrich for minicells an initial culture was grown in M9 media and incubated overnight at 30°C. The starting culture was then diluted back to an OD600 0.05 in fresh M9 media and returned to 30°C until the required growth phase was reached. To prepare the minicells the total culture was filtered through a 1.2 µm cellulose acetate Whatmann Puradisc 30 syringe filters where the resulting flow through was centrifuged (2500 xg, 10 minutes). The “pellet” of minicells was pipetted by taking 2 ml from the bottom of the tube and transferred to a sterile microfuge tube and mixed. A secondary centrifuge step was performed (8000 xg, 10 minutes) to pellet small cells and remove 1.95 ml supernatant to leave 50 µl of concentrated small cells applied to agarose pads for microscopy analyses or for mixing with PBS to run with flow cytometry.

## 2.11 Flow cytometry

Flow cytometry data was obtained using an analytical fluorescence flow cytometer (BD Symphony) as part of the flow cytometry core facility of Newcastle university. All data was acquired using BD FACS Diva software (BD Biosciences) counting 30,000 events per sample. 50 µl of *B. subtilis* and *E. coli* cultures were suspended in 500 µl filter sterile PBS, whereas 25 µl of *S. elongatus* cultures was mixed with 500 µl filter sterile PBS. All data and figures shown were analysed and gated using FCS Express 7 (De Novo Software).

## 2.12 Plasmid stability assay

### 2.12.1 Spot titre and plasmid stability assays

Spot titre assays were performed from cultures grown in flasks before transferring to a Falcon 96-well plates for dilution. The dilution series will be stated in figure legends, but usually 10-fold or 4-fold, with the media and supplements also stated. To inoculate, 5 µl aliquots were taken for each dilution using a multichannel pipette. These were then applied to appropriate plates which were incubated at 30/37°C (stated in the images or legends) overnight before imaging using Fujifilm FinePix S9500 digital camera to image.

The maxicell stability assay was performed in strains *B. subtilis* 168 and sGG115 with pAG-P08 plasmid. The strains were grown in LB media for 16 hours without antibiotic plasmid selection at 37°C. Cultures were diluted to an OD600 of 0.3 before 10-fold serial dilution and spot titre assays performed on media with and without erythromycin selection for the plasmid. Plates were incubated overnight at 30°C before imaging. Results were determined by comparing the colony forming units (CFU) which grew throughout the dilution series on control (no antibiotic) and selective plates (with antibiotic). Antibiotic selection plates would reveal any instability as those without a plasmid would not survive.

### 2.12.2 Plasmid stability assays in liquid media using CFU/ml

All strains were grown overnight at 37°C in complex defined or M9 media before diluting back to start the assay. For *B. subtilis*, the cultures were incubated at 37°C and *E. coli* were grown at 30°C. At each time point, an aliquot was diluted 10-fold and the 10<sup>-6</sup>-10<sup>-8</sup> dilutions were spread onto nutrient agar with and without selection. Plates were incubated overnight at 37°C and the colonies were counted to calculate CFU/ml. If the assay was every 24 hours, a 1/200 back dilution was done in fresh media before returning to 37°C. Control strains included those without plasmids, grown with and without selection. Colonies on plates were counted and the CFU/ml was calculated and plotted using GraphPad Prism 8, negative control was not shown on logarithmic scale.

## 2.13 Quantitative PCR for plasmid copy number

### 2.13.1 *B. subtilis* genomic DNA preparation

Methods were adapted from those provided by A. Johns (unpublished). Relative qPCR methods were used to determine fold change compared to a reference. In examples used within this study, plasmid copy number was monitored using the chromosome terminus as a reference. Copy number was determined using the assumption that each cell has 1 chromosome. There are limitations associated with this method as an accurate measure of the reference gene is not used for quantification and changes between chromosome and plasmid alone is relied upon. Consequently, changes to chromosome replication due to mutation or growth phase could affect relative qPCR fold changes. To harvest gDNA for qPCR experiments, bacteria were grown in CDM and 1.5 ml samples harvested at specific time-points via centrifugation at 8000 rpm for 10 minutes, discarding supernatant. The pellets were snap-frozen in liquid nitrogen before storage at -80°C. For processing, the pellets were first thawed at 4°C before resuspending in 200 µl lysis buffer (50 mM NaCl, 50 mM Tris, 10 mM EDTA, 1% Triton, 10

mg/ml RNase, 10 mg/ml Lysozyme and cOmplete™ EDTA-free Protease Inhibitor Cocktail, pH 7.0 as this is most suitable for lysozyme activity with 50 mM NaCl). Resuspended pellets were sonicated at 4°C in 10 second pulses for 100 seconds total, lysed samples were centrifuged 13,000 rpm for 5 minutes before collecting supernatant into a clean Eppendorf tube. Before use for qPCR, gDNA was diluted appropriately.

### 2.13.2 QIAgility robot set up

The pipetting was carried out using the Qiagen QIAgility automated pipetting robot with filter tips. The machine was calibrated for tube placement and height before each use to prevent liquid mishandling. The machine would pipette 14 µl of Luna qPCR mastermix with primer and 6 µl of diluted DNA samples. qPCR discs contain 100 25 µl wells, to balance the disc 20 µl of water would be dispensed into unoccupied wells.

### 2.13.3 Primers for qPCR

The primers used were either made in this study or synthesised from sequences provided in published literature. To probe for the chromosome, oligonucleotide sequences were provided by Stepan Fenyk (unpublished – red row in table 2.4) which anneal to the *B. subtilis* terminus. To probe for the plasmid, primers J10749 forward and reverse were synthesised from sequences provided by Lee *et al.*, 2006 which annealed within the ampicillin cassette (blue row in table 2.4). Primers were also designed in this study with the aid of Primer3Plus (<https://primer3plus.com/cgi-bin/dev/primer3plus.cgi>) to probe different areas of the plasmid.

**Table 2.4. Demonstration of oligonucleotide sequences used for qPCR experiments.** Oligonucleotide sequences for the chromosome were provided by Stepan Fenyk (unpublished – red), plasmid probes J10749 were provided by sequences published by Lee *et al.*, 2006, other probes were designed within this study (white rows) to probe different areas of the plasmid.

| Primer name | Sequence | Area probed | Tm | Product size (bp) | Source |
|-------------|----------|-------------|----|-------------------|--------|
|-------------|----------|-------------|----|-------------------|--------|

|                              |                                                        |                         |          |     |                                       |
|------------------------------|--------------------------------------------------------|-------------------------|----------|-----|---------------------------------------|
| qPCR57<br>qPCR58             | TTTGCATGAACTGGGCAA<br>TA<br>TCCGAACATGTCCAATGA<br>GA   | Terminus                | 55<br>53 | 112 | Murray<br>laboratory<br>(unpublished) |
| J10749 F<br>J10749 R         | CTACGATACGGGAGGGCT<br>TA<br>ATAAATCTGGAGCCGGTG<br>AG   | <i>bla</i><br>cassette  | 58<br>56 | 81  | Lee <i>et al.</i> ,<br>2006           |
| oGGalp1<br>oGGalp2           | ATTCCCGTCTCTTCGTTCC<br>TGTTTCTAATACCGAAGC<br>ACC       | <i>alp7A</i><br>operon  | 56<br>55 | 148 | This study                            |
| oGGlacZ<br>1<br>oGGlacZ<br>2 | AACGACATTGGCGTAAGT<br>GAAG<br>ATCAGCACCGCATCAGCA<br>AG | <i>lacZ</i><br>reporter | 58<br>60 | 137 | This study                            |
| oGGlacZ<br>3<br>oGGlacZ<br>4 | AAGCAAAACACCAGCAG<br>CAG<br>ATGACGGAACAGGTATTC<br>GC   | <i>lacZ</i><br>reporter | 59<br>57 | 83  | This study                            |
| oGGbla1<br>oGGbla2           | TTGCTCACCCAGAAACGC<br>TACCGCTGTTGAGATCCA<br>GTTC       | <i>bla</i><br>cassette  | 58<br>59 | 93  | This study                            |

#### 2.13.4 Standards

To perform quantitation of the samples, standards were run on the Qiagen Rotor-Gene Q real-time thermalcycler using a range of gDNA dilutions. The gDNA from the control strain was serially diluted 10-fold from 10x to 1000,000x. The gDNA was pipetted with the appropriate primer pairs and Luna qPCR master mix. Reactions were run on the Qiagen Rotor-Gene Q real-time thermalcycler, and data analysed using the respective Rotor-Gene Q software. Standard curves were generated and exported so they could be used for further quantification of test samples.

#### 2.13.5 qPCR experiments and quantification

To quantify the plasmid, all gDNA samples of control and mutants were diluted 1000x before pipetting 20 µl reaction mixtures using the Qiagility robot liquid handling system. Samples were run for 40 cycles on the Qiagen Rotor-Gene Q thermal cycler and data analysed using the



respective software (Qiagen). The standard curves were imported to give a Ct value which was used for quantification of terminus and plasmid target. The plasmid copy number was calculated as the fold change from terminus (counted as 1 per cell) to plasmid target.

## Chapter 3 - Characterisation of minicell producing *B. subtilis* as a synthetic biology chassis

### 3.1 Introduction

*Bacillus subtilis* is a rod-shaped Gram-positive genus of bacteria which consists of a thick cell wall, for division the rod elongates, segregates duplicated chromosomes before forming a cross wall to form two daughter cells. In industry, *B. subtilis* has been used frequently as it has the ability to secrete directly into the culture media making retrieval of products easier (Chen *et al.*, 2015). The strain 168 CA, used within this study, has a well-developed natural transformation system where exogenous DNA can be taken during certain growth conditions (Harwood, 1992; Anagnostopoulos and Spizizen, 1961).

Microbial bioreactor limitations, such as metabolic burden, are triggered when the host strains resources are diverted to recombinant gene expression which cause reductions in growth and expression levels. As a result, the Portabolomics chassis requires a host lacking chromosomal DNA in order to utilise the cytoplasmic materials while avoiding stress responses and transcriptional changes (see sections 1.5). Using an anucleated host would allow expression of industrial relevant compounds continuously while the growing population are unaffected, increasing yields and population fitness. Minicells have already shown the ability to express from plasmid DNA in both *E. coli* and *B. subtilis* (Shivarkumar *et al.*, 1979). Minicells are not able to divide like parental rod cells because of the lack of expression from genes encoded on the chromosome. However, they do have functional metabolic pathways and have been studied for polar surface composition (Mendelson *et al.*, 1974). Further to this, studies by Mertens and Reeve in 1977, revealed minicells from *B. subtilis* exhibit capabilities to transport amino acids and form cell wall constituents which demonstrated that minicells are biologically active. The authors also showed that minicell biosynthesis was resistant to chloramphenicol treatment, however, these cells were sensitive to high concentrations of penicillin but surprisingly

resistant to penicillin induced lysis (Mertens and Reeve, 1977). The ability to make mRNA and protein from either bacteriophage infection or plasmids made these minicells a prime candidate for biotechnology uses to exploit transcription and translation from DNA templates (Reeve and Cornett 1975; Frazer and Curtiss 1975).

Another form of anucleate bioreactors were considered for the project by exploiting chromosome replication. Replication of the chromosome must be coordinated with the cell cycle to ensure each daughter cell has a chromosome. Historically these larger anucleate compartments were made in DNA repair pathway mutants, DNA was removed by irradiation under UV light or enzymatic degradation (see section 1.3.4; East and Errington, 1989; Fan *et al.*, 2020). The work in this study was unique as the model was to make a strain that could continually produce anucleated cells within a growing culture, therefore, removing the need to process cultures to remove the chromosome. The maxicell would usually be considered an inviable cell as they cannot grow and divide, however, they can harbour plasmids and cytoplasmic machinery which may make them a useful industrial chassis.

Protein A is a cell wall associated protein native to *Staphylococcus aureus* and is composed of multiple domains for flipping over the cytoplasmic membrane, PG attachment and IgG binding (O'Halloran *et al.*, 2015). During translocation over the membrane by general secretion pathways, the signal peptide is cleaved off before sortase A processes the LPETG domain for covalent linkage to the PG (Sibbald *et al.*, 2006; Schneewind *et al.*, 1993; O'Halloran *et al.*, 2015). Some of the first studies of protein A were for the ability to bind to immunoglobulins, the five immunoglobulin binding domains bind to the Fc portion of IgG antibodies or the Fab region of IgM (Deisenhofer, 1981; Goodyear and Silverman, 2003). Protein A is of industrial relevance as it has application in antibody purification, immunoprecipitation assays, and diagnostic biosensors (Rigi *et al.*, 2019). Furthermore, protein A has been used within the pharmaceutical field for purification of monoclonal antibodies with great efforts being

implemented to increase stability throughout industrial purification techniques (reviewed Hober *et al.*, 2007).

## 3.2 Results: minicell compartments of *B. subtilis*

### 3.2.1 Data from the project before starting

Before this PhD project had started, there was already work within the *B. subtilis* Portabolomics chassis. Dr Aurélie Guyet had constructed minicell-producing strains of *B. subtilis* in accordance with the model proposed in chapter 1 (fig.1.10). The strain constructed was shown to work in a proof of principle using a GFP reporter controlled by a  $P_{spac}$  IPTG inducible promoter where minicells could be seen to independently express GFP (green arrows), whereas the parental sAG1333 strain showed negligible fluorescence in the absence of IPTG induction (fig.3.1B). This data was replicated from Aurélie Guyet (unpublished), using the strains available at the beginning of this study.

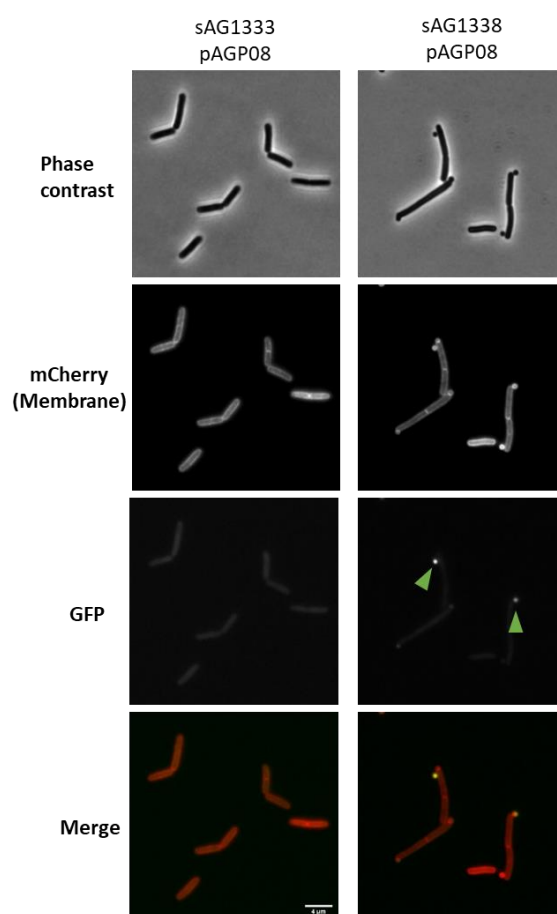


**Figure 3.1. Independently minicell expression of a fluorescent reporter.** Strains were grown in CDM at 30°C with continuous shaking. During mid exponential growth (4 hours) cells were mounted onto a 1.2% agarose pad

(agarose and water) and imaged using a Nikon Eclipse Ti with Cool LED pE-300 white light source and Photometrics Prime camera. Images were taken with use of Metamorph 6 software. Images were processed in ImageJ. Scale bar is representative of 4  $\mu\text{m}$  and is representative for all images.

As *B. subtilis* minicells displayed the ability to express GFP independently from the plasmid in the absence of IPTG induction, the next question was what proportion were able to do so. From microscopy images, populations were segregated into rods and minicells for counting and measuring fluorescence units to see how expression of GFP from the plasmid differed. To calculate the number of cells with GFP fluorescence brighter than parental rod cells, a threshold was set at the end of the normal distribution of rod cells GFP. Therefore, any anucleated cells with pixel intensity greater than this threshold was counted as GFP positive (threshold dotted red line fig.3.2A). It was evident from violin plots that there were more minicells over this threshold than rod cells, which corresponded to what was visualised in microscopy (fig.3.2A). Quantification revealed that 3% of the rod cell population were over the set threshold. However, it was encouraging to see that 48% of minicells were GFP positive. This was a reproducible result, with five repeats including the data shown in fig.3.2 showing that GFP positive minicells remained consistently at ~50%. In each repeat, >100 rod cells and minicells were counted respectively (>100 cells in 3 repeats, >300 cells in 2 repeats).

The percentage of minicells within the population were not able to be counted using microscopy imaging. This is because minicells were not distributed evenly throughout the cell culture spotted onto agarose pads. Minicells were found to be present in large quantities at the edges and therefore introduced bias when counting from different areas of the sample on microscopy slides.

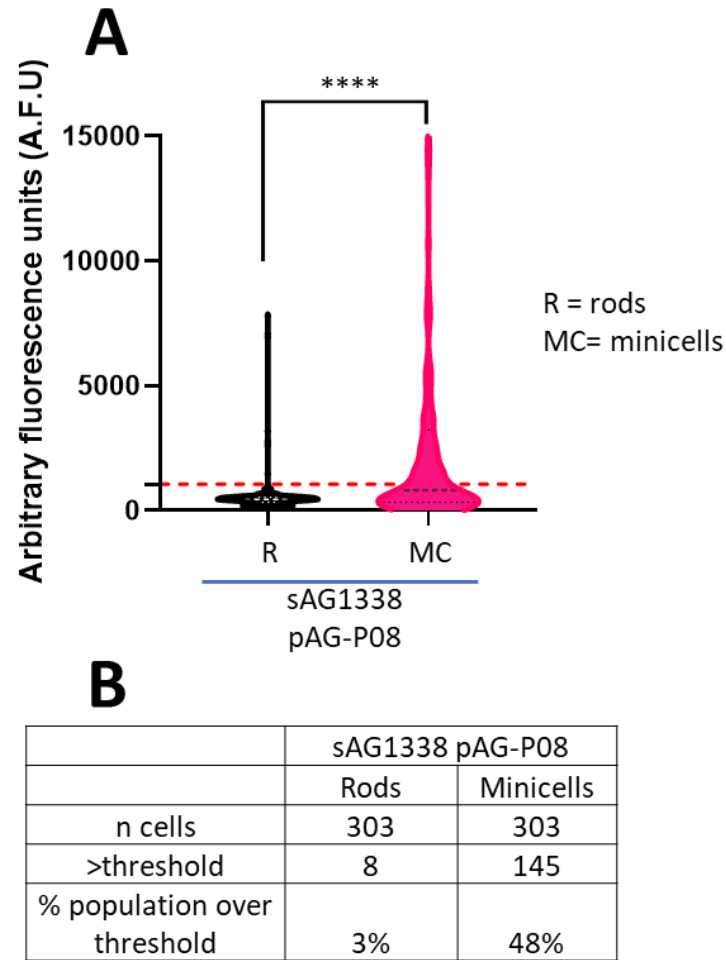


**Figure 3.2. Quantification of rod and minicell expression.** Strains were grown in CDM at 30°C with continuous shaking. During mid exponential growth (4 hours) cells were mounted onto a 1.2% agarose pad (agarose and water) and imaged using a Nikon Eclipse Ti with Cool LED pE-300 white light source, Photometrics Prime camera and images acquired with Metamorph 6 software. Images were quantified using ImageJ and further analysed using GraphPad Prism and Microsoft Excel. **A)** Violin plots of rod and minicell populations fluorescence intensity values. Statistical tests carried out was an unpaired t-test with Welch's correction ( $P < 0.001$ ). **B)** Counting of fluorescence populations.

### 3.2.2 Expression of $\beta$ -galactosidase and activity assays

As both Aurélie Guyet (unpublished) and this study had demonstrated minicell independent expression using a GFP reporter, the assay was repeated with a *lacZ* reporter to quantify expression. The original backbone used for microscopy in *B. subtilis* (pAG-P08; fig.3.3A) was modified to replace the *gfp* gene with *lacZ*, for  $\beta$ -galactosidase expression (pGG08Z fig.3.3B). The  $P_{spac}$  IPTG inducible promoter was maintained for LacI repression in rod cells. The plasmid was transformed into *B. subtilis* mutants such as sAG1331 (fig.3.3C) and sAG1338 (fig.3.3D and E). Transforming into different strains was used to determine if the plasmid was functional and repressed/induced in the correct conditions. It was obvious that in the strain lacking LacI (sAG1331; fig.3.3C), there was a blue colouration without the presence of IPTG as the promoter was not repressed. On the other hand, the strains with LacI showed a significant difference in colouration in the absence and presence of IPTG induction (fig.3.3D and E). In the absence of IPTG (fig.3.3D), bacterial colonies had no blue colouration indicating that *lacZ* expression had been suitably repressed. In contrast, the presence of IPTG in nutrient agar resulted in blue colouration indicating that  $\beta$ -galactosidase was expressed with induction (fig.3.3E). This demonstrated that the system was functional as LacI was able to repress  $\beta$ -galactosidase expression, and with the presence of IPTG expression was permitted.



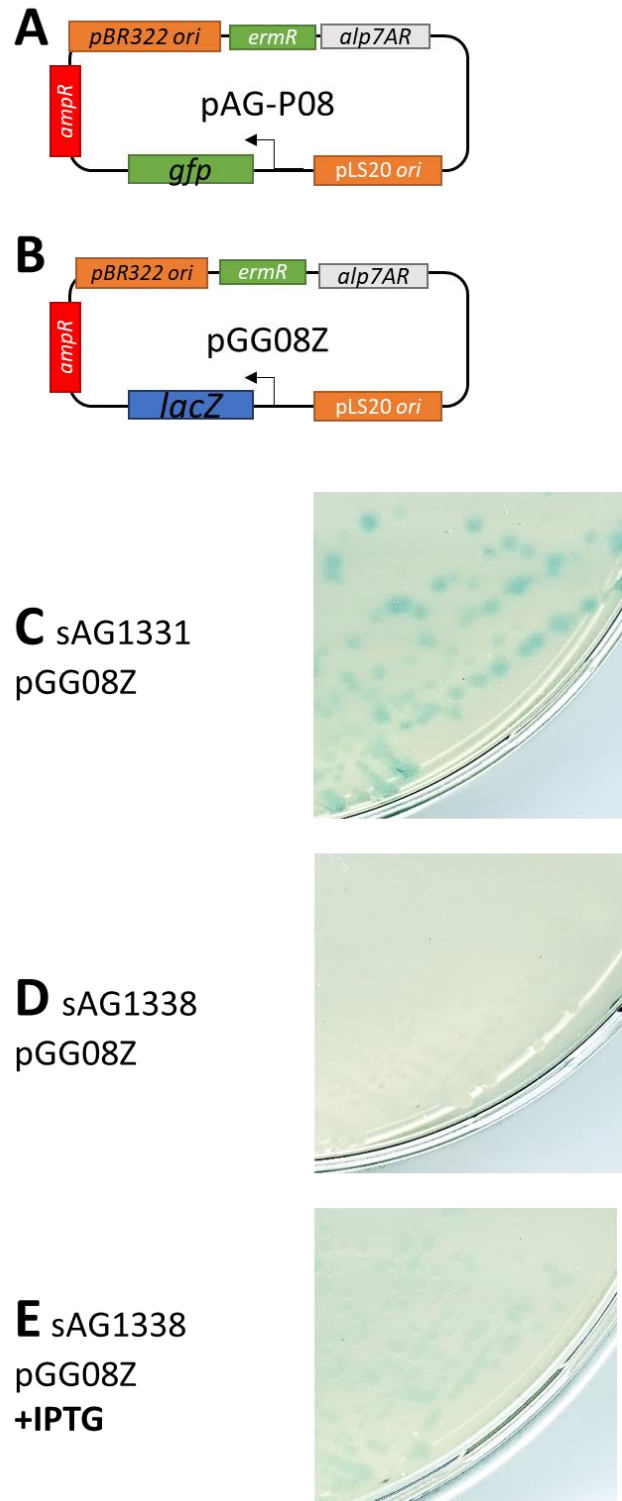


**Figure 3.3. Demonstration of plasmid encoded *lacZ* repression via chromosomal *lacI*.** A-B) plasmids used frequently within the study to demonstrate the genes present, reporters are either **A)** GFP fluorescence or **B)**  $\beta$ -galactosidase. **C)** the cloned  $\beta$ -galactosidase reporter plasmid (pGG08Z) within a *B. subtilis*  $\Delta minC$  strain. **D)**  $\beta$ -galactosidase reporter plasmid (pGG08Z) in a *B. subtilis*  $\Delta minC$  *lacI*<sup>+</sup> background without induction and **E)** the

same strain with 1mM IPTG induction to show blue colourimetric change. All plates were supplemented with 1 ug/ml erythromycin and 40 ug/ml X-gal. Inoculated plates were placed at 30°C for 22 hours.

The expression of  $\beta$ -galactosidase was quantified using activity assays to see if minicell expression was able to be estimated from whole cultures. Strains sAG1333 and sAG1338 with pGG08Z were grown in liquid media and sampled every hour for 6 hours. Optical density was measured at every time point and showed there was no significant difference in growth, regardless of minicell production or IPTG induction, indicating that there was no major disturbances to cell fitness (fig.3.4A). When measuring  $\beta$ -galactosidase activity using ONPG there was a minimal amount of activity from negative control strain sAG1333 with no plasmid, which demonstrated that *B. subtilis* had little background reactivity to ONPG (fig.3.4B - black bars). With the addition of pGG08Z to sAG1333, there was an increase in activity which resulted from leaky expression of *lacZ* from the  $P_{spac}$  promoter (Fig.3.4B magenta bars). By comparison, sAG1338 with pGG08Z presented an increase in  $\beta$ -galactosidase activity hypothesised to be from independent minicell expression (fig.3.4B teal bars). The increase was calculated across all time points in percentage which varied from 10-46% (fig.3.4D), when averaged across all time points it was revealed that sAG1338 pGG08Z had 35% greater  $\beta$ -galactosidase activity. Statistical analyses using a two sample t-test revealed that  $\beta$ -galactosidase expression was significantly greater in sAG1338 at some time points (fig.3.4D). With addition of IPTG (purple bars fig.3.4C), there was a significant increase in activity, which was expected as whole populations with a plasmid were expressing. Each time point, with exception to 240 minutes, showed that sAG1338 (fig.3.4C lilac) exceeded expression of sAG1333 (fig.3.4C dark purple), potentially a result of minicells providing an advantage at the start of growth. This was reproducible across triplicate experiments. Units displayed are miller units plotted in figure.3.4B and C.

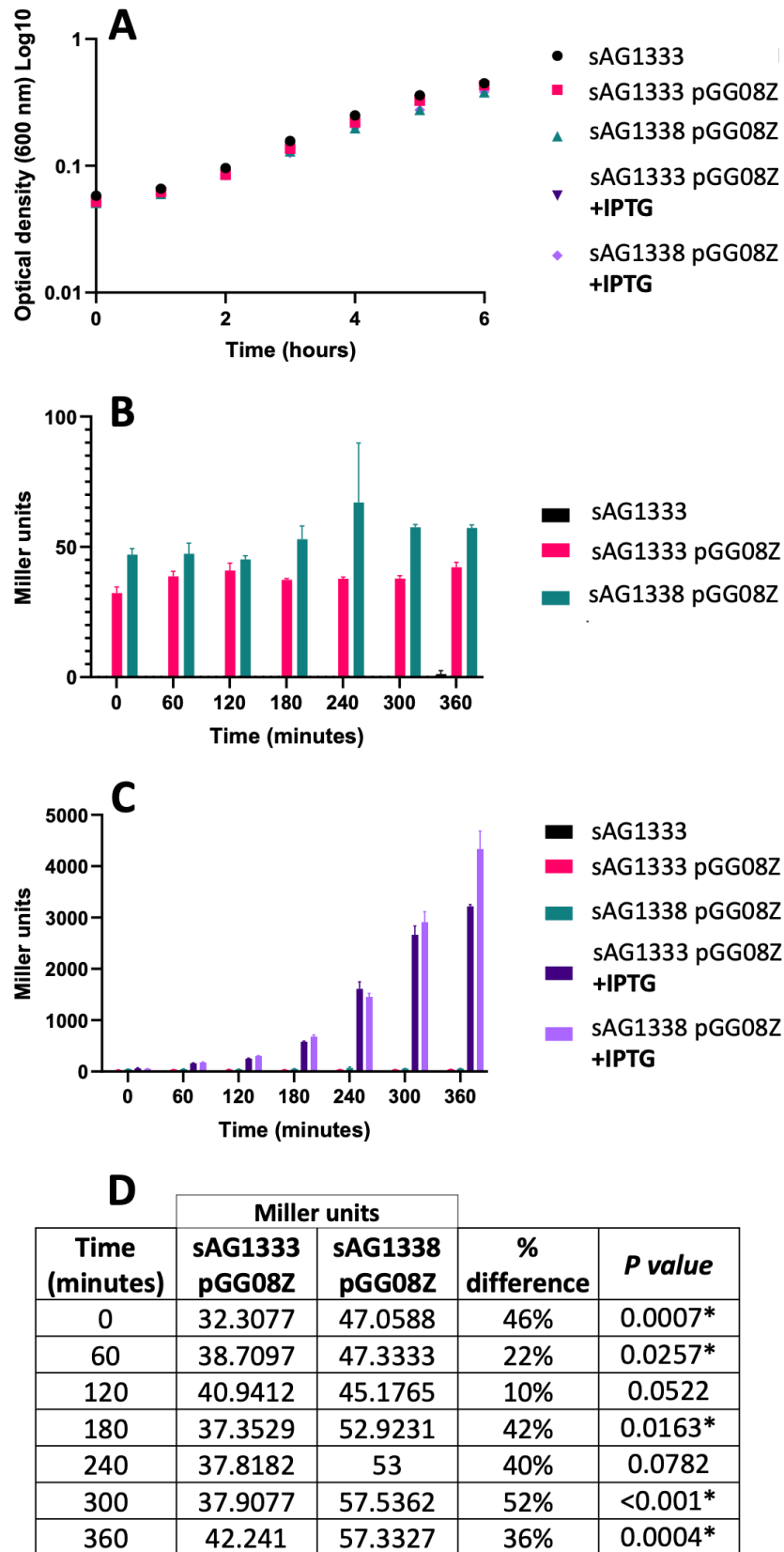


**Figure 3.4. Enzyme activity in mother and minicells.** Strains were grown over a 6-hour time course in CDM at 30°C with shaking. **A)** Growth curves of samples over 6 hours (optical density 600 nm) **B and C)**  $\beta$ -galactosidase

activity calculated in Miller Units, 200 µl aliquots were taken for lysis and β-galactosidase assay using ONPG.

**C)** Addition of IPTG. Technical repeats n=2, experimental repeats n=3. **D)** the difference between sAG1333 and sAG1338 with % difference calculated in Microsoft excel. Bars represent the mean of the technical repeats carried out and error bars represent the standard deviation of these repeats. Statistical analyses were performed using a 2-sample t-test, values <0.05 were considered significant (noted by \*).

### 3.2.3 LacI degradation in *B. subtilis*

To understand the mechanism by which minicells express *lacZ* and *gfp* from the plasmid, a LacI degradation assay was performed. Commercial α-LacI antibodies (Abcam) were used, whereas, chloramphenicol treatment was utilised to inhibit protein synthesis and measure the time for LacI to degrade in a *B. subtilis* strain expressing *lacI* from the chromosome (sGG106). The half-life of LacI was unknown in a *B. subtilis* background, therefore this experiment was performed to demonstrate the time it took for LacI to degrade. Which would demonstrate the time it would take for a minicell to be able to start expressing from the plasmid. PBP2B detection was used as a control to show equal protein loading from lysates. Firstly, a test was carried out to ensure if the antibody signal decreased linear in accordance to the lysate dilution. For both α-PBP2B and α-LacI there was a linear reduction as lysates became more dilute, this was evident as band intensity reduced (fig.3.5A). This was quantified and also demonstrated a linear reduction (fig.3.5B), however, it was evident that PBP2B was more abundant across all dilutions (black circles).

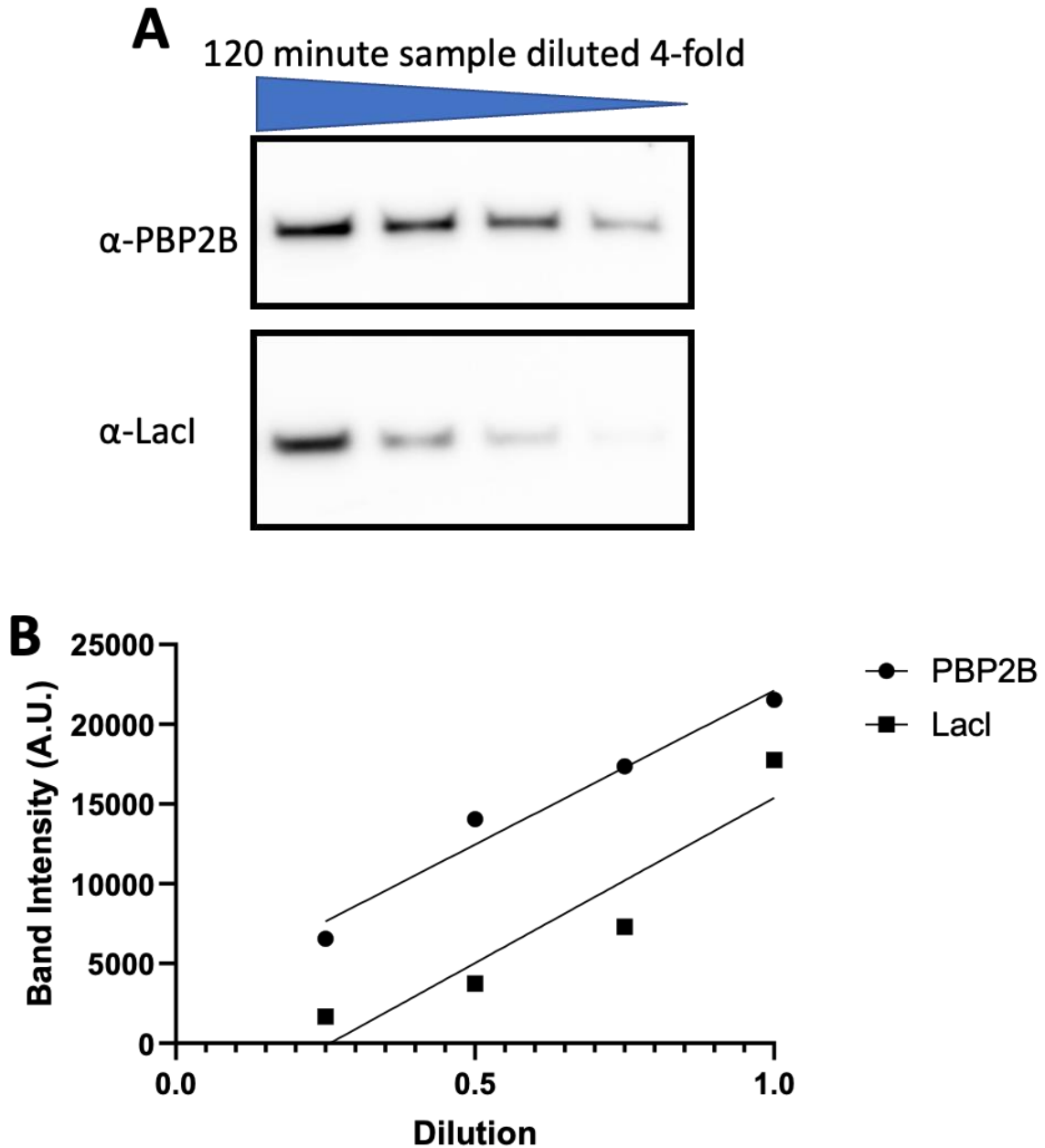


**Figure 3.5. PBP2B loading control for LacI degradation.** Cell lysates processed in SDS were separated on SDS-PAGE before diluting 4-fold and transferring proteins to 0.45  $\mu$ M PVDF membranes. To detect protein bands,  $\alpha$ -PBP2B polyclonal (1:5000) and  $\alpha$ -LacI (Abcam (1:2000) were incubated with membranes in blocking buffer (PBS, 5% milk powder). Secondary HRP labelled  $\alpha$ -rabbit and  $\alpha$ -mouse antibodies were used respectively and detection was achieved using the ECL western blotting substrate (Thermofisher). **A)** Western blots of bands detected for PBP2B and LacI lysate dilutions **B)** Quantification of dilutions with linear trend lines. Quantification and Graphs were calculated/constructed in Microsoft Excel/GraphPad prism 9.

When sGG106 were not treated with antibiotic, the amount of PBP2B remained relatively constant across all time points (fig.3.6A). LacI also did not appear to deviate across time points without antibiotic treatment, this indicated that LacI was consistently maintained throughout growth. This was also prevalent when quantified, as the band intensity remained uniform for PBP2B and LacI (fig.3.6B & C circles). Treatment with 50 µg/ml did show deviations in band intensity for LacI, though, PBP2B did not seem to be affected greatly and band intensity showed consistent numerical values across all time points (fig.3.6B squares). LacI quantity decreased greatest between 60-80 minutes after chloramphenicol addition but after 80 minutes, showed evidence of increasing (fig.3.6C squares). Trends from the first 100 minutes were analysed further, with the treatment of chloramphenicol, PBP2B quantities remained stable (fig.3.6D). However, by comparison the quantity of LacI was affected by chloramphenicol as it halved after ~80 minutes indicated in fig.3.6E. After the first 80 minutes, it was observed in fig.3.6C that the LacI quantity started to increase once again. Rational for this was hypothesised to be either a consequence of the antibiotic degradation that no longer took effect, or there was a significant turnover of LacI over an 80-minute period.

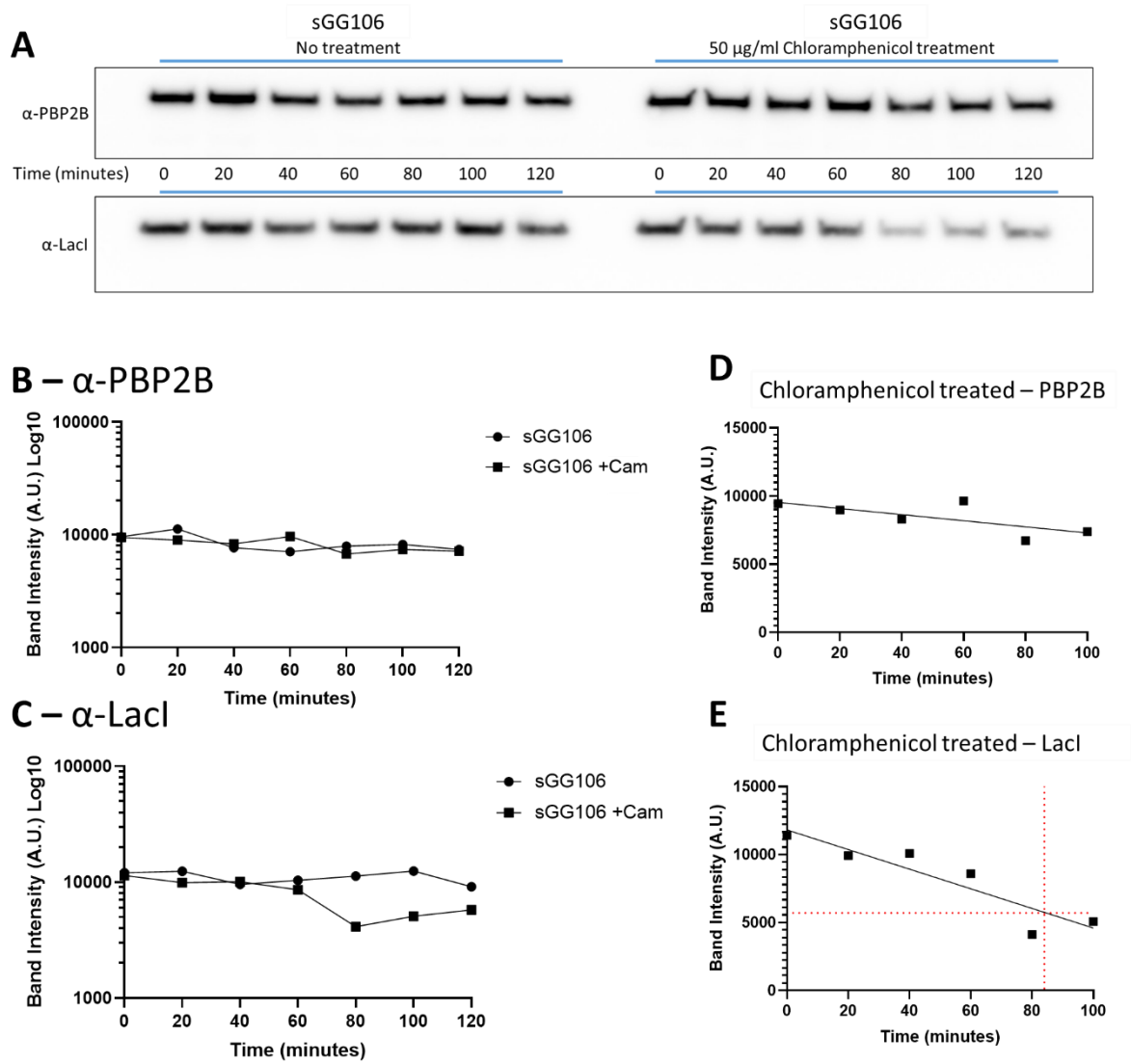


**Figure 3.6. Western blot band quantification.** Mid-exponentially growing cultures were treated with and without 50 µg/ml chloramphenicol. 2 ml samples were harvested every 20 minutes after addition and cell pellets processed in SDS before running cell lysates on SDS-PAGE. Western blotting consisted of using α-PBP2B polyclonal (1:5000) antibodies with α-rabbit HRP conjugated (sigma) (1:10,000) secondary antibodies. For LacI, α-LacI (Abcam (1:2000)) antibodies as primary and secondary antibodies were α-mouse HRP conjugated (sigma) (1:10,000). Detection was achieved using the ECL western blotting substrate (Thermofisher). Time shown within the x-axis indicates time in minutes after the addition of chloramphenicol to the test culture. Band quantification was carried out using Fiji and data points plotted in Graph pad Prism 9. **A)** immunostaining showing PBP2B and LacI bands from separated lysates. **B)** PBP2B protein quantification. **C)** LacI protein quantification. **D)** PBP2B quantification of chloramphenicol treated culture only. **E)** LacI quantification and half-life calculation using trendline of chloramphenicol treated culture only. **B & C)** Y-axis scales are logarithmic 10, **D & E)** Y axis show linear scales to show how the half-life was calculated.

### 3.2.4 Plasmid copy number in minicell producing strains

As it was obvious that LacI had a long half-life, it was still to be understood how minicells overcame transcriptional repression in order to start expressing independently from the plasmid. One hypothesis was that minicells could be replicating plasmids, which imbalanced the ratio of LacI/ $P_{spac}$ . To see if there could be replication in minicells, the copy number of the plasmid (pGG08Z) was analysed during mid-exponential growth. The plasmid and genome were both amplified using primers with specific targets to ampicillin antibiotic resistance cassette (tab.2.4 primer pair J10749 F + R; Lee *et al*, 2006) or chromosomal terminus (primers qPCR57 and qPCR58; tab.2.4). This experiment used the hypotheses that each cell contained one genome, plasmid copy number was therefore calculated in accordance to the terminus. Determining the ratio of terminus:plasmid revealed that sAG1333 had ~4 copies of pGG08Z per cell, whereas, there was a significant increase of copy number in sAG1338 (~30% greater; fig.3.7A). Significance was determined using a pairwise T-test with Welch's correction ( $P=0.003$ ). This suggested either that: plasmids were replicated within minicells after asymmetric division, or the  $\Delta minC$  mutation had affected plasmid replication in rod cells.



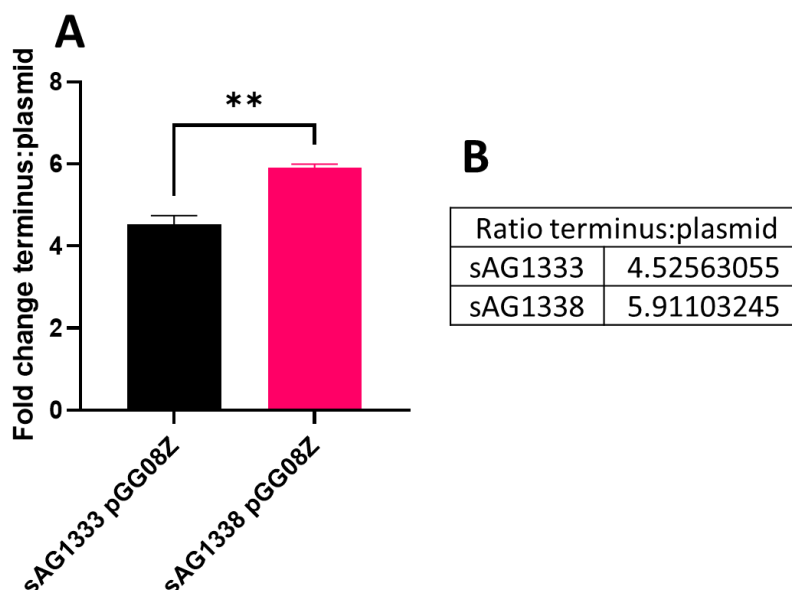


**Figure 3.7. Plasmid copy number in sAG1333 and sAG1338 strains.** Cultures were grown in CDM at 30°C for 4 hours when 5 ml aliquots were harvested during mid-exponential growth. Genomic DNA was extracted using lysozyme treatment and sonication before centrifugation and supernatant aspiration, supernatant was kept for qPCR experiment. gDNA was diluted 1000x before mixing with NEB Luna polymerase and primers for qPCR. **A)** Plasmid ratio to terminus calculated using Ct values **B)** averaged ratio of terminus:plasmid plotted. Error bars are representative of 3 technical repeats, although 3 biological repeats all show the same trend and significant difference.

### 3.3 Development of an anucleated system in *B. subtilis* using a DNA replication mutant

#### 3.3.1 Introduction to the maxicell model

To make larger anucleate compartments in accordance to the model described in Chapter 1 (fig.1.11B), a *B. subtilis* strain with a chromosome replication initiation mutation was retrieved from Prof. H. Murray (Murray, unpublished). The mutant contained a DnaA mutation where glycine at position 152 was substituted for serine, this subsequently reduced the affinity of the protein (sGG107 and sGG115). Chromosome replication was still permitted, however at a

much slower rate. While the divisome worked at wild type speeds, the chromosome was not replicated fast enough to occupy all compartments and resulted in anucleated daughter cells.

### 3.3.2 Construction and characterisation of the maxicell producing mutant

To analyse if maxicells would be a feasible anucleated chassis, first the strains were constructed. In accordance to the model presented in figure.1.11, *P<sub>pcn</sub>-lacI* was integrated into the chromosome to provide the transcriptional repressor at the *aprE* locus (sGG106 and sGG113). The mutant DnaA was integrated after *lacI* incorporation and colonies were screened for maxicell production using a DNA specific DAPI stain. When visualised using epifluorescence microscopy, sGG106 had regular nucleoids across the rod which corresponded with single cells when compared to merged images. As expected, the *dnaA* mutant (sGG107) showed anucleate cell formation, indicated by red markers, but there was also a secondary phenotype seen where nucleated cells appeared more filamentous (fig.3.8). Chromosomes of sGG107 were not as organised as the sGG106 and seemed decondensed. This may be a result of DNA replication initiation delays causing chromosomes to occupy more space within the cell. Chromosomes spreading across larger compartments of the cells may affect maxicell size, producing anucleated cells of heterogeneous sizes as the Noc system still prevents FtsZ polymerisation in spaces the chromosome occupies.

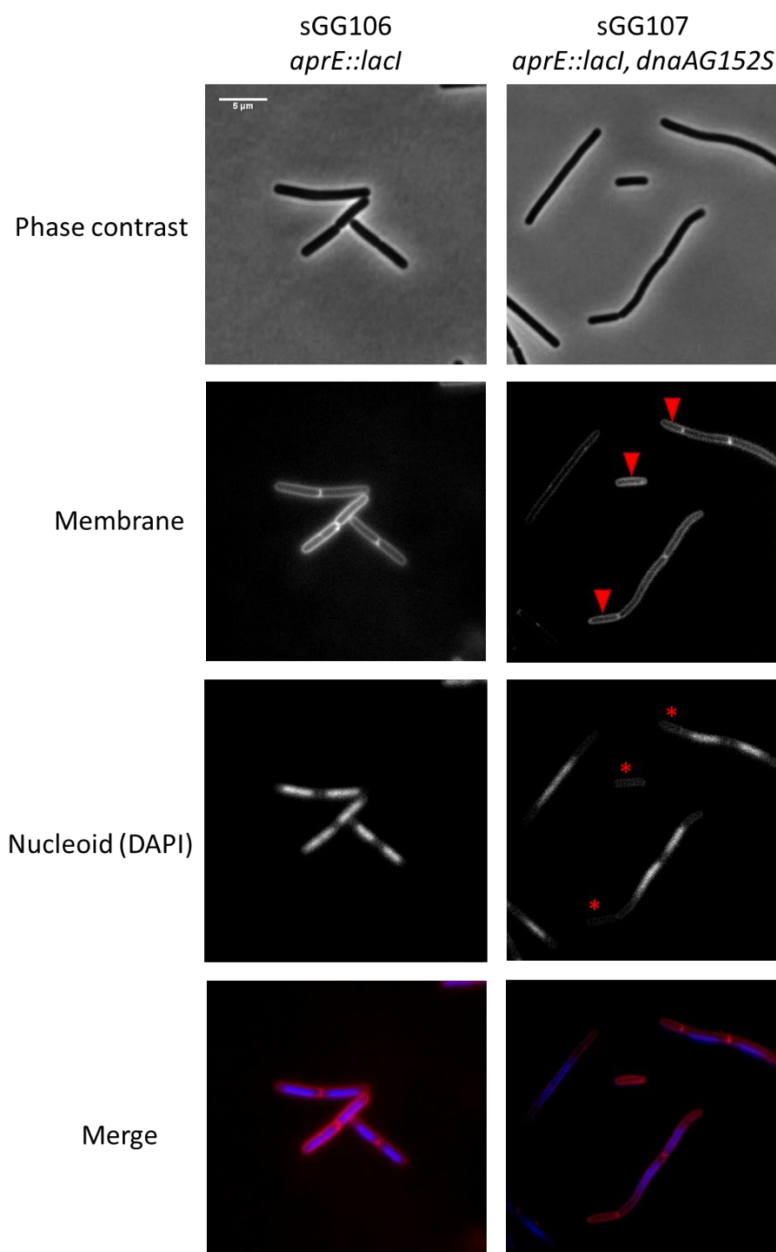


**Figure 3.8. Integrating the LacI repression system into the maxicell model.** Cultures were grown at 30°C in CDM to mid-exponential phase before treating with 1 µg/ml DAPI and applying to an agarose pad supplemented with 0.6 µg/ml FM5-95. DAPI shows where the nucleoid is localised and the mCherry (FM5-95 fluorescence) shows membrane staining. Anucleate cells are indicated by red markers (arrows and \*). Scale bar is representative of 5 µm and applicable to all images.

### 3.3.3 Portabolomics vector transformation and expression

#### Testing GFP independent expression from maxicells and repression in mother cells.

After a strain that produced maxicells had been constructed with the appropriate repression system (sGG107), pAG-P08 (fig.3.3B) was then transformed into the strain to detect independent expression from maxicells using the GFP reporter. Observing growth in liquid media was utilised to see how the production of maxicells affected phenotype. sGG107 had a longer lag phase and exponential growth was found to be slower. sGG107 had a 1.5-fold decrease in doubling time compared to sGG106, which was attributed to the reduced chromosome replication mutation as anucleate cells cannot replicate themselves (fig. 3.9A). During mid-exponential growth, it was evident that in sGG106 there was an even chromosomal distribution and negligible GFP expression, demonstrating good repression (fig.3.9B). Maxicells were identified using membrane (FM5-95) and nucleoid (DAPI) stains to show if DNA was present in cellular compartments. The phenotype presented by sGG107 was still apparent where small anucleated cells were either free floating or still attached to mother cells (fig.3.9C). Some maxicells of sGG107 pAG-P08 displayed strong GFP signals. Demonstrating that maxicells were able to express independently from the plasmid in a way that was similar to the equivalent minicell chassis (fig.1.10).

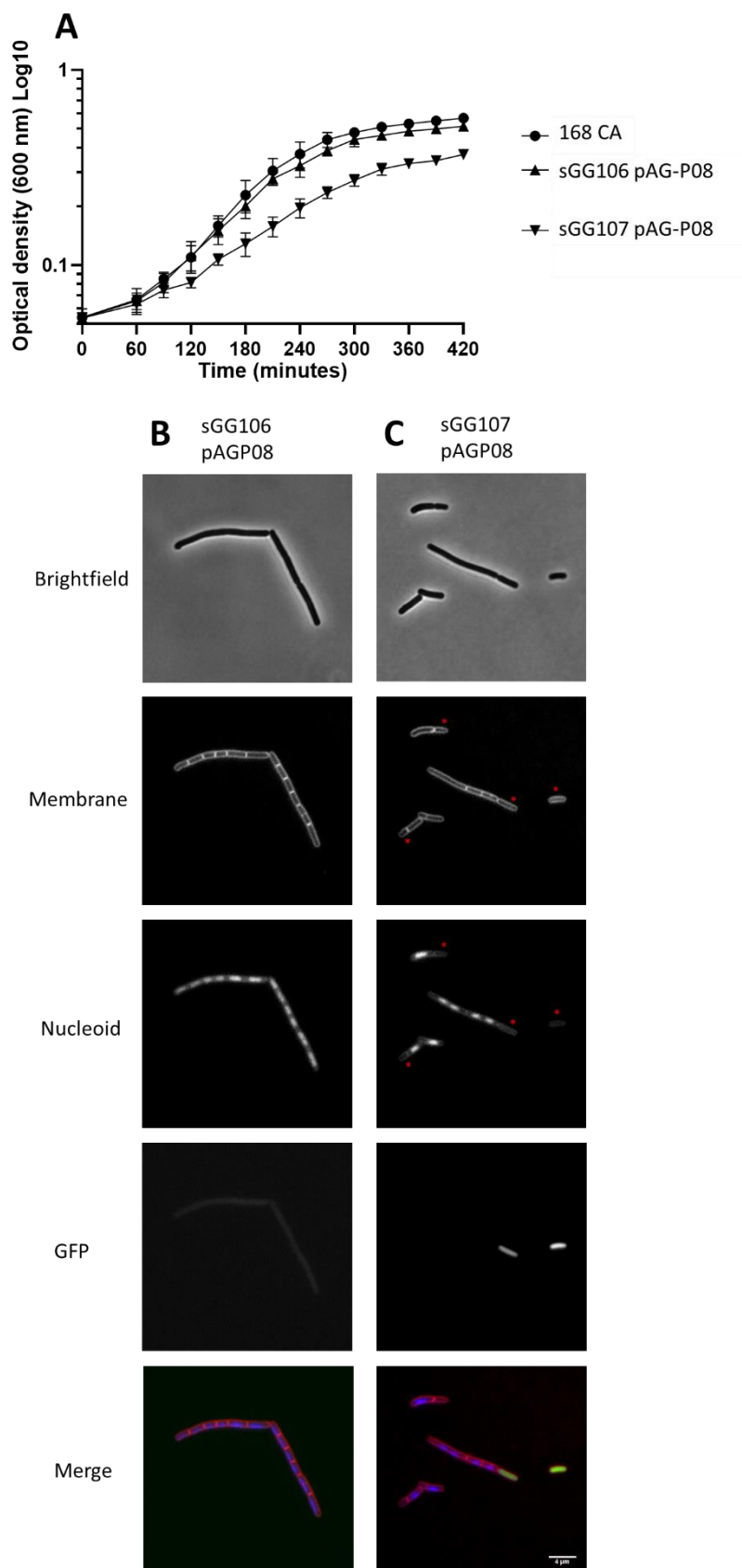


**Figure 3.9. Independent expression of GFP from maxicells.** Strains were grown in CDM at 30°C. **A)** Growth curve of the parental and maxicell producing strains monitored every 30 minutes. Optical density values are

plotted onto a logarithmic base 10 scale. Error bars signify the standard deviation between two biological repeats. n=2 **B)** Parental strain with plasmid pAG-P08 plasmid (GFP reporter) imaged on 1.2% agarose pads during mid-exponential growth (4.5 hours). **C)** maxicell producing strains with GFP reporter plasmid (pAG-P08) imaged during mid-exponential phase. FM5-95 was within the agarose pad for staining the membrane at a concentration of 0.2 µg/ml, nucleoid staining was facilitated with 1 µg/ml DAPI and GFP was expressed from the plasmid. Graphs were made with GraphPad prism 9, and images were examined using Fiji. Scale bar is representative of 4 µm and applicable to all images.

Fluorescence measurements of cell populations showed there was a notable difference between nucleated and anucleated cells (fig.3.10A). The red dotted line is the ‘reference line’ and served as a threshold for anything over to be counted as GFP positive. The line was determined by doubling the average (mean) value of nucleated cells fluorescence intensity and lies at the end of the normal distribution. In the violin plot, and seen in the table (fig.3.10B), there were only 6% of the nucleated cells over the threshold indicating good repression. Whereas, the anucleated cells fluorescence was much greater and the population was not grouped as tightly which showed variance within maxicell expression levels. Analyses of the anucleated populations fluorescence revealed that 52% of the maxicells were over the set threshold, meaning they were expressing GFP independently (fig.3.10A and B). A comparison of the means tested for significance using a Welch’s 2-sample t-test revealed the fluorescence intensity between the two populations were significantly different ( $P < 0.0001$ ). A test for equal variance was conducted to confirm which statistical tests should be performed. The analysis of variance (ANOVA) showed that the intervals did not overlap and the Levene’s test was significant ( $P = < 0.0001$ ). This method of counting populations could be flawed as it only described a single time-point and did not account for new anucleated cells that may have a plasmid but have not yet been able to express GFP from the plasmid. To determine how many cells of the population were nucleated or anucleate, microscopy images were used to count 5455 cells, of which 39% were anucleate as they did not have chromosome labelling

(fig.3.10C). This shows that overall, during mid-exponential growth, maxicells account for almost 40% of the whole culture and, out of this population, 52% were expressing GFP from the plasmid. This means that in the whole culture 20% were anucleated GFP producers.

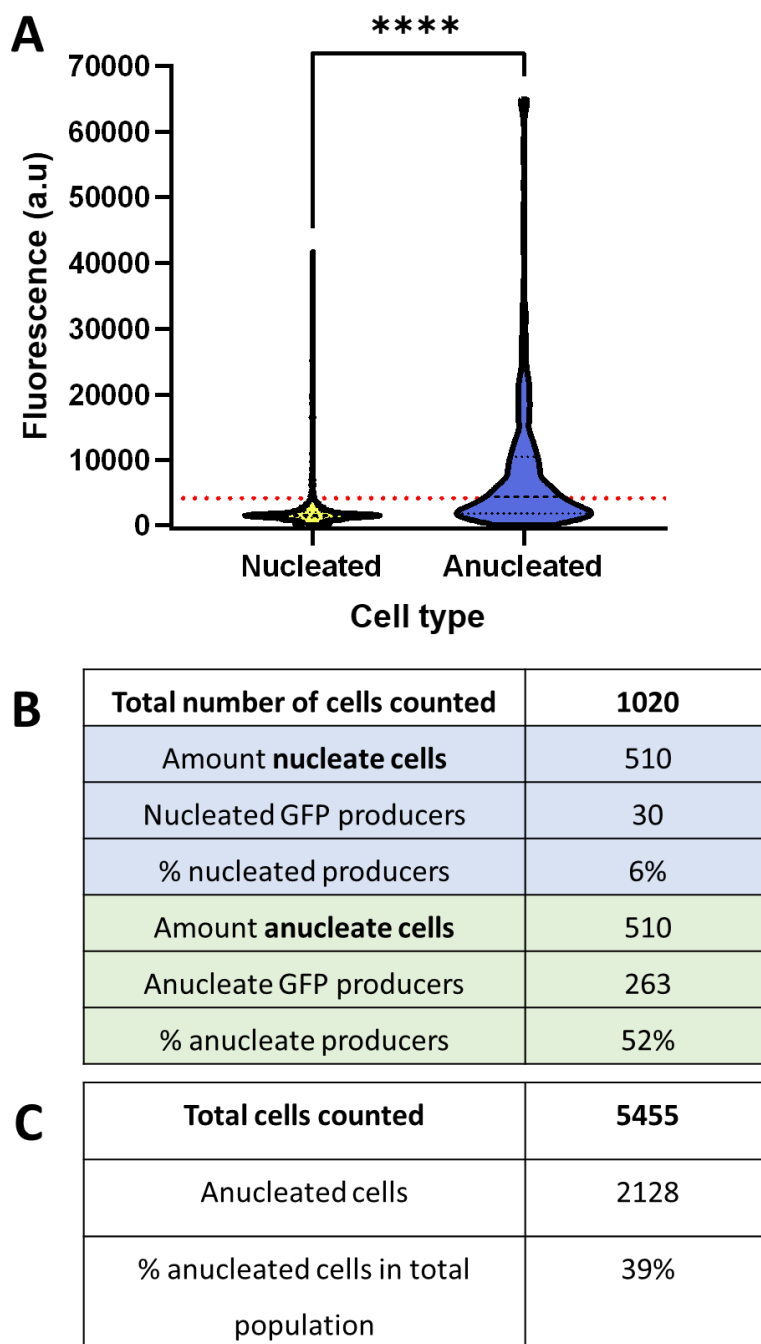


**Figure 3.10. Fluorescence intensity of nucleated and anucleate strains with a GFP reporter.** **A)** Cultures were grown in CDM at 30°C to mid-exponential phase in the presence of erythromycin for selection of the plasmid. Cells were mounted to a 1.2% agarose pad for imaging using DAPI stain to locate chromosomes and

GFP was expressed exclusively from the plasmid. Images were examined using Fiji counting 510 cells in each population randomly, violin plots and statistics were made using GraphPad Prism 8.  $P < 0.001$  in all tests run for parametric and non-parametric tests.  $N = 510$  cells per population **B)** Fiji software was used to calculate the fluorescence average per cell by drawing a line through each cell and calculating the average fluorescence intensity per cell. Nucleated and anucleated cells were counted as separate populations randomly by using DAPI stain determine the populations. GFP “producers” were calculated as anything over the reference line in Figure.3.10A, which is 2-fold greater than the mean of nucleated cells fluorescence (4170 a.u). **C)** Using microscopy images all cells were counted and ranked as nucleated or anucleated in accordance to the DAPI DNA staining. Fiji software was used to count and sort into 2 populations. Percentages of the population were then calculated in Microsoft excel.

It had been hypothesised that reduced genome replication could affect the amount of LacI expressed within maxicell producing strains. As all imaging thus far had taken place during mid-exponential phase, cells were imaged during stationary phase and fluorescence measured to ensure the nucleated cells were capable of maintaining repression throughout later stages of growth. The images showed an abundance of small anucleated cells present with varied fluorescence intensities (fig.3.11A GFP panel). It was obvious that repression in nucleated cells from sGG115 were not as tightly grouped compared to sGG113, where the whole population were all nucleated. However, there was still little evidence of GFP expression in nucleated cells from sGG115 (fig.3.11B). During stationary phase, the frequency of anucleated cells expressing GFP significantly increased (fig.3.11B purple plot). The statistical significance was determined using a non-parametric T-test with Welch’s corrections and represented  $P < 0.0001$ . Encouragingly, maxicells of sGG115 pAG-P08 were regularly over the set threshold and further analysis showed that 73% of the population were independently expressing from the plasmid. The nucleated cells of sGG115 with pAG-P08 did show some outliers, however, from the analysis this only accounted for 1% of the population over the threshold (fig.3.11B & C). Overall, it was clear that during stationary phase nucleated cells



were able to maintain good repression while it seemed the frequency of maxicells expressing GFP increased significantly.

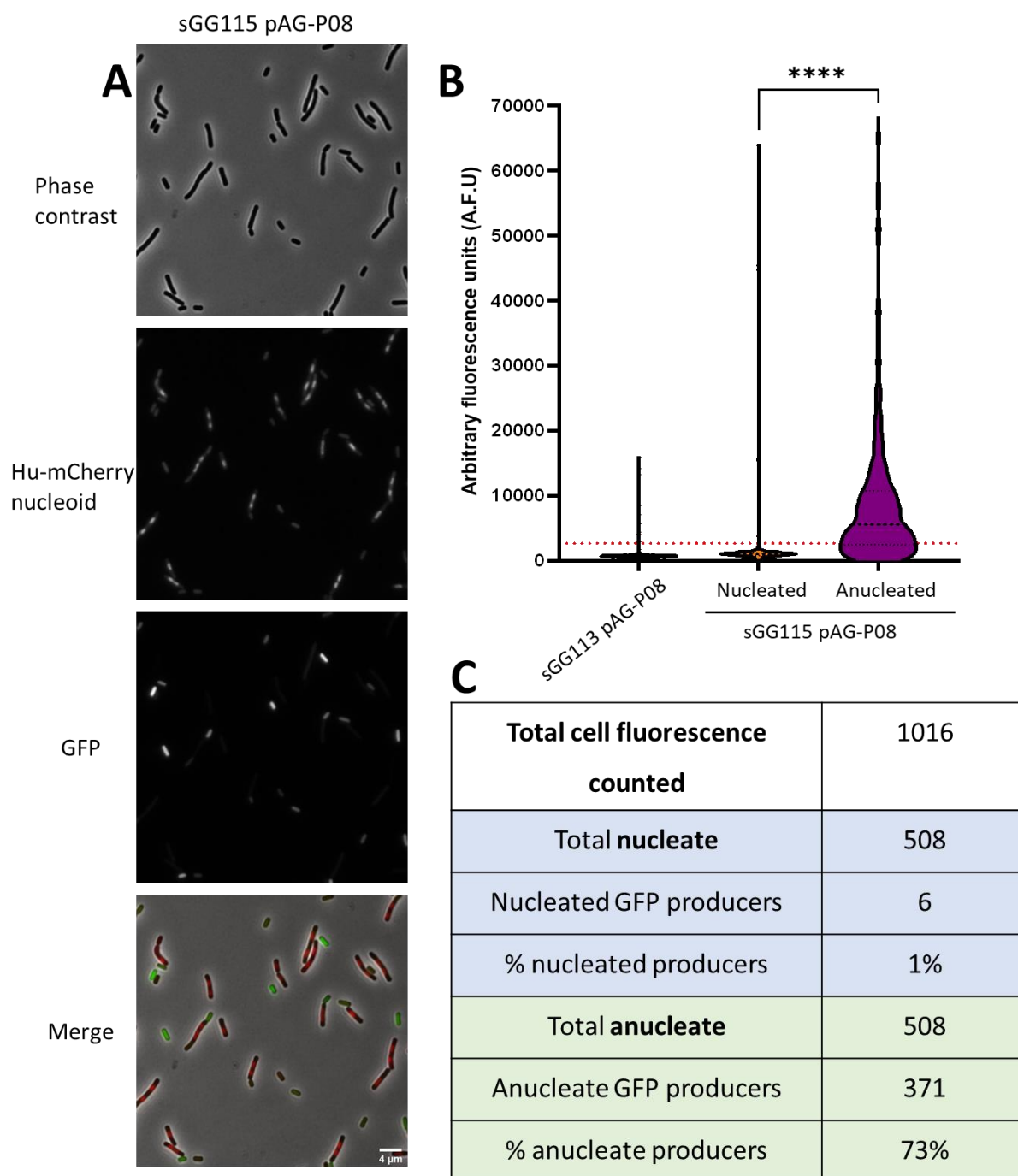


**Figure 3.11. Analysis of populations with GFP reporter during stationary phase.** Cultures were grown in CDM with erythromycin selection at 30°C until stationary phase (6.5 hours). Cells were applied to a 1.2% agarose pad before imaging using a Nikon Eclipse Ti with Cool LED pE-300 white light source and Photometrics Prime

camera. **A)** microscopy images of cells during stationary phase with different fluorophore excitation. Scale bar is representative of 4µm and is applicable to all images. **B)** fluorescence was counted using Fiji ImageJ software and plotted into a violin graph using GraphPad Prism 9. T-tests were applied with Welch's corrections. Red dotted line is representative of the threshold set to count 'GFP positive' cells. **C)** Cell populations and their percentages of GFP positive cells (over the threshold shown on the violin plot). N = 500 cells per population.

### 3.3.4 Time lapse microscopy and monitored expression of GFP

Time lapse microscopy was used to understand how maxicells grow, express and how long the fluorescence was maintained. For time lapse microscopy, a maxicell producing strain was constructed to stain the nucleoid constitutively. A Hu-mCherry tag was integrated into the chromosome (*amyE* locus) to label the nucleoid red fluorescent, pAG-P08 was also transformed to see maxicells express GFP during growth. Hu is a histone-like protein which bound to non-specific DNA sequences, therefore, when labelled with a fluorescent protein the nucleoid could be localised. Yellow arrows indicate when a maxicell was formed after the first 2 hours of growth, this cell was then monitored and found to exhibit increasing GFP fluorescence after 36 minutes. After a further 24 minutes (3:00 hours) the maxicell was clearly independently fluorescent and reached its maximum fluorescence intensity 1 hour 40 minutes after initial fluorescence was observed. The last panel images showed that the maxicell started to become noticeably dim 5 hours after first seeing fluorescence increase. It was notable that the chromosome started to decondense after 4 hours of growth, this may be attributed to the chromosome replication initiation mutation, stress and/or starvation. After this time-point the nucleoid localised Hu-mCherry fluorescent label was no longer fluorescent, suggesting the protein was no longer expressed. The merged images showed the centre time-point, as evidence there was no chromosome present in the GFP expressing compartment. Other cells from the time-lapse were examined and a trend was seen that after ~30-40 minutes maxicells with a plasmid started to fluoresce. A trend was also seen that maxicells were generally fluorescent for ~4-5 hours before fluorescence decreased.

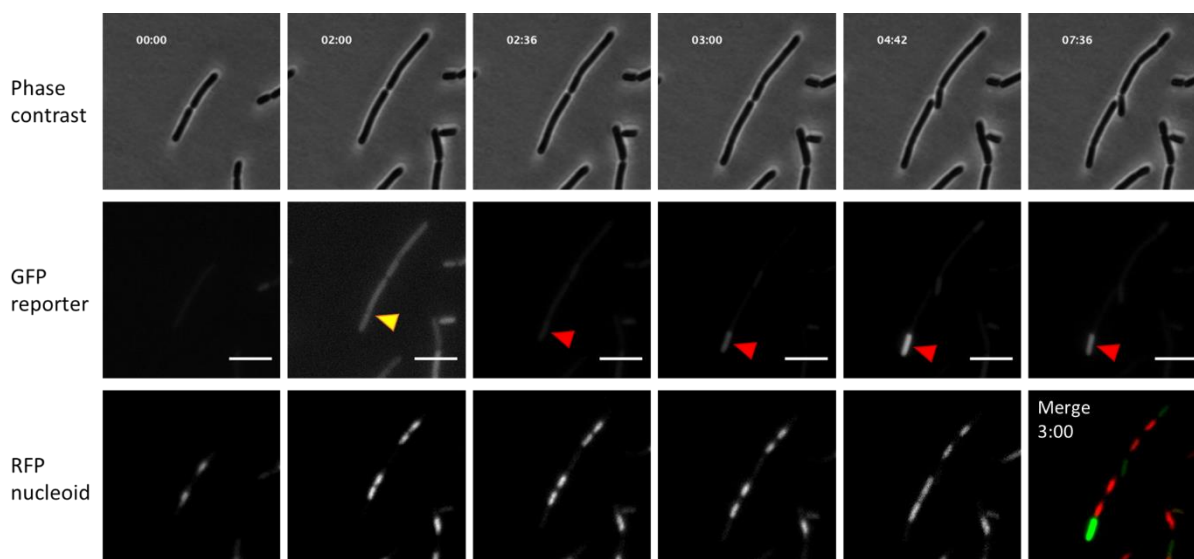


**Figure 3.12. Time-lapse of maxicell GFP independent expression.** Cultures were grown in CDM at 30°C to early-exponential phase in the presence of erythromycin for selection of the plasmid. Cells were mounted to a 1.2% agarose pad supplemented with minimal nutrients for imaging over time. Hu-mCherry expressed from the chromosome to identify the chromosome localisation and GFP was expressed exclusively from the plasmid as a reporter. Images were examined using Fiji. Scale bar is representative of 5  $\mu$ m and applicable to all images.

To obtain a more quantitative understanding of the changes, 3 cells of each nucleated (red) and anucleate (blue) population were examined in more detail quantifying fluorescence signals over time. The fluorescence values were measured using imageJ software and revealed that, over an 18-hour time period, maxicells started to express at a similar time after the starting culture was applied to the agarose pad (~2-2.5 hours) (fig.3.13A). There were varying strengths of GFP fluorescence between maxicells, where maxicell 1 was clearly much brighter than 2 and 3. All maxicells fluorescence peaked highest after around 4 hours 30 minutes of growth on the agarose pad. After exponentially increasing, the fluorescence remained at the peak for ~3 hours. Overall, from starting to increase expression to returning to the same as the nucleated cells, maxicells were found to be fluorescent for ~14.5 hours of the 18-hour time lapse. Although cells became noticeably sick and started lysing in later time points after nutrients began to run low on the agarose pad. Maxicells appeared to lyse after ~16 hours of growth and this was

reflected as fluorescence decreased rapidly after ~975 minutes. On a smaller Y axis scale, the nucleated cells (red shades) show an initial increase in fluorescence peaking after 3.3 hours from nucleated cells 1-2 and 4.5 hours for nucleated cell 3. The initial fluorescence decreased rapidly back down to a basal level (fig.3.13B). These fluctuations in fluorescence were never as high as that seen in maxicells, this was encouraging to show that the plasmid seemed to be suitably repressed in nucleated cells (fig.3.13A).

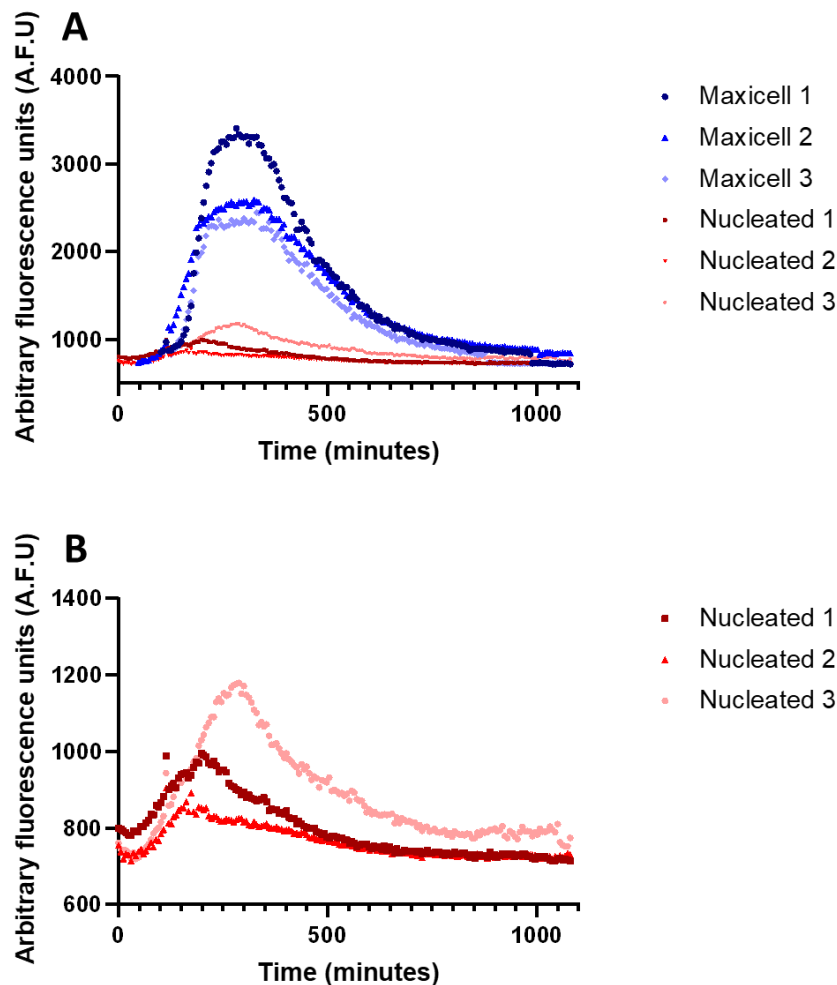


**Figure 3.13. Quantification of maxicell expression from time-lapse microscopy.** Cells were grown on a CDM agarose pad at 32°C while imaged for 18 hours. Images were taken every 6 minutes of phase contrast, GFP to monitor expression from the plasmid and mCherry to detect the nucleoid. Images and fluorescence were analysed with Fiji software. **A)** nucleated and anucleate GFP fluorescence **B)** nucleated cells only. Graphs were constructed in Graphpad prism 9.

### 3.3.5 Quantification of expression using $\beta$ -galactosidase

As it was seen that maxicells could independently express GFP from the plasmid, further experiments which would allow quantification were required. Therefore, the  $\beta$ -galactosidase assay was used as it shows enzyme activity from whole cultures. To simulate the same microscopy conditions, the strains were each grown in CDM with and without IPTG supplementation. The addition of IPTG induced the whole population to express the *lacZ* reporter on the plasmid (pGG08Z fig.3.3B). When there was no plasmids present (black and pink bars) there was negligible activity seen which showed there was little background in these strains (fig.3.14A). When sGG113 and sGG115 strains carried pGG08Z, there was an increase in  $\beta$ -galactosidase activity from both strains. An increase from sGG113 demonstrated that there was leaky expression from the plasmid (fig.3.14A teal bars). There was a significant increase in expression from sGG115, hypothesised to be from maxicell independent expression (fig.3.14A – purple bars). This was expected as the microscopy data eluded to the independent expression from anucleated compartments at high levels. On average, across all time points, there was an 11-fold increase in expression from sGG115 by comparison to sGG113 with plasmid (fig.3.14A purple and teal bars). The addition of IPTG to sGG113 (fig.3.14B lilac bars) demonstrated a large increase in activity which was a result of the whole population expressing from the plasmid. Interestingly, when sGG115 pGG08Z was induced with IPTG,  $\beta$ -galactosidase increased much greater than induced sGG113 pGG08Z. This significantly higher enzyme activity was hypothesised to be from maxicells providing an advantage at the start of growth and from overnight cultures, or this may indicate that there were more maxicells expressing from the plasmid which would usually still contain LacI as data suggests it can take 80 minutes to halve LacI concentrations (fig.3.6). Growth was different between sGG113 and sGG115 because of the DnaA mutation, however, it was evident that IPTG induction in both strains slowed growth further (fig.3.14C; black open circles and squares). Growth defects with

IPTG induction may indicate that there was a burden imposed on cells as they expressed  $\beta$ -galactosidase at high levels.

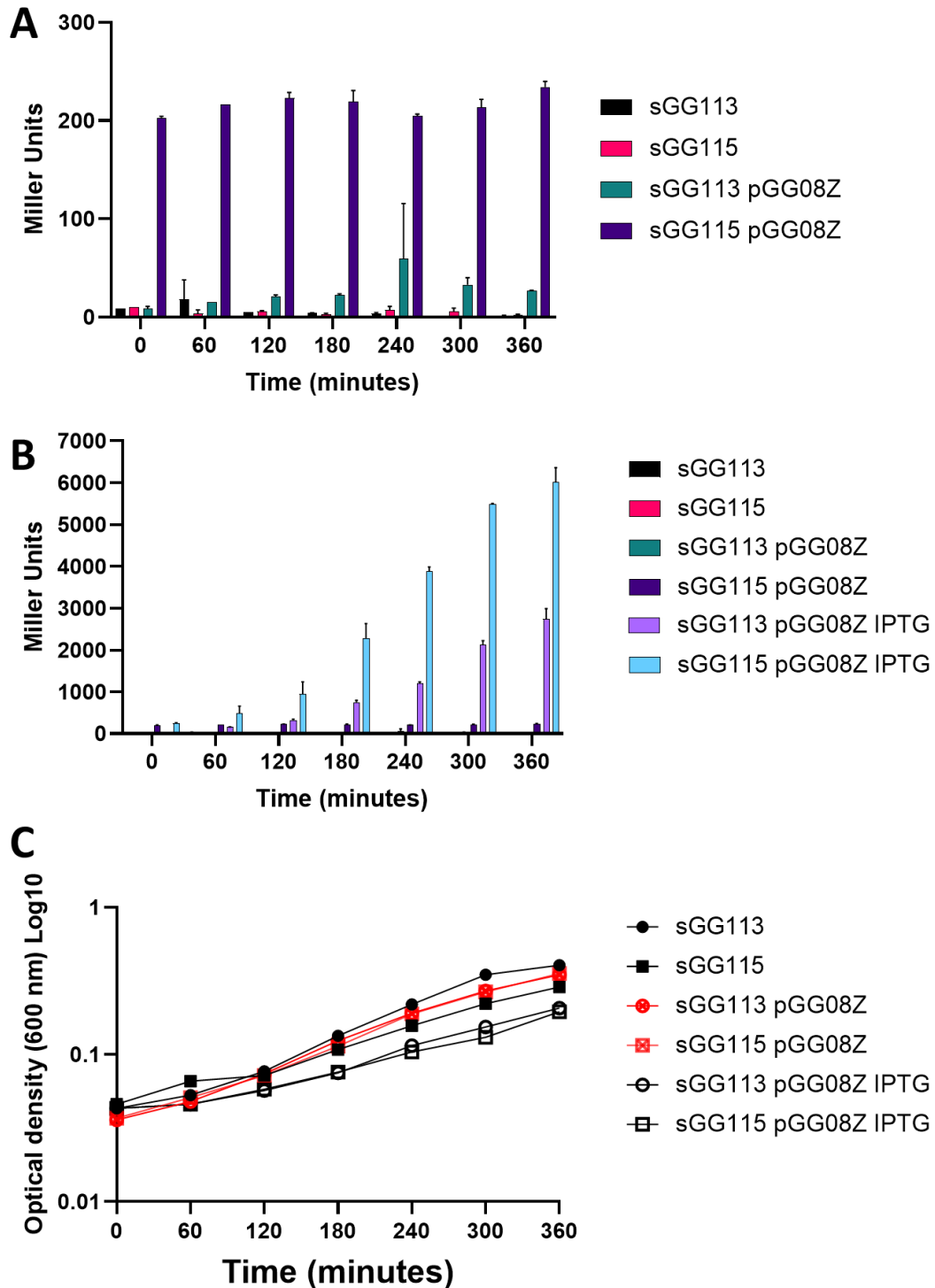


**Figure 3.14. Enzyme activity in maxicell producing strains whole population.** Enzyme activity from maxicell producing strains. All cultures were grown in CDM at 30°C with or without inducers (as stated in the legends). For each experiment the miller units were calculated to reflect the enzyme activity from colourimetric change

regarding the optical density of the culture. All strains from the assay represented with and without IPTG induction, IPTG shows a significant increase in  $\beta$ -galactosidase activity. **A)** Miller units shown where no strains were induced with IPTG **B)** IPTG induces strains included with an altered scale for miller units. **C)** growth curves of strains grown in CDM followed every hour. Error bars represent the standard deviation from three technical repeats. The experiment has been repeated three times as biological repeats.

The same assay was carried out in the strains grown in rich media to see how strains performed with accelerated growth. Without the addition of IPTG, the maxicell producing strain with pGG08Z had a constant increase in  $\beta$ -galactosidase expression (fig.3.15A – orange bars). This showed that, regardless of media, the maxicell phenotype exhibited a consistent increase in  $\beta$ -galactosidase activity. However, leaky expression was present from the sGG113 pGG08Z control strain in both media conditions (fig.3.15A – purple bars). On average there was a 26-fold increase, averaged across all time points, in  $\beta$ -galactosidase activity from sGG115 pGG08Z compared to the sGG113 pGG08Z control. This demonstrated that growth in rich media had a more profound effect on expression from the maxicell producing strain, especially when compared to the data in the CDM (fig.3.15A).

The addition of IPTG to sGG115 pGG08Z showed consistently greater  $\beta$ -galactosidase activity (fig.3.15B - yellow bars), the starting activity was higher in maxicell producing strains from the overnight carryover but exponentially increased overtime. This could also have indicated that, when lacking a chromosome, maxicells were able to express uncontrollably and therefore yield much more product. Trends were comparable for sGG115 pGG08Z grown in minimal or rich media, however, rich media did have a greater effect on  $\beta$ -galactosidase activity. Additionally, IPTG induction did have an effect upon growth phenotypes, growth curves showed that there was notable slower growth (open squares and circles) compared to non-induced strains (fig.3.15C). This was indicative of cellular stress as a significant load was applied with high levels of expression from the plasmid.

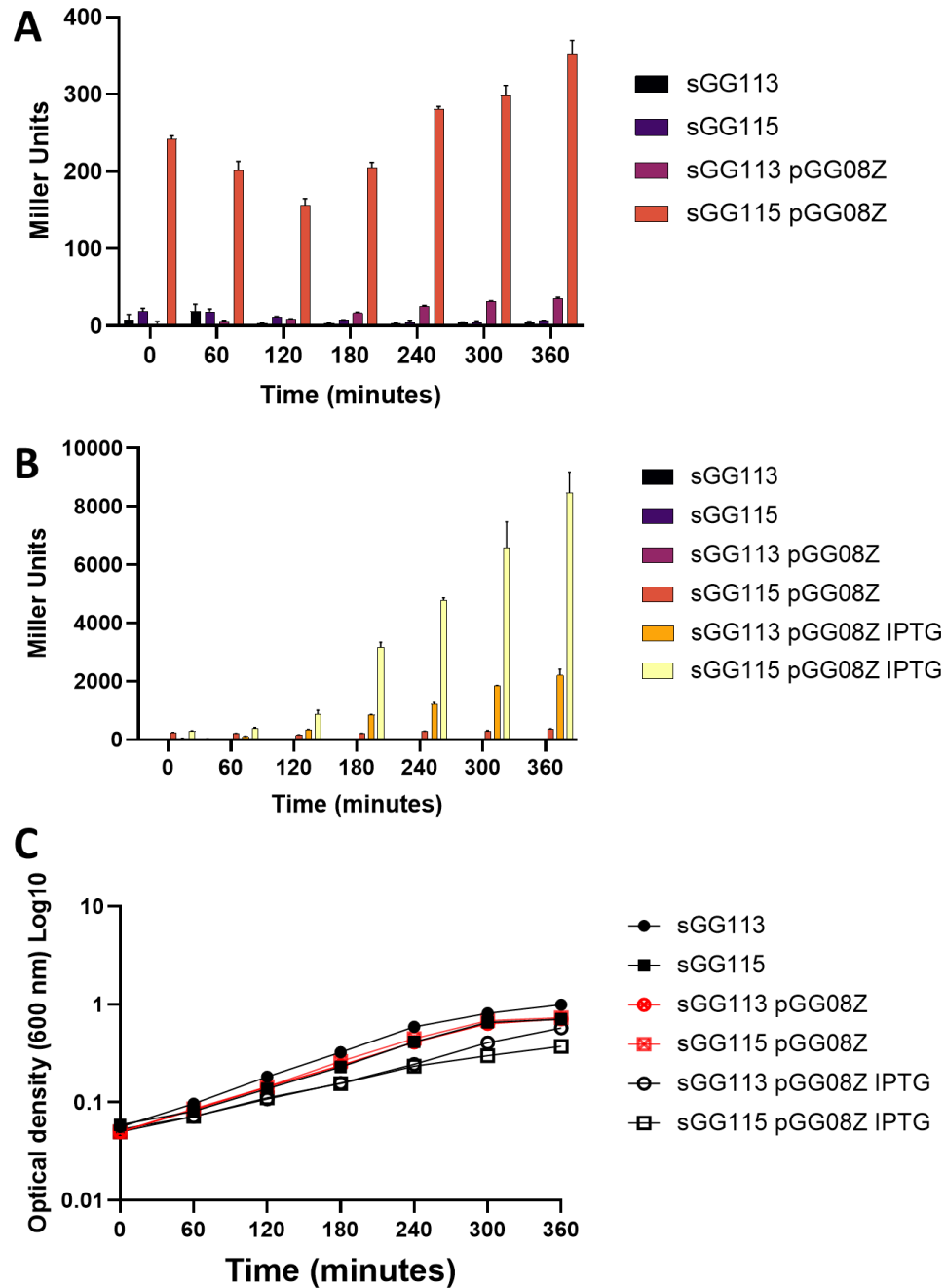


**Figure 3.15. Enzyme activity in maxicell producing strains whole population in rich media.** Enzyme activity from maxicell producing strains. All cultures were grown in rich media (lysogeny broth – LB) at 30°C with or without inducers (as stated in figure legends). For each experiment the miller units were calculated to reflect the enzyme activity from colourimetric change regarding the optical density of the culture. All strains from the assay represented with and without IPTG induction, IPTG shows a significant increase in  $\beta$ -galactosidase activity. **A)** Miller units of cultures with no IPTG inducer present **B)** Miller units scale altered to represent IPTG induced strains. **C)** growth curves following optical density (600 nm) every 60 minutes. Error bars represent the standard deviation from three technical repeats. The experiment has been repeated three times on separate occasions.



**Localisation of gene expression.** To ensure that  $\beta$ -galactosidase activity was exclusive to anucleate compartments, an FDG assay was performed using the ImaGene Green FDG kit (Invitrogen). Quantification was not able to be gained from these data sets as staining did not appear to be consistent, therefore, these images were used for qualitative analyses to show  $\beta$ -galactosidase expression was specific to anucleated compartments. The manufacturers instructions yielded low fluorescence signals, therefore, a range of concentrations were applied. Overall at a concentration of 5 mM, without the presence of IPTG, maxicells were brighter than the nucleated cells. Nucleated cells did present weak signals at most concentrations, likely a result of leaky expression seen in sAG1333 and sGG113 controls. IPTG addition induced expression in the whole population, however, it was revealed that across all concentrations expression was not homogenous.

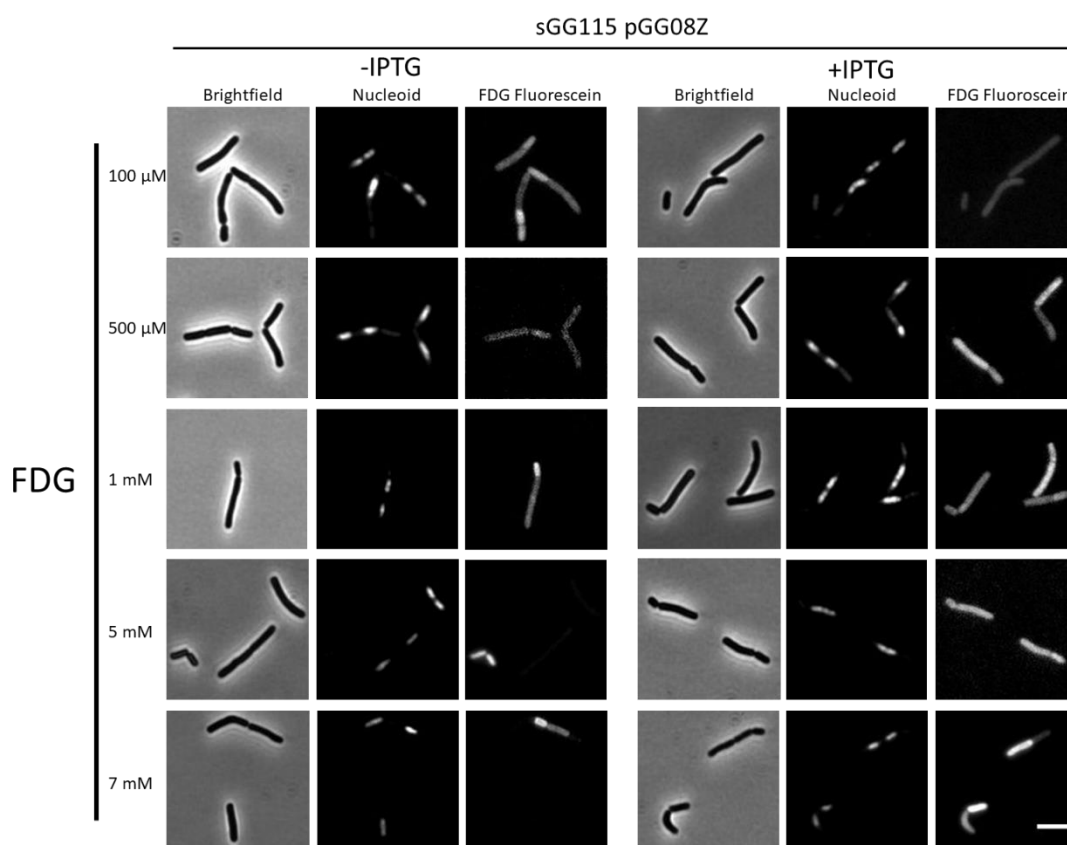


**Figure 3.16. Localisation of enzyme activity optimisation assay.** sGG115 pGG08Z were grown to mid-exponential phase in CDM before incubating with various concentrations of ImaGene Green FDG substrate (invitrogen). Cells were incubated with the substrate for 30 minutes at 37°C before mounting onto a 1.2% agarose

pad. Nucleoid stain was from Hu-mCherry expressed from the genome. Images were examined using Fiji. Scale bar is representative of 2  $\mu\text{m}$  and applicable to all images.

### 3.3.6 Population analyses using flow cytometry

Although microscopy was able to provide a detailed understanding of single cells, flow cytometry was used to attain a better analysis of greater populations. When performing the flow cytometry the size and granularity of the populations were first looked at to see if the maxicell producing strain displayed a phenotype. The side scatter (SSC-H – indicating granularity) showed there was 2 populations of cells in the sGG113 parental strain (fig.3.17A) where there were some less granular cells accounted for <10% of the population (gate 2). In contrast, sGG115 exhibited a different phenotype where the density of counts had shifted more into gate 2 accounting for 33% of the population (fig.3.17B). This indicated an increase in the population of cells with lower granularity. The forward scatter (FSC-H) of both strains were comparative although the maxicell producing strains (fig.3.17B) did show more smaller particles within gate 2. However, these phenotypic changes were not able to specifically discriminate between nucleated and anucleated populations.

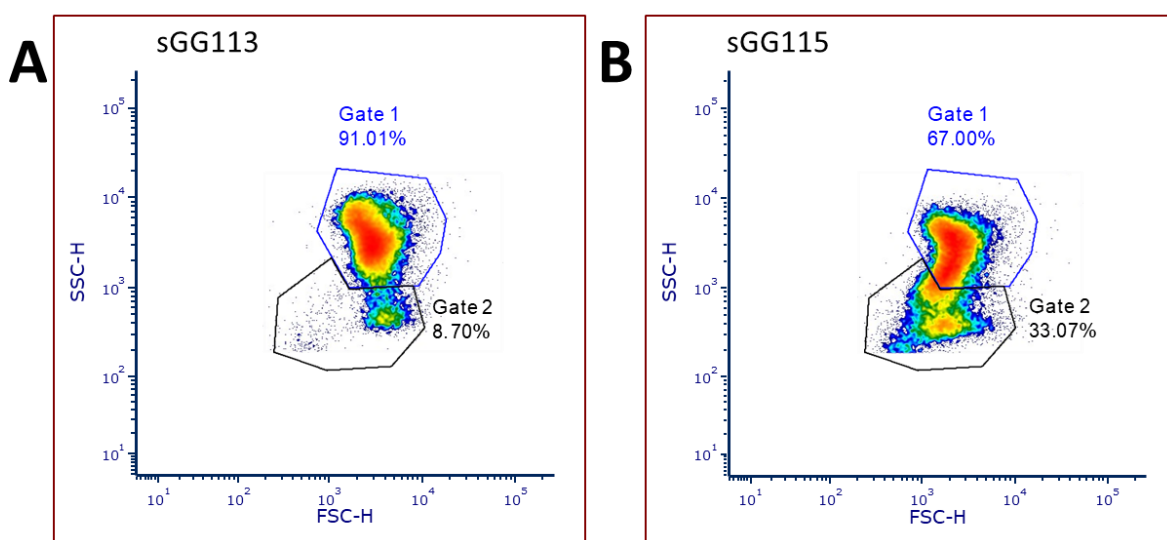


**Figure 3.17. Cell size and property analysis of wild type and maxicell producing strains.** 50  $\mu\text{l}$  of mid-exponentially growing cultures were mixed with 500  $\mu\text{l}$  filter sterile PBS sheath fluid. Density plot colours represent population concentration - red showing high densities decreasing to yellow and blue populations

showing lower densities respectively. Strains were analysed by light to detect forward (FSC-H) and side scatter (SSC-H), 30,000 events were recorded each strain. Gating and other analysis were completed using FCS express 6.

To make clearer conclusions about maxicell populations, the red stained chromosome (Hu-mCherry) was utilised to define nucleated and anucleated compartments. Gating was decided using a negative control (fig.3.18A), the control strain used (sGG106) had no fluorescent markers to serve as a benchmark for any background signals. Then gating outside these zones indicated a specific fluorophore expression by shifting into the red, green or double positive gates. Cell populations shifting into red florescent gates was demonstrated well by the parental strain, where all cells were nucleated, and 96% of events were red fluorescent cells (fig.3.18B). There was 2.9% of events residing in the negative fluorescence area, this was assumed to be cellular debris or old cells with dimmed chromosome labels. Constructs with the Hu-mCherry integration and production of maxicells was found to have 2 clear populations of cells. The first population observed accounted for 46% and clearly exhibited red nucleoid signals, the second population were within the 'negative' fluorescence gate which indicated 53% were anucleated cells (fig.3.18C). This was significantly greater than 39% counted using microscopy that may be a result of cell debris counts within the population or chromosome decondensation in older cells reducing chromosome associated red fluorescence intensity. It was reassuring to see that there was a notable difference in the fluorescence phenotype and that chromosomal red fluorescence could be used to count nucleated cell and 'free' maxicell populations.

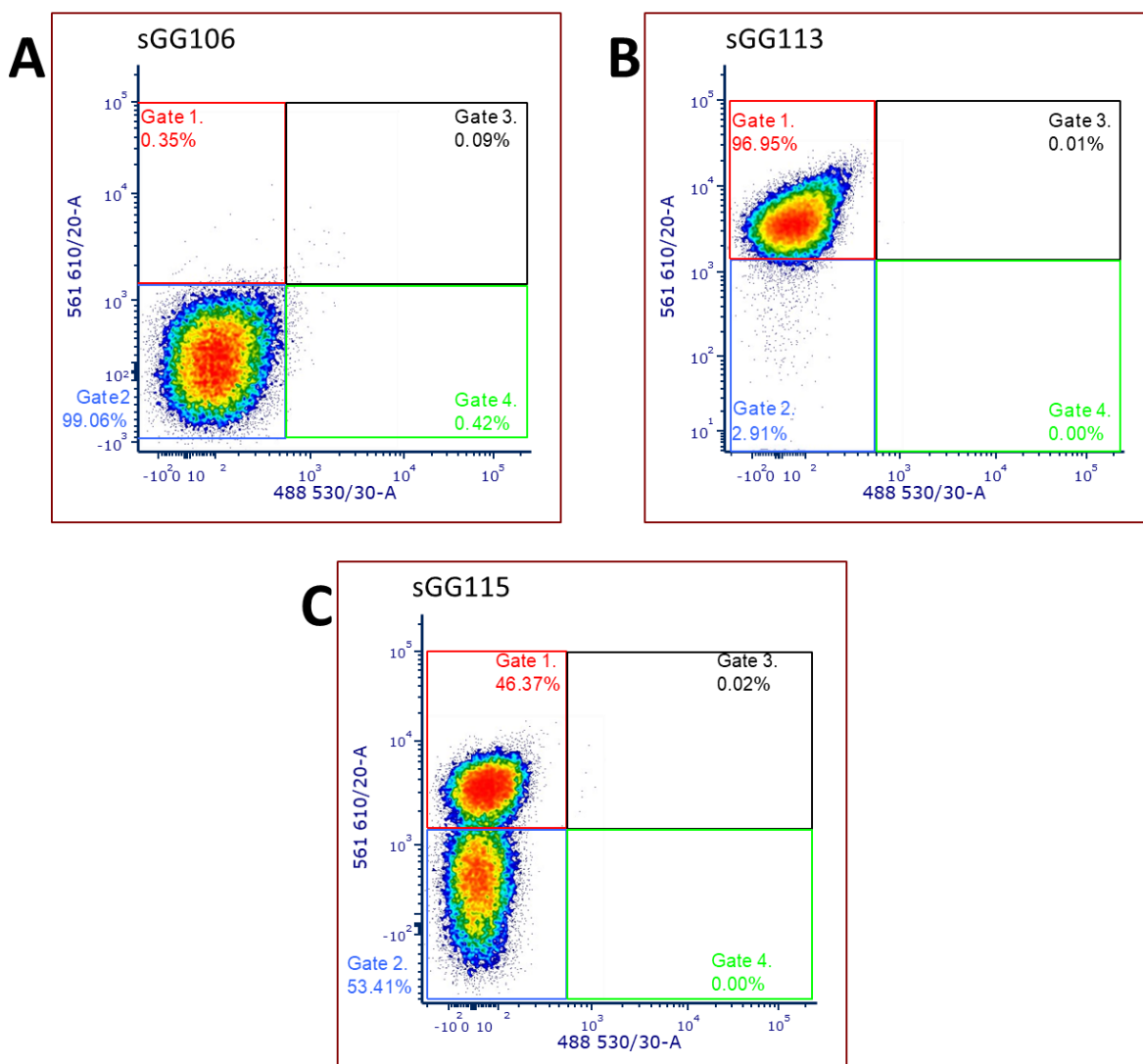


**Figure 3.18. Fluorophore analysis of maxicell producing strains using flow cytometry.** 50  $\mu$ l of mid-exponentially growing cultures were mixed with 500  $\mu$ l filter sterile PBS sheath fluid. Density plot colours represent population concentration - red showing high densities decreasing to yellow and blue populations showing lower densities respectively. Strains were analysed under lasers which excite red and green fluorophores (561 nm and 488 nm respectively). 30,000 events were counted per strain. Gating and other analysis were completed using FCS express 6.

As it was clear that, using Hu-mCherry, maxicells were able to be detected, flow cytometry fluorescent analyses were used further to see if the amount of GFP positive maxicells could be quantified. Gate 3 represented ‘double positive’ populations, where cells counted within this field were both showing GFP and mCherry fluorescence from plasmid expression and chromosome labelling. Analyses of sGG113 with pAG-P08 confirmed that the strain was able to repress expression of GFP from the plasmid, as less than 1% of the population were in gates 3 or 4 (fig.3.19A). When the plasmid was introduced into sGG113, there was a decrease in the number of cells which exhibited red fluorescence by comparison to the strain without the plasmid (fig.3.18B). Cells in the ‘negative’ gate accounted for 8.47% of the population which could have been counts reflecting cell debris or other particles. sGG115 strains with pAG-P08 revealed a more prominent shift in the populations fluorescence profiles where 26% of cells were GFP positive and negative for mCherry, indicative of maxicells independently expressing GFP (fig.3.19B – gate 4). There was 24% of anucleated cells which were negative for both fluorescence profiles (fig.3.19B – gate 2). Nucleated cells, indicated in gates 1 and 3, showed the majority of nucleated populations (36.35%) were repressing GFP expression from the plasmid (fig.3.19B - gate 1). Although it was suggested that maxicells accounted for larger populations of GFP expressing cells, consistent with microscopy data, there was also a population shift into the ‘double positive’ gate 3. This did not correspond with microscopy data, which suggested only 6% of nucleated cells were expressing GFP (fig.3.10). This discrepancy within the flow cytometry and microscopy data when counting ‘double positive’ nucleated and GFP positive cells may be a result of flow cytometry methods not being able to discriminate between cells in chains. It was assumed these counts could be chains of cells as shown in fig.3.19C where a chain may be counted as both nucleated and GFP positive but there are different cell populations within the one event (red arrows indicate anucleate cells expressing GFP). The amount of anucleated producers was similar to the microscopy data

which showed that there would be ~50% of the maxicell population expressing GFP, calculated to account for ~20% of the overall population (fig.3.10). Flow cytometry data agreed as it showed that 26% of the population were GFP positive maxicells, however, as it was identified that flow cytometry populations were not as refined because of chaining this could increase. Nevertheless, it was concluded that 26% of the ‘free’ maxicell population were positive for GFP expression.

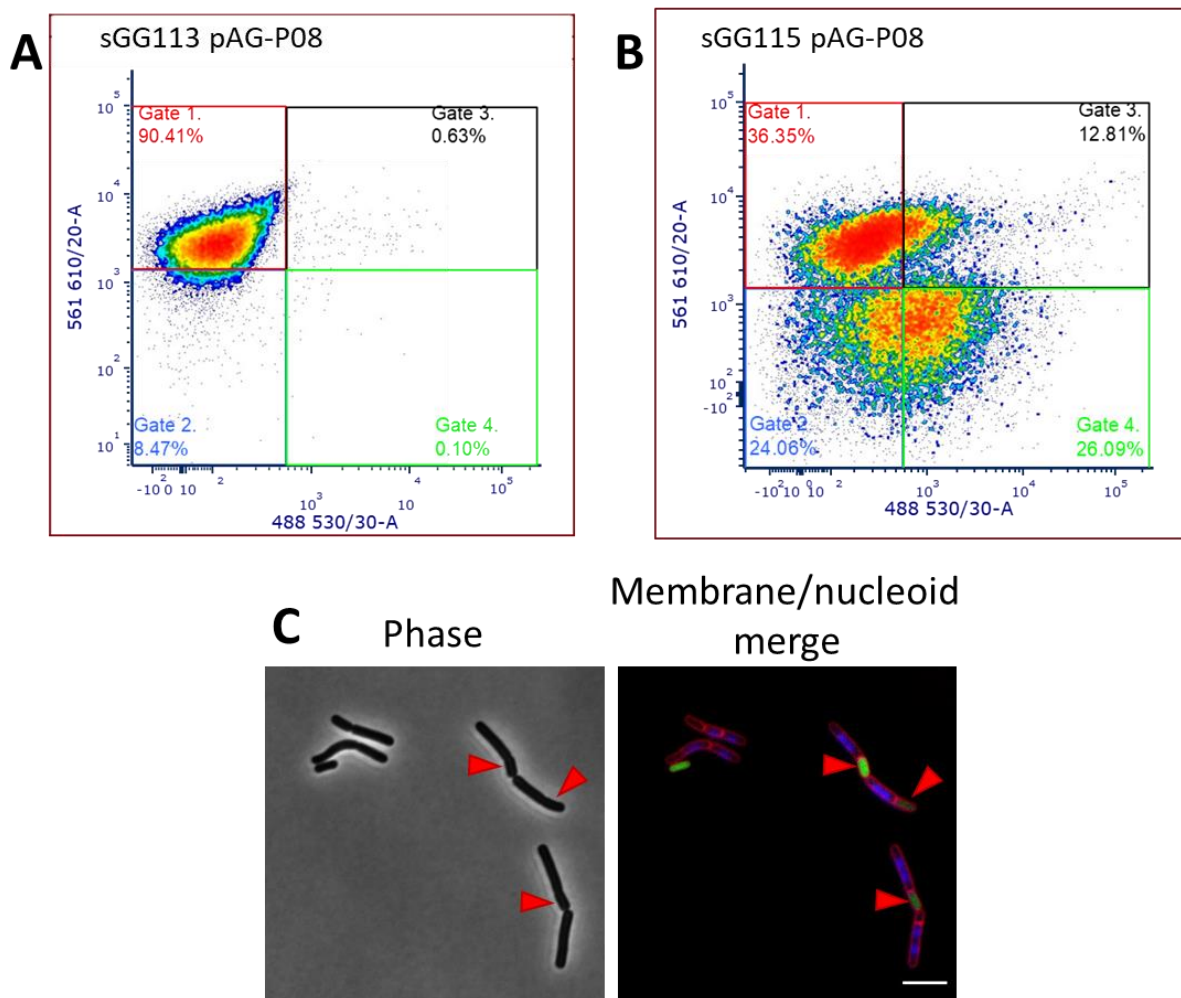


**Figure 3.19. Dual fluorophore detection in strains with pAG-P08 plasmid.** Mid-exponentially growing cultures were mixed with 500  $\mu$ l filter sterile PBS sheath fluid. 30,000 events were counted for each strain. Strains were analysed under lasers which excite red and green fluorophores (561 nm and 488 nm respectively). Density plot colours represent population concentration - red showing high densities decreasing to yellow and blue populations showing lower densities, respectively. Gating and other analysis were completed using FCS express 6. **A)** Wild-type strain with GFP plasmid to show repression efficiency in nucleated cells. **B)** Maxicell producing

strain with GFP reporter plasmid (pAG-P08) identifying nucleated and anucleate cells expression of the reporter.

C) Represent maxicell growth during mid-exponential growth that chains of cells are present. Membranes were stained with 0.6  $\mu\text{g/ml}$  FM5-95 dye and the nucleoid was detected using 1  $\mu\text{g/ml}$  DAPI, cells were images using a Nikon Eclipse Ti with Cool LED pE-300 white light source and Photometrics Prime camera. Scale bar is representative of 4  $\mu\text{M}$  and is applicable to all microscopy images.

### 3.4 DnaA is not required for pLS20 driven plasmid replication

#### 3.4.1 Plasmid behaviour in maxicell producing strains.

As little was understood about the pLS20 replicon and its mode of action, the role of DnaA was considered. Within the pLS20 origin, there was a DnaA box found during bioinformatics analyses by A. Guyet (unpublished). As the maxicell producing strain suffered with a DnaA mutation, it was unclear if there would be decreased plasmid replication or stability if the box was functional. After growth for 16 hours without antibiotic selection, cultures were serially diluted and colony forming units (CFU) observed on media with and without antibiotic selection. sGG115 pAG-P08 does not grow as dense as the wild type as a result of slower growth rate, however, it was still shown that the plasmid was stable. CFU were able to grow across all dilutions, regardless of antibiotic supplementation. As it appeared the plasmid was stable over 16 hours without selection, it was hypothesised that the DnaA box was redundant for plasmid replication.

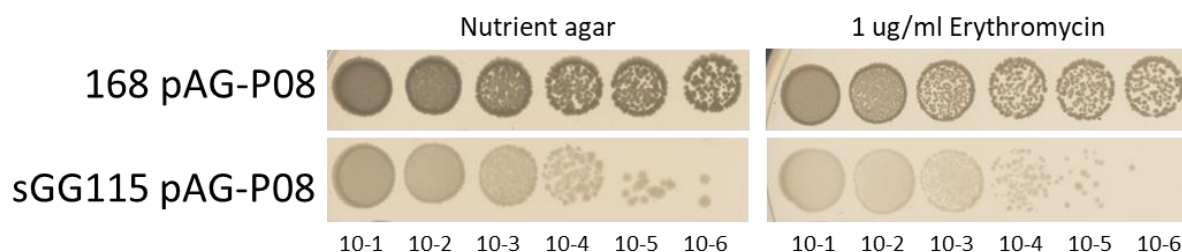


**Figure 3.20. Stability of the pLS20 replicon in maxicells.** Cultures were grown in a rich media for 16 hours at 37°C without the presence of antibiotic. No antibiotic selection allows the plasmid to be lost if there is an instability. After 16 hours, the cultures were diluted to an optical density (600 nm) of 0.3. This was followed by

10-fold serial dilution before spotting 5 µl onto nutrient agar plates without and with erythromycin selection. Images were taken after growth on plates at 37°C for ~24 hours with FujiFilm FinePix camera.

To see if there was an alternate role for the DnaA box, qPCR analyses were employed to see how the plasmid reacted when DnaA was mutated. The plasmid copy number was determined by comparison to the genome, therefore it was assumed that one cell contained one genome. Then the number of plasmids was identified via the ratio of terminus:plasmid. In the control strain (sGG113), the pLS20 replicon during mid-exponential growth was present at 2-copies per cell (fig.3.21 – teal bar; Meijer *et al.*, 1995). In sGG115, when maxicells were produced, there was a significant increase in copy number (fig.3.21 – purple bar). As chromosome replication was slower, it was expected that there would be a change in copy number. However, as it was indicated by flow cytometry and microscopy analyses that ~50% of the population were maxicells, then it was only expected that the copy number would increase by ~50% when compared to sGG113. This dramatic increase in copy number suggested a few possibilities such as DnaA may be working as a negative regulator, there was significant replication of the plasmid in maxicells or both may have occurred as DnaA was diluted out



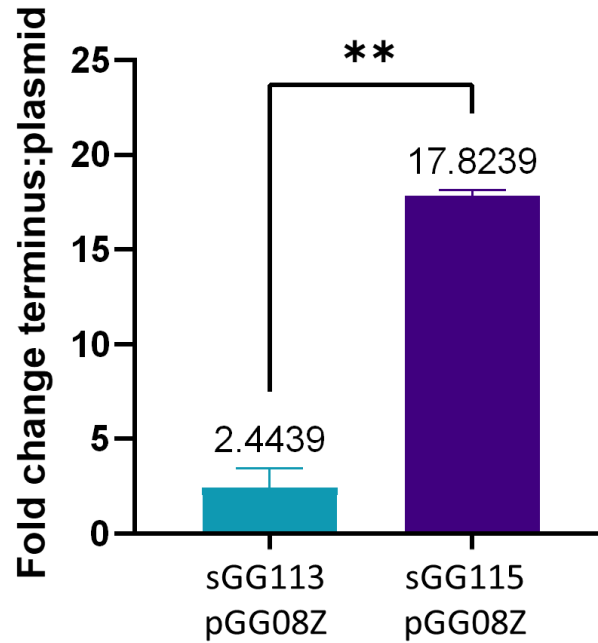


**Figure 3.21. Plasmid copy number in maxicell producing strains.** Strains carrying the pGG08Z plasmid were grown in CDM at 30°C, a 5 ml aliquot was taken during mid-exponential growth, processed in lysis buffer and sonicated before centrifugation. The lysates supernatant was diluted 1000x before depositing into tubes using the Qiagen Qiagility Robot with NEB Luna universal qPCR master mix and appropriate primers to detect chromosome terminus or plasmid. The qPCR samples were run and data was analysed using Rotor-Gene Q software. Terminus:plasmid ratios were calculated using Microsoft Excel. Error bars represent triplicate experiments.

### 3.4.2 pLS20 DnaA box mutagenesis

**DnaA box mutagenesis cloning.** As mentioned previously, there was a single DnaA box identified by A. Guyet (unpublished) as shown in fig.3.22A. This box was mutated in order to retain the same number of nucleotides but replacing the sequence for that of a restriction site (XbaI) (fig.3.22B). There were successful clones attained of pGG018 and sequenced before transformation into the Portabolomics strains. Transformations with the  $\Delta dnaA box$  plasmid were successful within all *Bacillus* Portabolomics strains, showing that this box was not required for replication of the pLS20 origin. Although assumed to be redundant, it was still

questioned if the DnaA box could have a role in copy number regulation as it was seen in the DnaA mutant that copy number increased (fig.3.21).

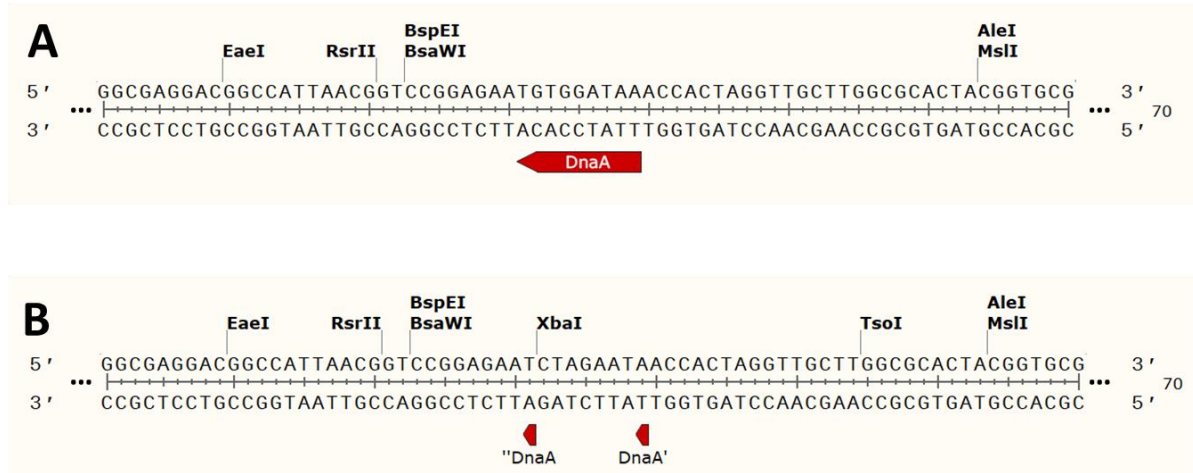


**Figure 3.22. Demonstration of the DnaA box mutagenesis.** The mutant plasmid was made via PCR with specific oligonucleotides to change the DnaA box sequence for that of a palindromic sequence recognised by XbaI restriction enzyme. The linear PCR fragment was cut and religated with T4 DNA ligase (NEB) to produce pGG018. **A)** the native DnaA box within the pLS20 sequence (whole sequence not shown) **B)** the mutated DnaA box after replacing with palindromic sequence. Images from SnapGene.

Plasmid quantification was used once again to see if the DnaA box mutation had effected copy number. In this experiment, a control strain was used (sAG1392) and a minicell producing strain (sAG1338) to see how the mutant plasmid affected replication in these chassis compared to pAG-P08. In sAG1392, the copy number of pAG-P08 was ~2 copies per cell (fig.3.23A black bar), however, the DnaA box mutation produced significant reduction in copies per cell (fig.3.23A grey bar). A 2-sample T-test was carried out between both plasmids with Welch's corrections as variances were not equal, which demonstrated a significant difference between pAG-P08 and pGG018 in sAG1392 ( $P=0.02$ ). When the plasmids were introduced into sAG1338, there was an even greater significant decrease in copy number with pGG018 ( $P<0.001$ ). pAG-P08 showed a copy number of ~4 copies per chromosome, whereas pGG018 was only present at ~1 copy per cell. The significantly greater reduction in pGG018 replication in sAG1338 may have confirmed the hypothesis that plasmids were replicating in minicells.

Reduced copy number meant that less minicells gained a plasmid, therefore replication within minicells would not occur or effect copy number detected. Overall, it was suggestive that the DnaA box may have a role in regulating plasmid copy number.

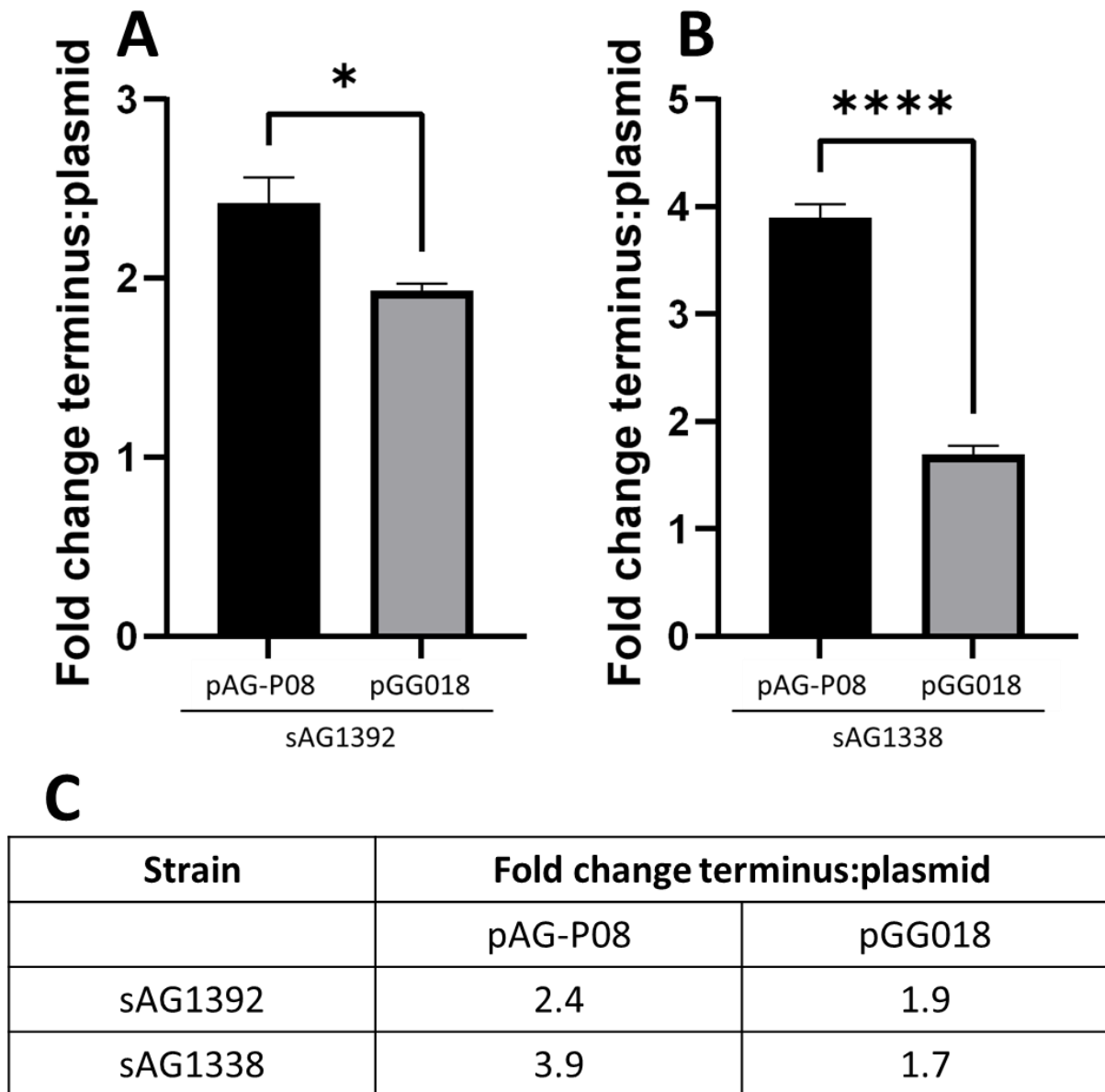


**Figure 3.23. Quantification of plasmid per chromosome with WT *oriLS20* and *Adna.Abox oriLS20*.** Cultures were grown in CDM at 30°C where aliquots were taken during mid-exponential growth. At the time point, 5 ml of culture was harvested and gDNA extracted using lysozyme and sonication. Supernatant was diluted 1000x before performing qPCR with NEB Luna polymerase. Terminus:plasmid ratios were calculated using Microsoft Excel and graphs plotted with GraphPad prism 9. **A)** comparisons of the plasmids in the same sAG1392 background **B)** comparisons of plasmids in the minicell producing Portabolomics strain sAG1338. **C)** plasmid to

chromosome ratios. Error bars are representative of 3 technical repeats, although qPCR data was run in duplicate on separate occasions.

To see if there were any effects within sGG115 with *dnaAG152S* mutations, the same plasmid quantification was carried out. In the control strain, sGG113, there was a significant reduction in pGG018 detected (fig.3.24A;  $P=0.002$ ). pGG018 was counted as <1 copy per cell which was likely to be a result of loss of plasmid, potentially with such low copy number the plasmid is diluted out and skewing the amount terminus:plasmid ratio (fig.3.24C). Interestingly, the same reduction in copy number was not matched within sGG115 (fig.3.24B). There was no significant difference in the copy number between pAG-P08 and pGG018 ( $P=0.45$ ), this indicated that DnaA had a role in controlling copy number as the DnaA box deletion did not have any effect with mutant DnaA. That could also support the hypothesis that maxicells were gaining a plasmid and replicating them independently as the copy number is significantly higher than that of nucleated strain sGG113 (fig.3.24C). Potentially, maxicells had a limit for how much plasmids were replicated, and this is why there was no significant difference between pAG-P08 and pGG018. It was already hypothesised that DnaA may be regulating plasmid copy number, so the lack of the DnaA box likely does have a role in anucleate compartments. Significance was determined the same as mentioned previously using a T-test with Welch's corrections.

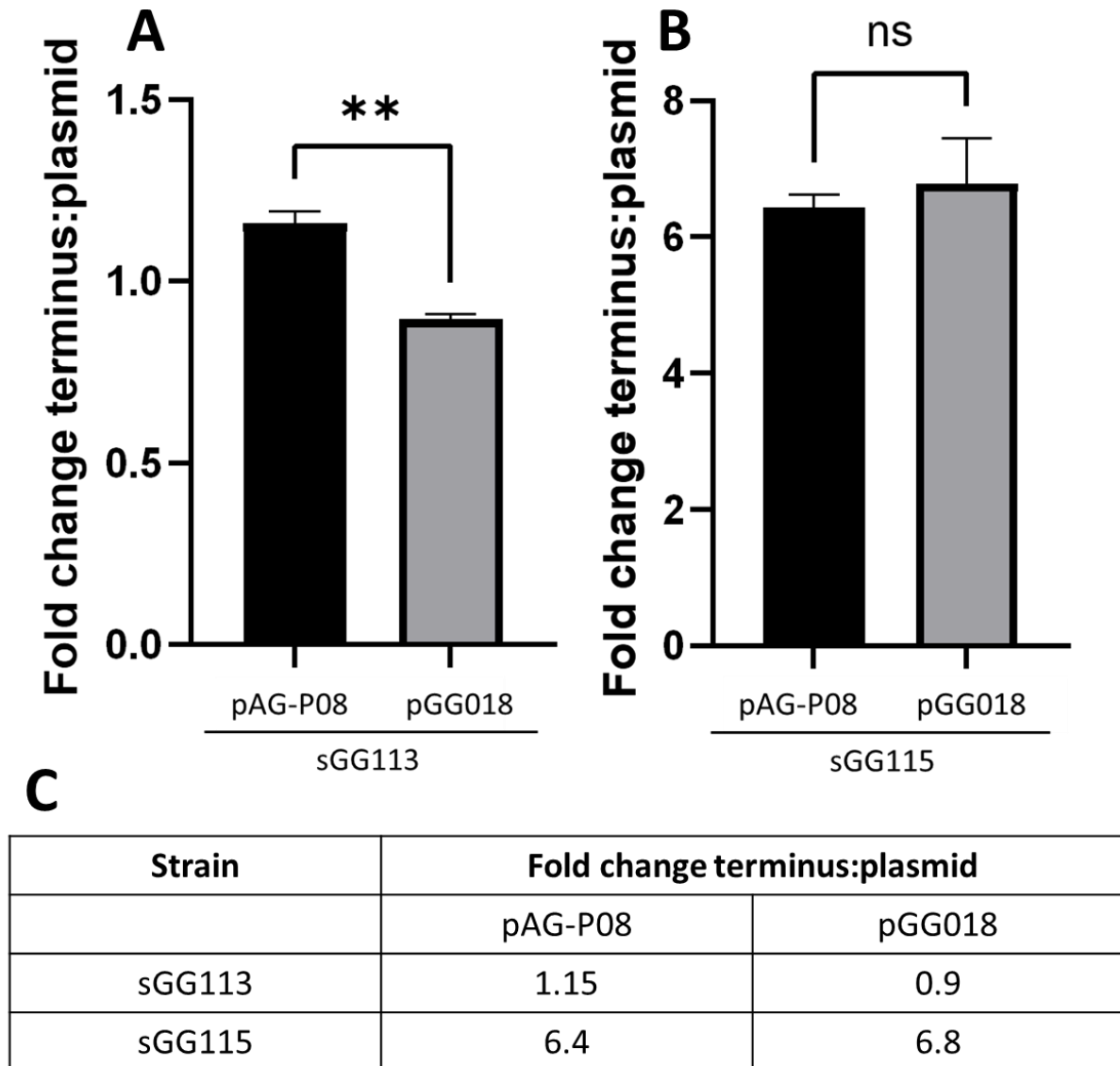


**Figure 3.24. pLS20 DnaA box mutagenesis in maxicell producing strain.** Strains with plasmids were grown in CDM with the presence of erythromycin for selection. During mid-exponential phase at 30°C 5 ml aliquots were harvested and lysed in lysozyme buffer with subsequent sonication. DNA preparations were diluted 1000x before running qPCR with NEB Luna polymerase. Plasmid copy numbers were calculated in Microsoft excel by determining ratio of terminus:plasmid. Graphs were plotted using GraphPad prism 9. **A)** sGG113 background strain comparison of plasmids **B)** Maxicell Portabolomics strain sGG115 comparison of plasmids **C)** fold change of plasmid to chromosome. Error bars are representative of triplicate technical repeats.

## 3.5 Industrial uses for anucleated chassis of *B. subtilis*

### 3.5.1 Using protein A constructs

To see if these chassis were able to show a proof of concept for industrial applications, protein A was used as a reporter. As mentioned within the introduction, protein A is native to *S. aureus* and therefore it would be interesting to see if compartments developed in this study could express, secrete and attach heterologous proteins to the cell wall. Both mini- and maxicell chassis were transformed with protein A reporter plasmids. The constructs of Amirah Alofi (unpublished) were used where  $P_{spac}$  inducible promoter (fig.3.25A), similar to previous plasmids pAG-P08 and pGG08Z, controlled *spa* expression (fig.3.3A & B). pAM011 (fig.3.25A) was transformed into *B. subtilis* Portabolomics strains, and Western blot analyses showed that without induction bands were present but faint (fig.3.25B). On the other hand, the addition of IPTG to induce expression from the whole population revealed 2 bands were present, this was assumed to be where the signal peptide was cleaved off when the protein was translocated over the cell wall. When exposed for a greater period of time, the strains with no induction did show visible bands (fig.3.25C). The longer exposure was required as the uninduced strains did not express the protein as abundantly, this was expected as, if the model worked correctly, only mini- and maxicells should express from the plasmid. All strains displayed 2 bands similar to the IPTG induced strains, the sGG115 pAM011 showed a denser band which may be a result of greater expression.

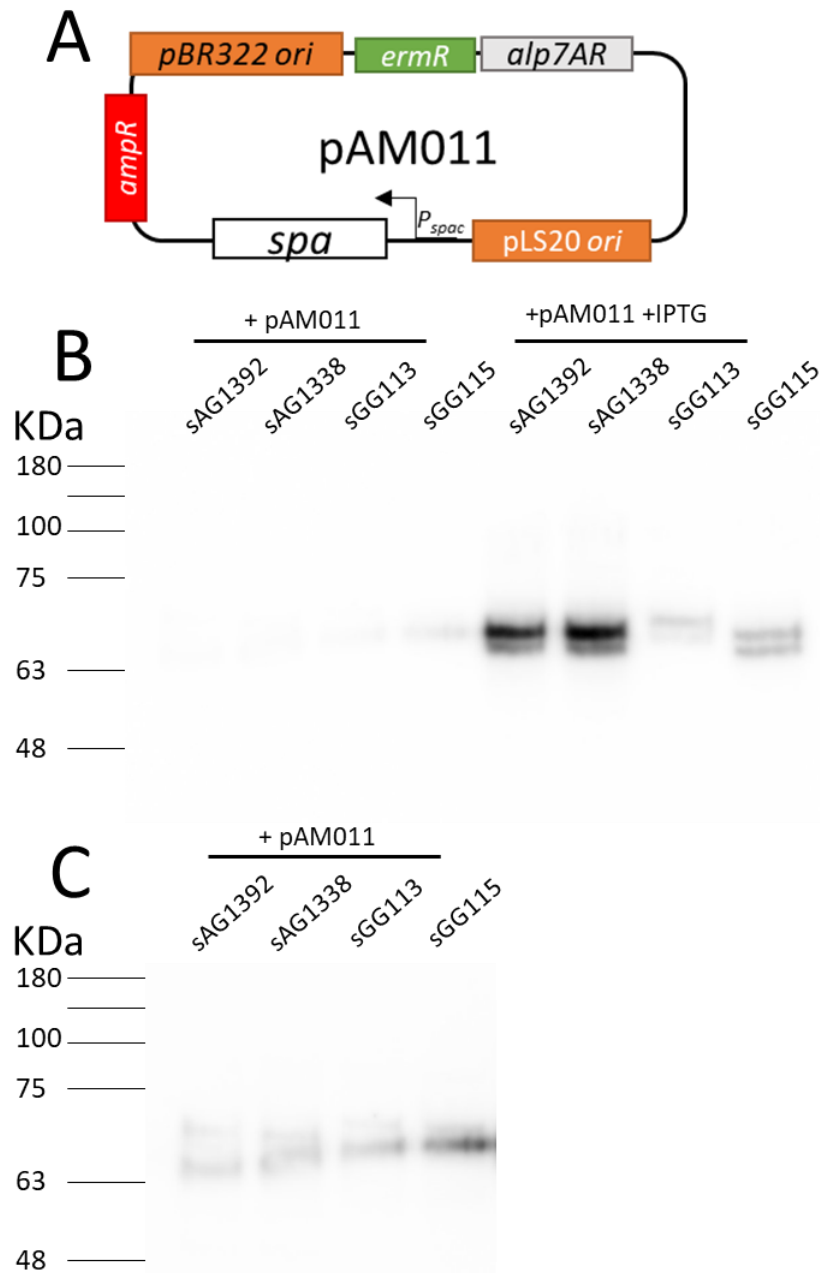


**Figure 3.25. Protein A expression from wild type, mini- and maxicell strains.** **A)** the plasmid is the same backbone as others described but replaces *gfp/lacZ* for the protein A encoding gene *spa* amplified from *Staphylococcus aureus* RN4220. The gene was under the control of  $P_{spac}$  inducible promoter and therefore should be repressed in nucleated cells with LacI production. **B)** Cell lysates were separated on a 4-12% gradient SDS-PAGE before transferring to PVDF membrane.  $\alpha$ -Protein A (Abcam) was used to label the protein with  $\alpha$ -rabbit HRP secondary labelling (1 minute exposure). **C)** the same immunostained image with greater exposure for weaker bands (10 minutes) Images were analysed using Fiji imageJ software.

It was clear that protein A was expressed, however, it was hypothesised that the carbohydrate binding domain (CBD) of a native autolysin could enhance cell wall attachment. Therefore, a fusion protein was made. The native autolysin *lytC*'s CBD was used to replace LysM of protein A (shown in blue in fig.3.26A). After transformation into the Portabolomics *B. subtilis* strains, the proteins expression levels were investigated using western blot. Without the addition of the IPTG inducer, there were bands present in minicell and maxicell producing strains (fig.3.26B red arrows). However, these bands were not very intense, so a greater exposure time was used for better analyses of expression. With greater exposure to non-induced strains, the bands were clearer and demonstrated that anucleated compartments were expressing the proteins more than parental controls. This was encouraging as it demonstrated that mini- and maxicells were likely expressing the fusion protein from the plasmid independently (fig.3.26C). With the addition of IPTG to induce expression from all cells with a plasmid, there was a clear increase in the fusion protein's abundance (fig.3.26B). It was clear in all strains that, unlike native protein A (fig.3.26B & C), there was only one band present. This was potentially suggesting that, since the signal peptide was occluded by the addition of the *lytC*(CBD), there was no peptide cleavage leading to translocation over the cell membrane.



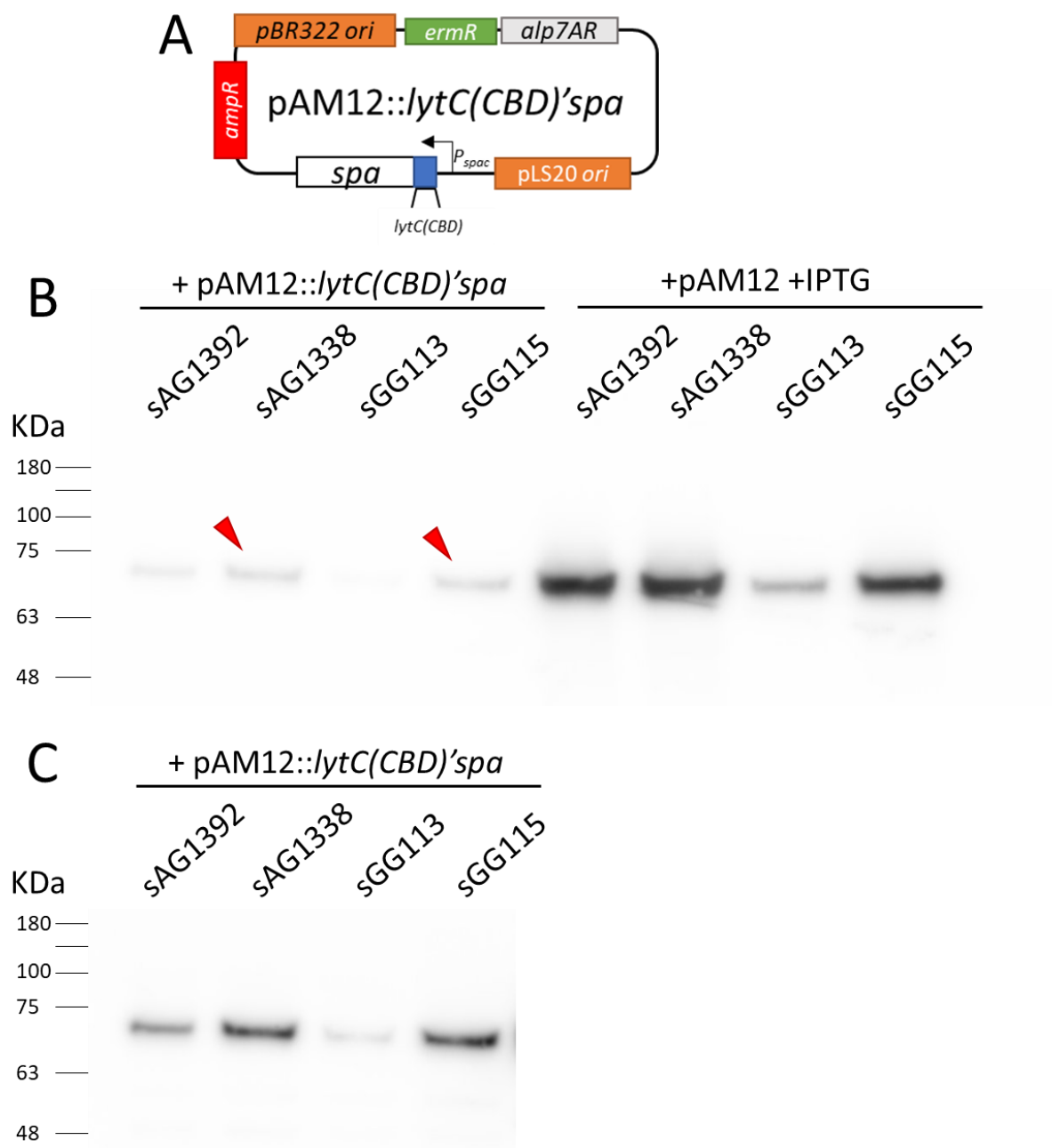


**Figure 3.26. Attachment of a native *B. subtilis* carbohydrate binding domain.** **A)** the plasmid with the same backbone as others described but replaces *gfp/lacZ* for a chimeric protein with autolysin *lytC* carbohydrate binding domain replacing LysM of protein A. The chimeric gene was under the control of  $P_{spac}$  inducible promoter and therefore repressed cells with LacI production. **B & C)** Cell lysates were separated on a 4-12% gradient SDS-PAGE before transferring to PVDF membrane.  $\alpha$ -Protein A (Abcam) was used to label the protein with  $\alpha$ -rabbit HRP secondary labelling (**B**) 1 minute exposure; **C**) 10 minute exposure). Images were analysed using Fiji imageJ software.

### 3.5.2 Expression and secretion of protein A

As it was known that protein A was expressed and was hypothesised to be translocated over the inner membrane, localisation assays were performed. Fixed cells were immunolabelled to see the localisation of protein A, in the sAG1392 control strain there was high signals associated with the cell wall when expression was induced with IPTG (fig.3.27A). This was assuring as it indicated that *B. subtilis* was able to secrete the protein and it looked as if it could be integrated into the cell wall (fig.3.27A). Without induction (fig.3.27B), there was some non-specific fluorescence between clusters of cells which was also seen in the no plasmid control (appendices 4). This indicated that the repression system was working well and any leaky protein A expression was not affecting immunolabelling.

In sAG1338 (fig.3.27C-D), IPTG induction did show that there was expression and intense fluorescence signals in all cells. The localisation of the protein appeared to be cell wall associated, which was prominent in minicells as well as rod cells. In the IPTG induced strain, it was obvious that there were some stronger septal associated signals which indicated where the majority of protein A translocation/insertion takes place (fig.3.27C). In sAG1338 without induction, there were minicells with strong cell wall like staining suggesting that minicells could be decorated with protein A expressed from the plasmid (indicated with yellow arrows – fig.3.27D). The nucleated cells did not have staining as bright by comparison to the minicells. However, the amount of protein A labelled minicells did not seem to reflect that of GFP expression which may be a result of minicells taking longer to secrete the protein, therefore the protein remained cytoplasmic.

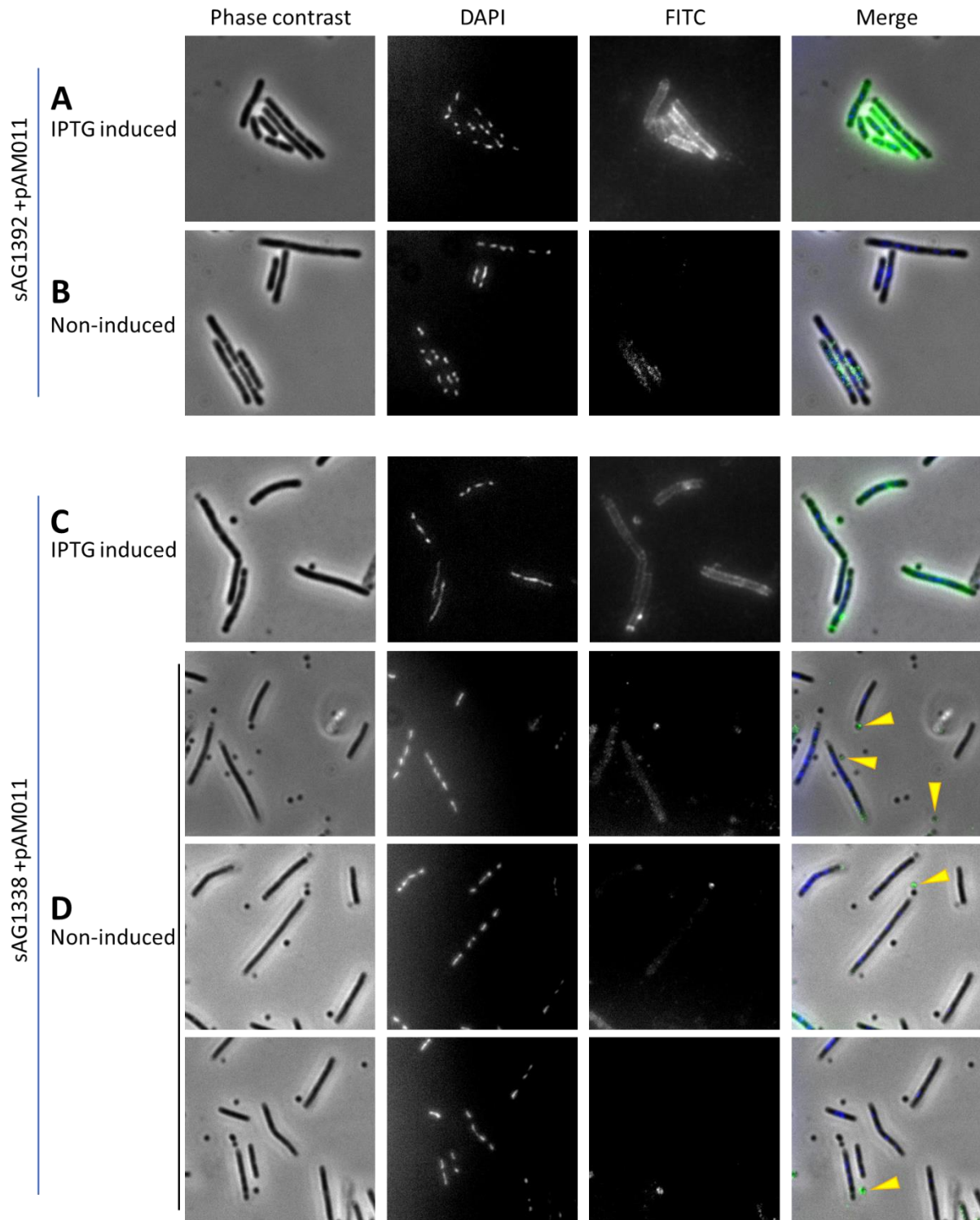


**Figure 3.27. Protein A expression from sAG1392 and sAG1338 with different induction conditions.** Cultures were grown in CDM at 30°C for 4 hours before aliquoting for fixation. Harvested cells were fixed in 1% paraformaldehyde/PBS before attaching to slides with 0.01% poly-L-lysine. The cells were incubated with the primary antibody overnight at 4°C before washing and incubating with the FITC-labelled secondary antibody for 1 hour 30 minutes at room temperature. After a second wash, mounting medium with 0.2 µg/ml DAPI was applied

and imaged using a Nikon Eclipse Ti with Cool LED pE-300 white light source, Photometrics Prime camera and Metamorph 6 software. A) sAG1392 with pAM011 and IPTG induction. B) sAG1392 with pAM011 and no induction. C) minicell producing strain with pAM011 and IPTG induction. D) minicell producing strain with pAM011 and no induction.

Western blot analyses showed that there were 2 clear bands from maxicell producing strains, therefore it was hypothesised that maxicells showed greater efficiency at expressing protein A. To see if maxicells were expressing and integrating protein A into the cell wall, immunostaining analyses were performed for localisation. With IPTG induction, the sGG113 control strain displayed membrane associated signals which appeared brighter at the septal PG (fig.3.28A). Without IPTG induction, non-specific low intensity signals were detected mainly in between clusters of cells (fig.3.28B). This was similar to the no plasmid controls shown in appendices 4 and represented good repression of the reporter gene in this strain. When maxicells were produced (sGG115; fig.3.28C & D), IPTG induction exhibited heterogeneous staining across both nucleated and anucleated cells (fig.3.28C). When cultures were grown in the absence of induction, there was obvious staining specifically associated with anucleated cells indicated by yellow arrows (fig.3.28D). With regards to expression, there was no difference between maxicells which were attached or detached to mother cells, examples shown with yellow arrows revealed that expression could happen in either incidents (fig.3.28D). Staining of maxicells appeared to be punctate around the cell wall, there did not seem to be any explicit patterning as the localisation in each maxicell looked different.

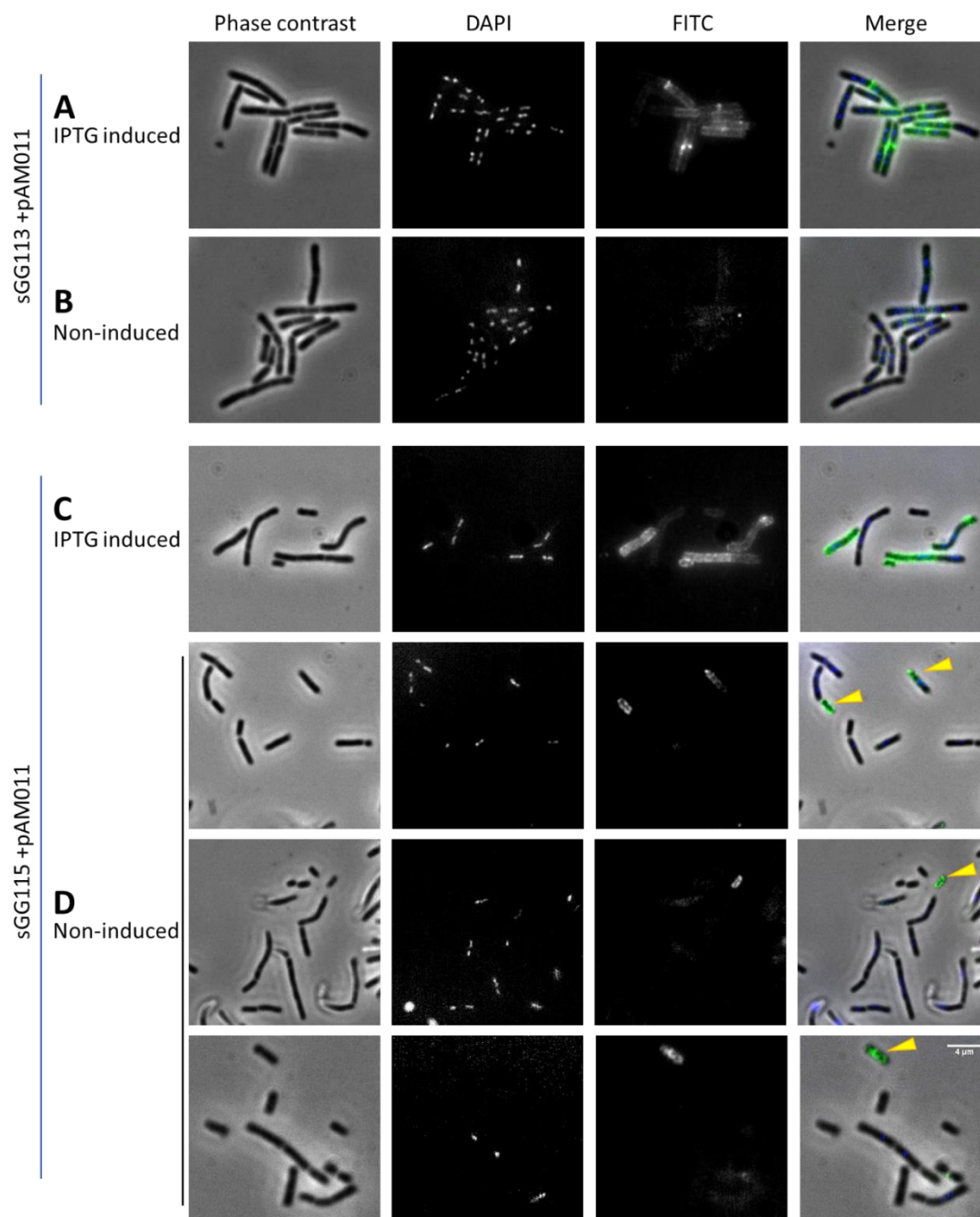


**Figure 3.28. Protein A expression from sGG113 and sGG115 strains in different induction conditions.**

Cultures were grown in CDM at 30°C for 4 hours before aliquoting for fixation in 1% paraformaldehyde/PBS. Fixed cells were attached to slides with 0.01% poly-L-lysine and incubated with  $\alpha$ -Protein A primary antibody overnight at 4°C. After washing, cells were incubated with FITC-labelled secondary antibody for 1 hour 30 minutes at room temperature. After a second wash, mounting medium with 0.2  $\mu$ g/ml DAPI was applied and imaged using a Nikon Eclipse Ti with Cool LED pE-300 white light source and Photometrics Prime camera, Metamorph 6 software. A) IPTG induced sGG113 with pAM011. B) sGG113 with pAM011 and no induction. C) sGG115 with pAM011 induced with IPTG. D) sGG115 with pAM011 and no induction.

The fusion protein outline in fig.3.26 was hypothesised to have greater attachment abilities to the cell wall as it contained the native CBD of autolysin *lytC*. There were worries from Western blot analyses that the protein was not secreted as only 1 band was present, therefore, localisation assays were performed. The *LytC* CBD fusion to protein A showed limited staining in cells with or without IPTG induction. In the absence of IPTG there was one cell (fig.3.29B) with mid-cell localisation but this was not prominent across the whole population of cells as shown in the image. Other cells did not show any specific staining. This supported the hypotheses that the protein was not translocated over the cell wall, and therefore must have remained in the cytoplasm.

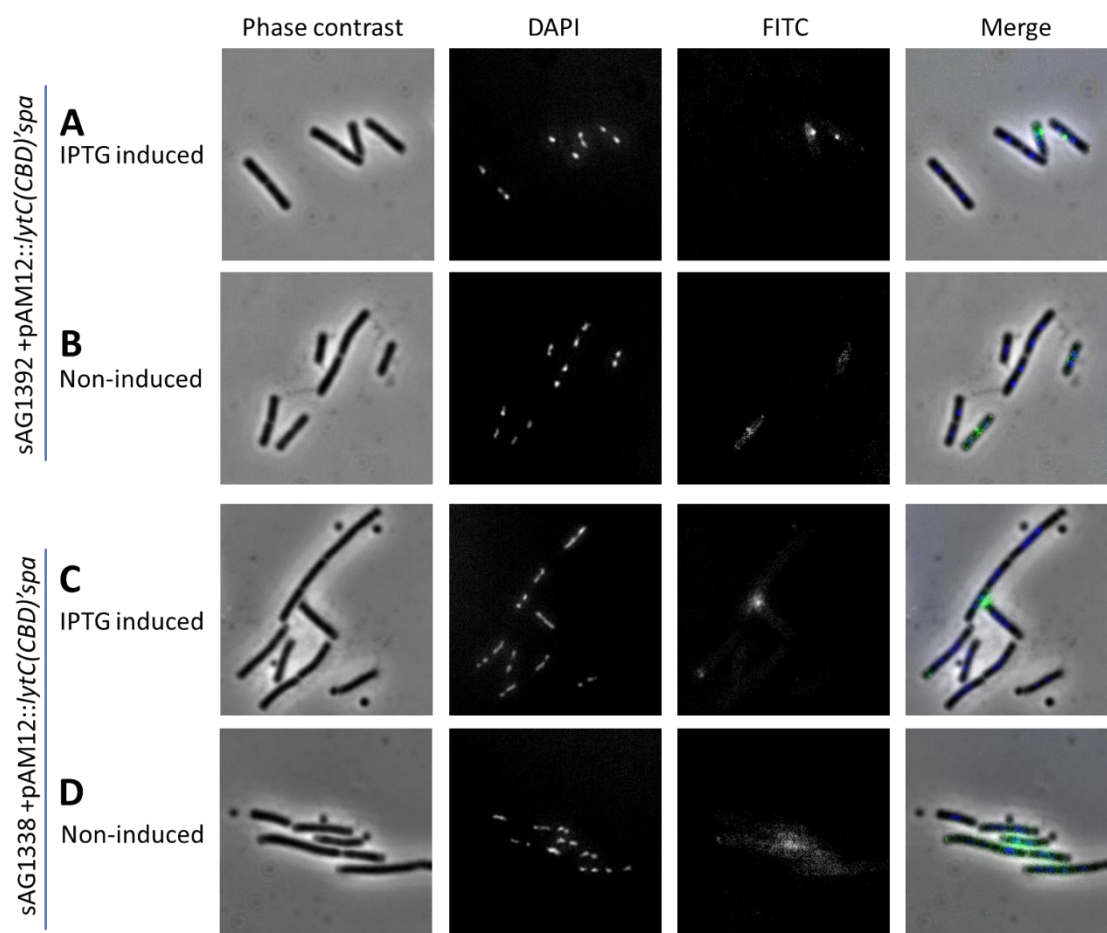


**Figure 3.29. Minicell strains expression of *LytC*-Protein A fusions.** Strains were grown in CDM for 4 hours at 30°C before harvesting and fixing in 1% paraformaldehyde/PBS solution. Cells were adhered to glass slides via 0.01% poly-L-lysine fixation and blocked with 2% BSA before overnight incubation with  $\alpha$ -Protein A at 4°C. Protein A was detected using a FITC labelled secondary antibody, incubated at room temperature for 1 hour 30

minutes before cells were washed and mounted in medium containing 0.2 µg/ml DAPI. A+B) sAG1392 population with plasmid A) with 1mM IPTG induction B) no inducer added. C+D) sAG1338 pAM12::*lytC(CBD)*'*spa* C) with IPTG induction D) no induction. Cells were imaged with Metamorph 6 software on a Nikon Eclipse Ti with cool LED light source.

To further investigate the localisation of the fusion protein, immunostaining was also carried out within sGG113 and sGG115. The rationale being that, although minicells did not express, maxicells could be more likely to have the appropriate machinery on the cylindrical body. Similar to what was described previous, in both sGG113 and sGG115 there was very little staining in any of the cells and any staining present did not form specific foci like wild type protein A (fig.3.28). IPTG induction in either strains did not feature any cell wall specific foci (fig.3.30A & C). This indicated that the protein had not been translocated over the membrane or attached to the cell wall. Since the protein was known to be expressed via Western Blot (fig.3.26B), it was likely that the protein was within the cytoplasm of the cell. This was also supported by the lack of signal peptide cleavage seen in the Western Blot analyses (fig.3.26B).

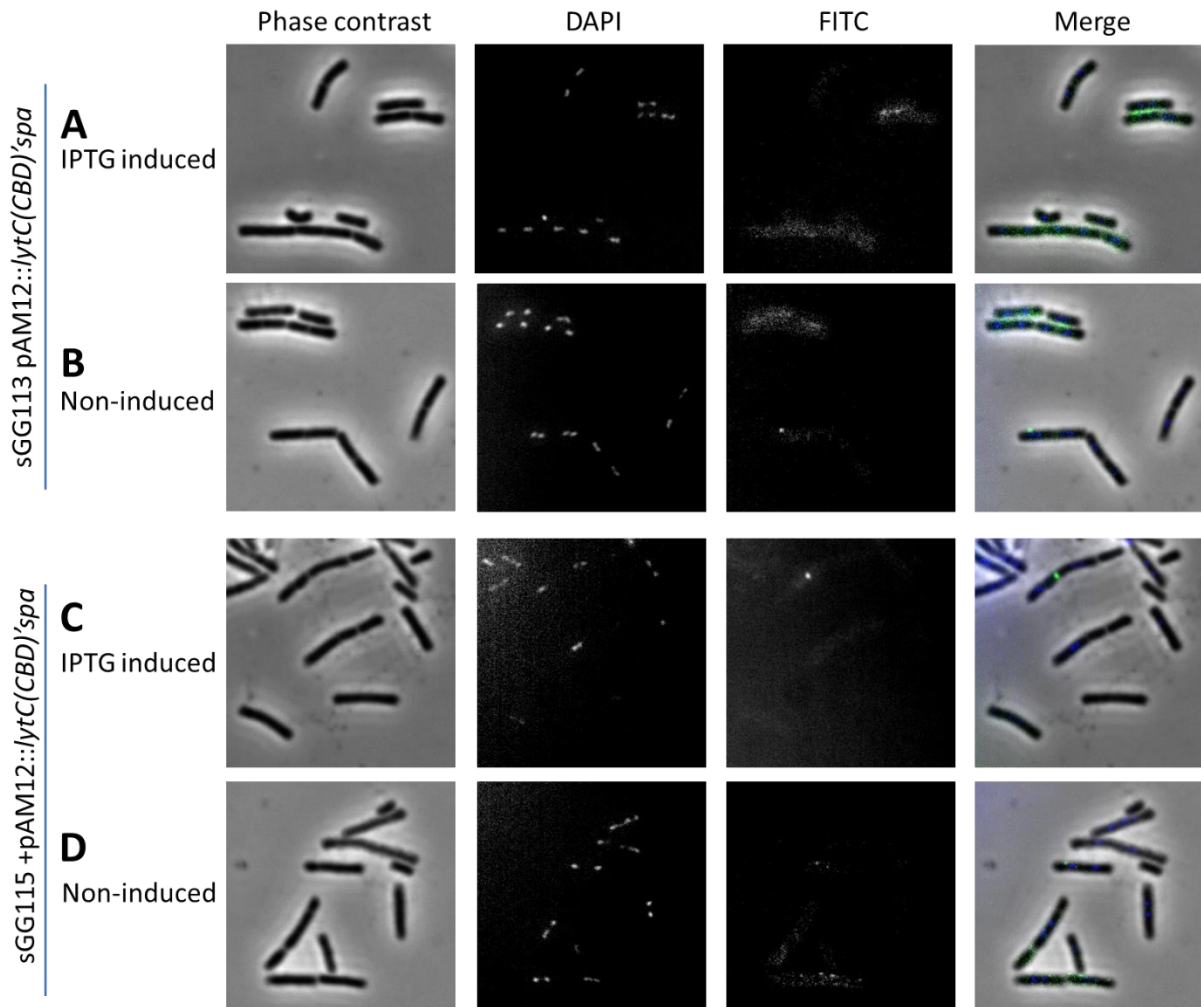


**Figure 3.30. Maxicell producing strains with LytC-Protein A fusion reporter.** A & B) sGG113 population with plasmid A) 1mM IPTG induction B) no inducer added. C & D) sGG115 C) with IPTG induction D) no induction. Strains were fixed in paraformaldehyde solution before mounting to glass slides with poly-L-lysine, blocking with 2% BSA. Primary antibodies were incubated overnight at 4°C and for detection FITC labelled secondary antibodies were used. Cells were imaged using Metamorph 6 software with a Nikon Eclipse Ti with cool LED light source.



## 3.6 Discussion

In this chapter, *Bacillus subtilis* anucleate cell producing strains were constructed and characterised. Different methods of anucleate cell production were explored to see if there were strains which differed in the proportion of compartment specific production and expression. Performance of the plasmid in these strains was also explored to understand how capable the vector was for segregating into anucleate compartments.

### 3.6.1 *Bacillus subtilis* minicell chassis characterisation

Minicells of *B. subtilis* had already been exploited for compartmentalised expression by Aurélie Guyet (unpublished) before this study had started. It was hypothesised that the LacI system would repress expression in the nucleated mother cells and, after an asymmetric septum formed, minicells would then allow expression (see section 1.5 for model). High copy number plasmids have been used frequently in previous studies with minicells as they were more likely to gain a plasmid from differential diffusion within the cytoplasm. A recent publication demonstrated this as, although greater quantities of minicells were gaining a plasmid, there was no control of expression within the rod cell and therefore expression was prevalent in all cells (Yu *et al.*, 2021). High copy number plasmids led to the inability to regulate expression in rod cells, however, there was also evidence to show that they can also impose a metabolic burden even before inducing expression (Ceroni *et al.*, 2015). To meet the aims of this study, control of expression was important which was why low copy number plasmids were used. However, low copy number plasmids do encompass limitations associated with instability when the plasmid is not efficiently segregated into daughter cells. Therefore, this study utilised Alp7AR operon as it was known to segregate pLS20 plasmids within *B. subtilis*, increase stability and force plasmids to the poles of the cells which would help minicells gain a plasmid (Derman *et al.*, 2012). Although plasmid copy numbers were low, it was still shown that 50% of the minicell population were GFP positive, this was an encouraging starting point but more work

was required to improve the number of minicells with a plasmid. Data from this study did show that there was some leaky expression from  $\beta$ -galactosidase activity assays, however, this was not detectable in microscopy analyses (see section 3.2.2).

Minicells were chosen for compartmentalisation as they were always abundant in cultures containing appropriate mutations to affect Z-ring placement, however, minicell production can be affected by growth phase and rich media (Frazer and Curtiss, 1975). Stationary phase is a stage where cells no longer grow but remain metabolically active, this phase is often triggered when nutrients become limited or other stressors are introduced and rapid growth is no longer optimal (Jaishankar and Srivastava, 2017). The optical density is maintained well during stationary phase as there are some divisions which is equal to the rate of cell death. However, it was evident from studies by Reeve *et al.* 1973 that minicell production frequency seemed to be highest during late-exponential to early stationary phase. With further studies suggesting that minicells were produced for up to 7 hours during stationary phase (Frazer and Curtiss, 1975). During stationary phase, cell chains are separated as cells shorten and become motile accommodated by autolysins *lytC* and *lytD* (Blackman *et al.*, 1998). Therefore, results described by Reeve *et al.* may be a result of greater autolysin activity which did not produce more minicells but released more from attachment to rod cells. Analyses of cultures in this study were mainly carried out during mid-exponential growth, this was because data by Vagner *et al.*, 1998 showed that  $P_{spac}$  induction was reduced 10-fold during stationary phase which, in turn, could affect the number of minicells expressing reporters. However, if enrichment techniques were required, it would be better to harvest during stationary phase as more free minicells would be yielded. For minicell enrichment waiting until stationary phase may not be optimal, therefore, growth media has been found to be an important factor in minicell production with liquid minimal media supplemented with 1.5% casamino acids and 0.5% glucose found best (Frazer and Curtiss, 1975), alongside rich medias such as Penassay broth

(Lennox, 1955). The concentration of casamino acids was reported to be important for minicell production, media with low amino acids equated to low yields of minicells (Frazer and Curtiss, 1975). Therefore, growth media should be considered to increase minicell production for any industrial applications as more minicells would yield more product.

To have self-regulated expression it was hypothesised that once a minicell was made, the LacI transcriptional repressor was depleted which would allow the reporter to be expressed from the plasmid. However, data from this study revealed LacI was relatively stable and had a long half-life (see section 3.5.1). This indicated that LacI likely remained in the cytoplasm and would actively repress expression from the plasmid, therefore, more minicells in the population could have a plasmid but unable to express. Similar experimentation in *E. coli* by Purcell *et al.* also revealed that, using chloramphenicol to inhibit protein synthesis, LacI was not readily degraded unless the protein was SsrA tagged. In fact, those without an SsrA tag did not deviate from time 0 and showed over 60 minutes that LacI is stable after protein synthesis inhibition (Purcell *et al.*, 2012). Studies of LacI localisation and single molecule tracking found that, in *E. coli*, LacI was mobile and mainly localised around the nucleoid (Garza de Leon *et al.*, 2017). If this localisation was the same in *B. subtilis*, then it could be likely that not many LacI molecule are entering minicells and does not require much degradation. Another hypothesis about minicells overcoming LacI repression was demonstrated when strains producing minicells increased plasmid copy number and potentially out competed LacI (see section 3.2.4). There was also a suggestion that DnaA may play a role in pLS20 copy number by acting as a negative regulator (section 3.3), if this was true then it is plausible that minicells depleted of DnaA were able to upregulate plasmid replication. The contents of the minicells cytoplasm are largely unknown, although it is reported that they contain ribosomes, protein, RNA and plasmids (Farley *et al.*, 2016). A further future direction would be proteomic analyses of minicells to understand their contents.

### 3.6.2 Maxicell chassis development and characterisation

Maxicells were of interest because their larger size and were assumed to increase likelihood of compartments gaining plasmids and having more cytoplasmic machinery to allow greater expression. However, data suggested that maxicells did have approximately the same number of GFP positive cells as minicells (50%; see section 3.3.3). This demonstrated that there may be a limit to the number of plasmids available to be segregated into anucleated compartments. Maxicells from the DnaA mutant did show the ability to independently express at high levels, with  $\beta$ -galactosidase assays showing that maxicell production boosted activity 11-26-fold, respective of minimal or rich media (see section 3.3.5). Time-lapse analyses did show that maxicells were able to express GFP for ~5 hours on a flat pad, although it was noted that this was not likely to represent the same conditions as liquid culture with constant aeration, therefore maxicells may remain GFP positive longer. Future work would be required to understand specifically how long maxicells of *B. subtilis* can actively express from the plasmid, though it was demonstrated in *E. coli* that ATP and NADH production was required for longevity of SimCells by Fan *et al.* The inclusion of a plasmid bound glycolysis pathway even extended SimCell expression up to 10 days (Fan *et al.*, 2020), whether this is comparable in *B. subtilis* would require further analyses.

As mentioned in the introduction, previous studies had made maxicells via irradiation or enzymatic degradation of the chromosome (East and Errington, 1989; Fan *et al.*, 2020). Both models relied on removing the chromosome from parental cells, while these methods are useful for making large quantities which can be stored (Fan *et al.*, 2020). This made the maxicell model in this study unique as maxicells were naturally and continuously produced throughout growth. Either method could be considered advantageous in accordance to the application, for example batch production may be useful as less nutrients would be required and less likelihood

of contamination. Whereas, continuous production of anucleate cells may be advantageous for nonstop supply of compartments, removing the need to re-culture and process cells.

While trying to determine the frequency of maxicells in a culture, flow cytometry analyses using fluorescently labelled nucleoids showed that ~50% of the population did not have a chromosome (see section 3.3.6). However, this method lacked specificity for counting GFP positive maxicells as it was clear chains of cells were counted as one event hence nucleated and anucleated cells were determined in a single event. This was confirmed by microscopy analyses where chains of cells were present and demonstrated the likelihood these single events were being counted as both GFP positive and nucleated, as shown in figure 3.19C. Therefore, this method was found not to be discriminatory enough between cells in chains. Nonetheless, flow cytometry did serve to confirm that ~26% of the free maxicells were expressing GFP and have had plasmids.

Interestingly, like the minicell strain, the maxicell producing strain also showed a significant increase in copy number (See section 3.4.1). As chromosome initiation was impaired in maxicell producing strains and flow cytometry revealed ~50% of the population were anucleate, it was expected the plasmid fold change to terminus would be ~50% greater than the control. However, in the maxicell producing strain it can be seen that there was a 7.4-fold increase. It could be stipulated that there is replication occurring in maxicells or that the DnaA protein affects replication of the pLS20 origin. Little is known about the pLS20 origin apart from it being low copy number and a theta replicator in *B. subtilis* (Meijer *et al.*, 1995). However, data in this study suggested that DnaA may be a negative regulator of the pLS20 replicon as the DnaA mutant of the maxicell producing strain had a profound effect on copy number. When the plasmid was compartmentalised within maxicells it was possible that DnaA depletion led to increased plasmid replication. As mentioned in section 2.13.1, there are limitations associated with relative qPCR methods as this study relied on the chromosome

terminus as a reference to determine copy number changes. The assumption that there was one chromosome per cell would likely have an effect within the maxicell producing strains as DNA replication initiation was affected which could provide uncertainty about changes observed. To overcome this, absolute qPCR could be employed as a more reliable option as the absolute copy number can be determined since the template quantity is accurately measured.

DnaA has not been described as a negative regulator before as it is the main initiator protein of chromosome replication, however, it seemed the *dnaAG152S* mutation has an affect where reduced DnaA functionality allowed the plasmid to replicate to a greater degree. Dr Aurélie Guyet bioinformatically found a DnaA box within the pLS20 origin (unpublished), two hypotheses were formed. The first was that deletion of the DnaA box would lead to high plasmid copy number like the maxicell strain displayed, the second was that the DnaA box was essential and deletion would not allow replication. Encouragingly, when the DnaA box of pLS20 was mutated the plasmid was able to be transformed and replicate in all Portabolomics chassis strains and subsequent sequencing showed that the mutation was retained. This showed that the DnaA box was not required for replication and refuted the second hypothesis as replication was permitted without this box. However, further analyses did show that deletion of the box reduced plasmid copy number. This was contradictory to the first hypothesis that had been established where it was thought DnaA could be a negative regulator of pLS20 replication. As there was evidence which suggested that the pGG018 mutant could be unstable, further work is required to test stability of the plasmid as instability could suggest that the DnaA box does have a role (see section 3.4.2). Whether the plasmid is stable or not could aid in understanding the role of DnaA in the pLS20 replication initiation or regulation.

There were 2 further hypotheses formed related to the mutated DnaA box, the first is that there are more DnaA recognition sites within the sequence which have not been found bioinformatically. Future work would also include bioinformatic work to potentially detect

more DnaA boxes or other iteron sequences which could aid in replication/stability. The second hypothesis is that the mechanism of pLS20 replication is independent of DnaA and the protein is not essential but does play a role in regulating copy number. This could be tested with the use of the constructed  $\Delta dnaA$  strain with xylose inducible *dnaA-dnaN* (see appendices 4) and maxicell producing strains.

### 3.6.3 Protein A expression and secretion

As mentioned in the introduction (see section 1.3.5), there is an increasing interest in decorating minicells with antigens for vaccination purposes. A recent novel example was the use of polar localised autotransporters specifically enriching minicells and decorating with an influenza hemagglutinin (HA) tag (Yu *et al.*, 2021). To see if polar compartments (minicells) or rod compartments (maxicells) had the ability to secrete proteins, which would be useful for industrial applications, Protein A was used as a heterologous reporter for expression. protein A was a useful reporter as it could be tethered to the PG cell wall and detected using commercial products. As well as being an industrially relevant proof of concept, protein A was also thought to be of use for maxicell purification. As maxicell compartments are much larger than minicells, it is not as easy to segregate them from mixed cultures. This is where the protein A expression could be useful as cell wall decoration with a detectable protein may allow for selective purification.

The Western blot of the native protein expressed from the plasmid showed that there was the presence of 2 bands, this was promising as it indicated that the signal peptide was cleaved off for translocation over the cell membrane. Immunostaining did show some maxicells and minicells with a decorated cell wall, some of which were still attached to the mother cell (section 3.5.2). It was unclear if protein A was linked to the PG of *B. subtilis*, however, experiments worked best without lysozyme treatment which is considered to be a result of peptidoglycan degradation affecting protein A anchorage to the cell wall which would

subsequently be washed away. When fixed cells were treated with lysozyme, most of the protein A signals seen were cytosolic.

It was recently reported in *Staphylococcus aureus* that protein A is only incorporated into the growing septum throughout the cell cycle (Zhang *et al.*, 2021). There were also other mechanisms discovered which would determine where the protein A is incorporated such as lipoteichoic acid synthesis (Zhang *et al.*, 2021). As minicells and maxicells are inactive cells, which cannot grow, it may be the reason why not many cells were labelled with protein A and most of the protein likely remained within the cytoplasm. Potentially, as there were some protein A labelled compartments, some anucleate cells may have been expressing from the plasmid during the cross-wall synthesis between nucleated and anucleated progeny. However, it could also be considered that some minicells were decorated with protein A expressed from nucleated cells during asymmetric septum synthesis.

It was hypothesised that the protein A LysM domain may not mediate strong attachment to the peptidoglycan of *B. subtilis*, therefore, the *lytC/spa* fusion was designed in order to resolve PG cell wall leakage problem. LytC is known to homogenously decorate the cell wall (Yamamoto *et al.*, 2003), and has been used in previous studies to anchor fusion proteins to the cell wall while retaining the anchored proteins normal activity (Tsuchiya *et al.*, 1999; Kobayashi *et al.*, 2000). LytC's cell wall binding domain is dissimilar to the LysM motif, it consists of three direct repeats (Yamamoto *et al.*, 2003). Unfortunately, this fusion did not show any signals from any of the Portabolomics strains. As the Western blot only showed 1 band, it was surmised that the signal peptide was not cleaved. Closer analyses of the protein domains provided by Amirah Alofi (unpublished) showed the LysM domain was deleted, however, the signal peptide was now inserted in between the new CBD and IgG-binding domain (fig.3.31). This may have affected the translocation over the inner cell membrane and therefore no signals were apparent.



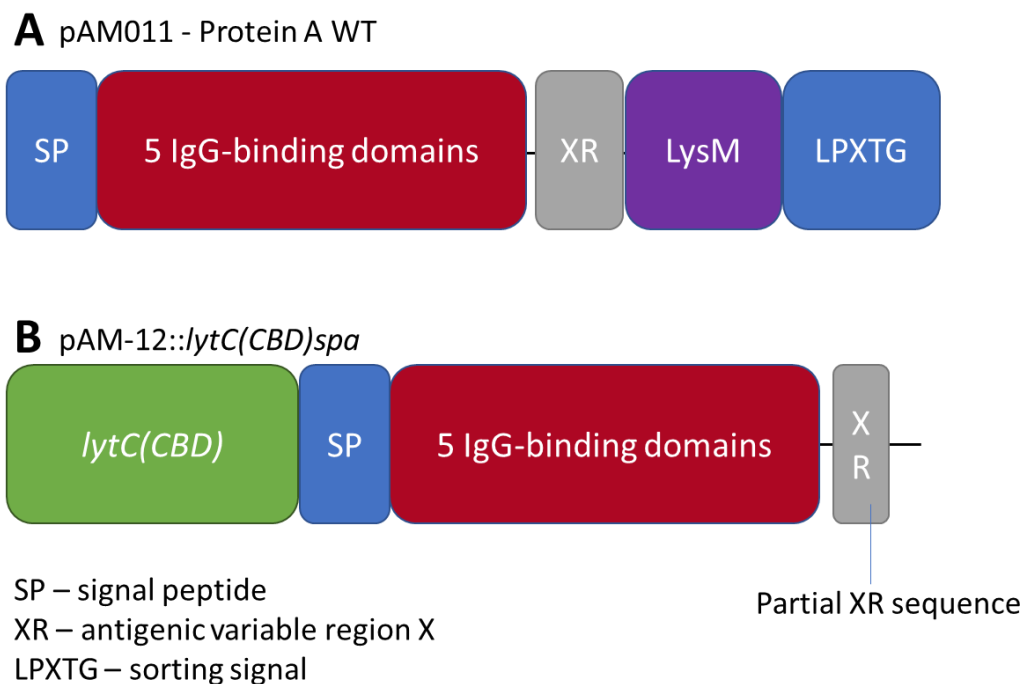


**Figure 3.31. Demonstration of the protein A structure and carbohydrate binding domain fusion.** **A)** native protein A expressed from pAM011. Each domain is labelled referencing O'Halloran *et al.*, 2015 and Zhang *et al.*, 2021. **B)** The product expressed and protein domain ordering from pAM012 from Amirah Alofi (unpublished).

To conclude and summarise, the mini- and maxicell producing strains appeared to show a similar amount of anucleate cells expressing from the plasmid (~50%). However, the maxicell chassis was found to be a much greater producer, likely because of being a larger compartment. It was highly suggestive that the plasmid replicated uncontrollably within anucleated compartments and this is how they overcome LacI repression from the cytoplasm, as LacI had a long half-life. Low copy number vectors were better to use to maintain regulation, although there was some leaky expression in control strains.

## Chapter 4 – modulating plasmid copy number to increase chassis expression in *Bacillus subtilis*

### 4.1 Introduction

As mentioned within Chapter 3, the low copy number plasmid was adequate for the system as nucleated cell expression was repressed, whereas anucleate compartments did display independent GFP expression. This was in keeping with the models in order to drive compartment specific expression (see sections 1.5). However, as demonstrated in chapter 3, only ~50% of anucleated cells with the pLS20 driven plasmid displayed GFP expression (plasmid map fig.3.3A). It was desirable to increase the number of minicells which were expressing, however, a balance needs to be formed in order for repression to remain strong in rod cells but great enough for more minicells to gain a plasmid.

#### 4.1.1 Plasmid replication apparatus split to modulate copy number

A system was developed in order to increase and decrease copy number using an inducer, attempting to find an optimal copy number for tolerable nucleated cell repression while increasing anucleate cells gaining a plasmid. The aim was to place the plasmid replication initiator protein on the chromosome, under a  $P_{xyI}$  inducible promoter, to see if the plasmid was maintained and if plasmid copy number could be modulated. The system is based upon a self-replicating minimal replicon, pLS32, isolated from *Bacillus natto* (Tanaka, 1998). The pLS32 replicon uses a replication protein (RepN) alongside the origin (*oriN*), the system does not require DnaA but relies on the RepN protein which is essential for replication initiation. This vector has a copy number of 2-3 plasmids per chromosome (Tanaka, 1998; Tanaka *et al.*, 2005). The DNA binding (iteron) sequences were identified as 5 AT-rich repeat sequences within the *repN* gene (Tanaka *et al.*, 2005). When the iteron sequences were sequentially altered there

was a change in plasmid copy number (sequences 1-3) or failure to replicate the plasmid at all (sequences 4-5), this indicated that the iterons are used in both replication initiation and regulation of the plasmid (Tanaka *et al.*, 2005). However, Tanaka *et al.*, 2005 found that when *repN/oriN* were on both chromosome and plasmid there was a strong incompatibility and no transformants or transformants died upon restreaking. But when the iteron sequences were deleted in pairs from the chromosomal copy of *repN*, it was found that iterons 1, 2 and 3 were responsible for the described incompatibility, which the authors suggested was a result of negative regulation (Tanaka *et al.*, 2005). The model proposed to utilise pLS32 is based on segregation of the initiator protein and the iteron sequences in order to control protein expression, thus controlling copy number. The plasmid would contain the 5 iteron sequences only to provide the minimal region required for replication initiation (fig.4.1 ticks). To prevent a second replication bubble occurring on the chromosome or a homologous recombination event, the RepN protein must be mutated to remove iteron sequences (fig.4.1). Splitting the replication initiation protein would also be beneficial as there would be no potential for the plasmid crossing between different species, unless it had the appropriate RepN protein.

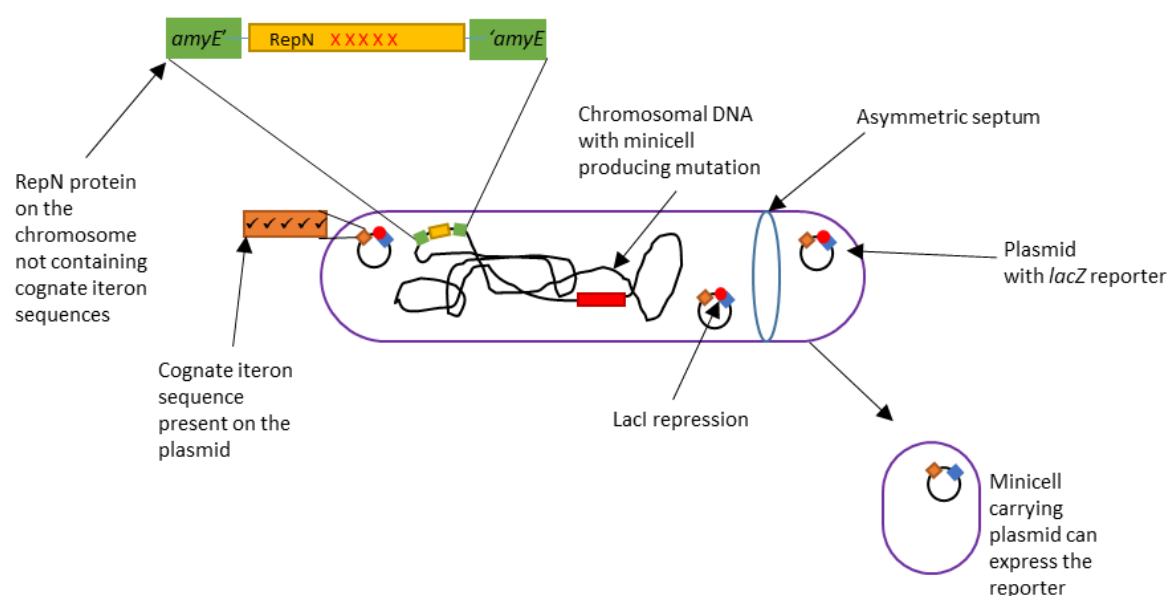


**Figure 4.1. Demonstration of the proposed host restrictive region in *B. subtilis*.** The model shows the use of the pLS32 replicon and separating the protein from the cognate iteron sequences. The mutated protein will no

longer have the AT-rich iteron regions, the iterons will be on the plasmid specifically removing all possibility of transfer to unwanted hosts and allow regulation of copy number using a  $P_{xyI}$  promoter.

#### 4.1.2 pLS20 replicon random mutagenesis

The pLS20 replicon, used within the *B. subtilis* chassis in chapter 3, is not well understood. It is known that pLS20 is a Theta type replicon derived from a naturally occurring plasmid isolated from *Bacillus subtilis* IFO3335 (Theta replication demonstrated fig.1.8) (Meijer *et al.*, 1995). pLS20 produces 2-4 copies per chromosome, however, the method of replication was not elucidated and is suggested to be a new class of theta-type replicon (Meijer *et al.*, 1995). A theta-type replicon uses bi-directional replication and is favourable as it is said to be more stable than rolling circle as it does not produce single stranded intermediates (Meijer *et al.*, 1995; Tanaka, 1998). The coding sequence of the pLS20 origin has detected a DnaA box present, suggesting it might depend on host initiation proteins for replication (A. Guyet, unpublished). As little is known about pLS20, mutagenesis of the origin should allow better determination of copy number control and may help determine a number of copies optimal for anucleate cells to gain a plasmid.

The plasmid system described in chapter 3, used an actin like filament segregation system to retain stability of the plasmid known as the Alp7AR operon. Alps mechanically move plasmids to the poles of the cells to improve segregation into daughter cells (Derman *et al.*, 2012). The Alp7AR operon was isolated from native pLS20 which is composed of an actin polymer (Alp7A) and a regulator (Alp7R) (Derman *et al.*, 2012). Unpublished work by Aurèlie Guyet shows that, in sAG1333, the pLS20 driven plasmid can be lost quickly when grown in liquid media without antibiotic selection. This was seen in spot titre assays where there was a lack of colony forming units (CFU) across dilutions (fig.4.2A & C). However, when Alp7AR was present stability improved as all dilutions showed CFU on solid media with and without erythromycin selection (fig.4.2B & D).

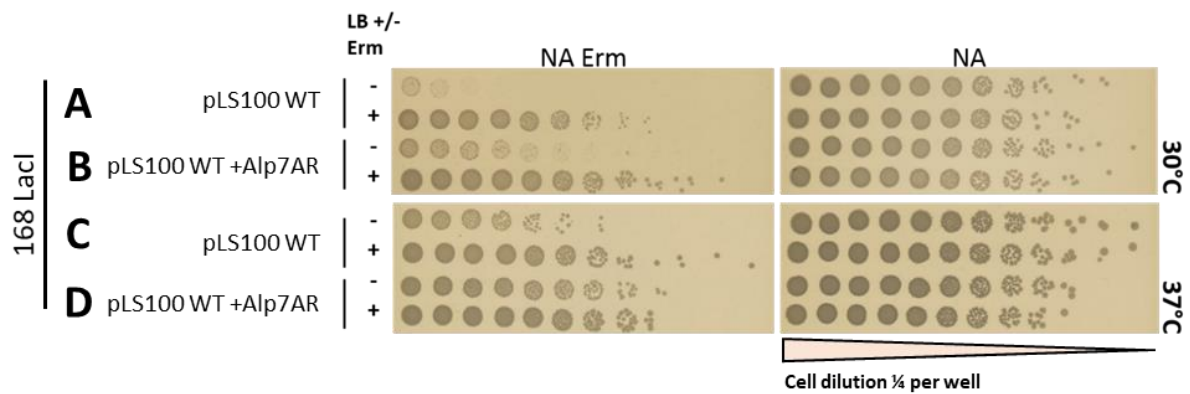


**Figure 4.2. Alp7AR operon increases the stability of low copy number plasmid.** Strains were grown in rich LB media with and without erythromycin selection (1 µg/ml) at 30°C for 18 hours. Cells were diluted before 1/4 serial dilution, 5 µl of culture dilutions were spot onto nutrient agar with and without erythromycin selection (1 µg/ml). Plates were incubated at varying temperatures (indicated by the figure) for 24 hours before imaging. **A)** Wild-type plasmid with no Alp operon grown at 30°C. **B)** Plasmid with Alp operon grown at 30°C. **C)** Wild type plasmid with no Alp operon grown at 37°C. **D)** Plasmid with Alp operon grown at 37°C. Unpublished work by Aurèlie Guyet.

The aim within this chapter was to increase copy number enough to yield more anucleate cells with a plasmid, but to retain good repression in nucleated cells. The concern was that increasing copy number would out-compete LacI and therefore compromise repression in mother cells. However, there were also limitations to adjusting copy number in relation to Alp7A as it was known to be toxic when overexpressed or when the regulator is deleted (Alp7R) (Derman *et al.*, 2012). Therefore, copy number required fine-tuning to enable increased copy number without causing lethality.

## **4.2 Results: Plasmid replication apparatus split to control copy number via expression of replication protein.**

### **4.2.1 Design of the RepN iteron mutant and integration into the vector**

As described by Tanaka *et al.*, the RepN protein cannot be present on both plasmid and chromosome simultaneously (Tanaka *et al.*, 2005). To combat this, a gene was synthesised which lacked the 5 AT-rich DNA binding sequences while retaining the proteins amino acid sequence (GeneArt – Thermofisher). The *B. subtilis* codon usage table was used to choose the most common codons used with the strain for each amino acid. This way the protein could reside on the chromosome and not induce replication via the iteron sequences. The gene was synthesised to include a P<sub>xyI</sub> promoter so expression was inducible and copy number of the plasmid could be modulated by increasing protein concentrations. Base changes, highlighted in red, integrated more GC to reduce AT-rich regions (fig.4.3). Iteron sequences, underlined, were the main focus but any base substitutions were carefully selected to ensure the amino acid sequence was not disturbed. This mutant version of RepN was able to be cloned into *B. subtilis* and integrated into the *amyE* locus using an integration vector.

|                    |                                                                                 |
|--------------------|---------------------------------------------------------------------------------|
|                    | ...R L N N E V D D L L I K A E E T A K N I                                      |
| RepN-Iteron mutant | CGG TTA AAC AAT GAA GTT GAT GAC CTT TTA ATT AAA GCA GAG GAA ACA GCC AAG AAT ATC |
| Wild-type RepN     | CGG TTA AAC AAT GAA GTT GAT GAC CTT TTA ATT AAA GCA GAG GAA ACA GCC AAA AAC ATA |
|                    | D K N W M G Q N E L S S N E R I G Q N E                                         |
| RepN-Iteron mutant | GAC AAG AAT TGG ATG GGG CAG AAC GAG CTG AGC TCG AAC GAG CGC ATC GGC CAG AAC GAG |
| Wild-type RepN     | GAT AAA AAC TGG ATG GGT CAA AAT GAA CTT TCC AGT AAT GAA CGG ATA GGT CAA AAT GAA |
|                    | L S G K V Q I E P S R E V K L N H P D G                                         |
| RepN-Iteron mutant | CTG TCG GGC AAG GTC CAG ATC GAG CCG AGC CGG GAA GTG AAG CTG AAT CAC CCC GAC GGC |
| Wild-type RepN     | CTT TCC GGG AAA GTT CAA ATT GAA CCA TCA AGA GAG GTC AAA TTG AAC CAT CCG GAT GGT |
|                    | S K R T T N N N N S I K N N F N N N E N...                                      |
| RepN-Iteron mutant | TCC AAG CGG ACG ACC AAC AAT AAC AAT TCG ATC AAA AAT AAC TTT AAT AAT AAT GAG AAT |
| Wild-type RepN     | TCA AAA AGA ACC ACT AAT AAT AAT AAC TCT ATT AAA AAT AAC TTT AAT AAT AAT GAG AAT |

**Figure 4.3. Demonstration of iteron mutations.** The nucleotide base changes introduced into RepN to produce mutated iteron sequences. Red indicates where nucleotides have been substituted, underlined sections indicate the 5 iteron sequences identified (Tanaka *et al.*, 2005). The protein amino acids sequence remains the same for both.

To make the plasmid with iteron only sequences, the starting plasmid (pGGZ32) was reduced using sequential PCR's with restriction digestion and ligation. This was performed sequentially to remove the iteron regions upstream and downstream sequences in two steps, leaving iteron sequences only (fig.4.4A – iterons depicted as XXXXX). Upstream of the iteron sequences, 683 bp were removed including the native promoter and ribosome binding site (RBS), this made the pGGP01 vector where the downstream bases were still present. Downstream of the iteron sequences, a further 147 bp were deleted to leave the iteron sequence only, this plasmid was name pGGP02. The size changes were evident with a diagnostic PCR using primers binding outside the origin sequence illustrated by the plasmid maps (fig.4.4C). When DNA was migrated on an agarose gel, it was evident that the plasmid replicon reduced in size as the protein sequence around the iterons were removed.

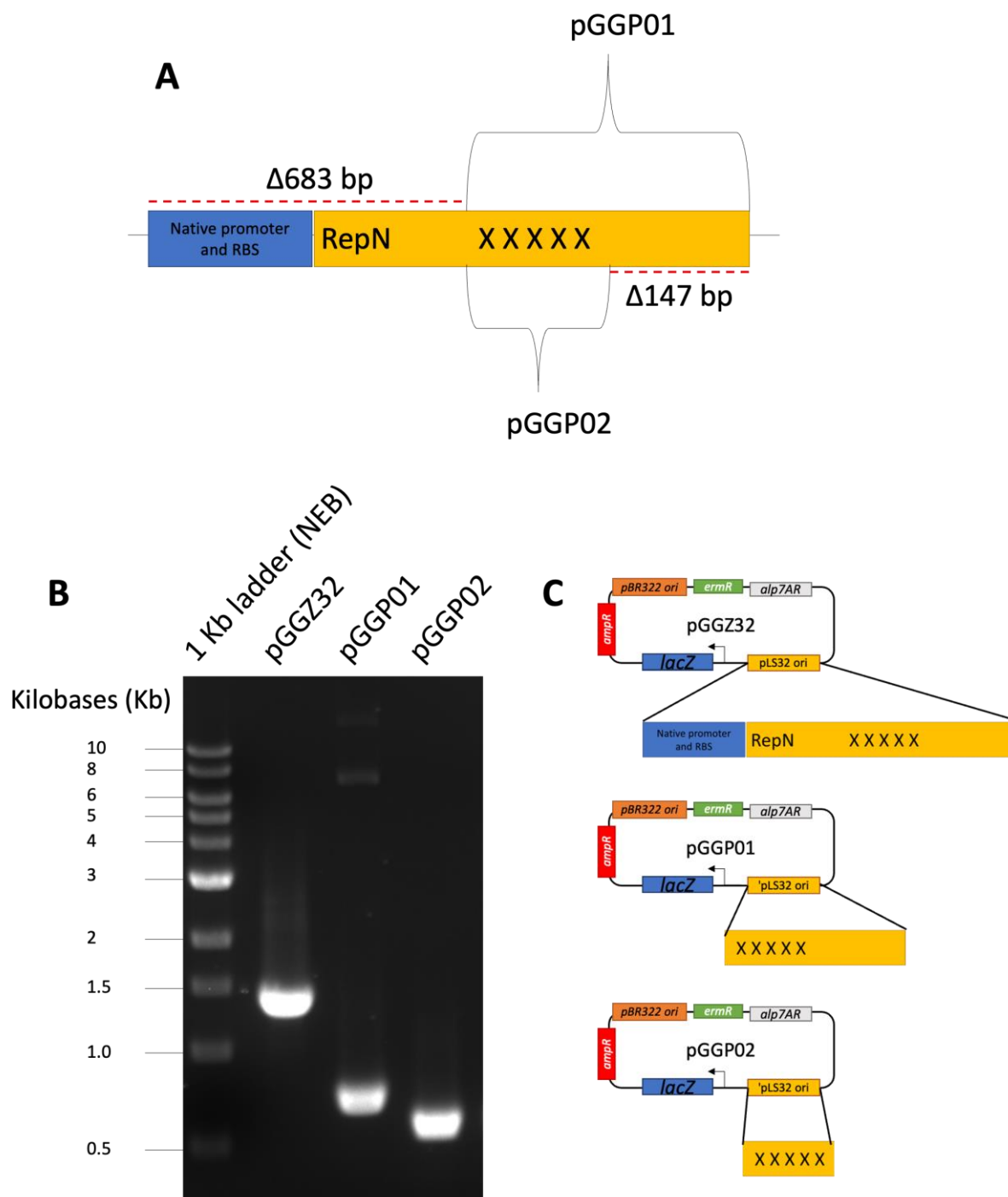


**Figure 4.4. Plasmid production for the host-restrictive system.** **A)** Schematic of deletions within the RepN/*oriN* replicon. XXXXX indicates the 5 iteron sequences within the proteins code. Red dotted lines indicate deletions and brackets indicate what part of the origin was left in each vector. **B)** Plasmid sizes run on a 0.8% agarose gel after progressive cycles of PCR, restriction digest and ligation in order to remove large sequences of the RepN protein. DNA was stained with SYBR gold (Thermofisher) before running on the gel at 100 volts (V) for 45 minutes. **C)** Plasmid maps to illustrate the vectors constructed for the RepN/*oriN* split system in which the origin of replication is reduced to the 5 iteron sequences required for replication.



### 4.2.2 Characterisation of transformants

To see if the plasmid was present and active, transformants were selected by IPTG induction of the  $P_{spac}$  driven  $\beta$ -galactosidase reporter which would show blue colouration in the presence of X-gal on solid media. In the sGG104 background, the iteron only plasmids should express the reporter if the plasmid is present and replicating. With X-gal addition, there was a colourimetric change on plates with IPTG induction, indicating that the  $P_{spac}$ - $lacZ$  was functional (fig.4.5A blue boxes). Interestingly, when pGGZ32 was introduced, with the whole pLS32 origin, most of the patches showed a dark blue colorimetric change even in the absence of IPTG. This shows that the pLS32 replicon, shown in fig.4.4C, was compatible with the RepN-iteron mutant but high colourimetric change may be a result of increased plasmid copy number from 2 RepN proteins expressed. Alternatively, this could be from strong promoters identified bioinformatically surpassing  $P_{spac}$  (Appendices 5).

To further identify if the plasmids were present, strains which indicated the presence of  $lacZ$  by exhibiting blue colouration with IPTG induction were lysed and plasmid DNA extracted. The plasmid DNA was used as a template for diagnostic PCR using oligonucleotides specific to the plasmid backbone, amplifying outside the  $repN/oriN$  area (same as fig.4.4B). Encouragingly, the PCR revealed that the different origin sizes were present from the miniprep which indicated that the plasmids were present within extracted DNA (fig.4.5B).

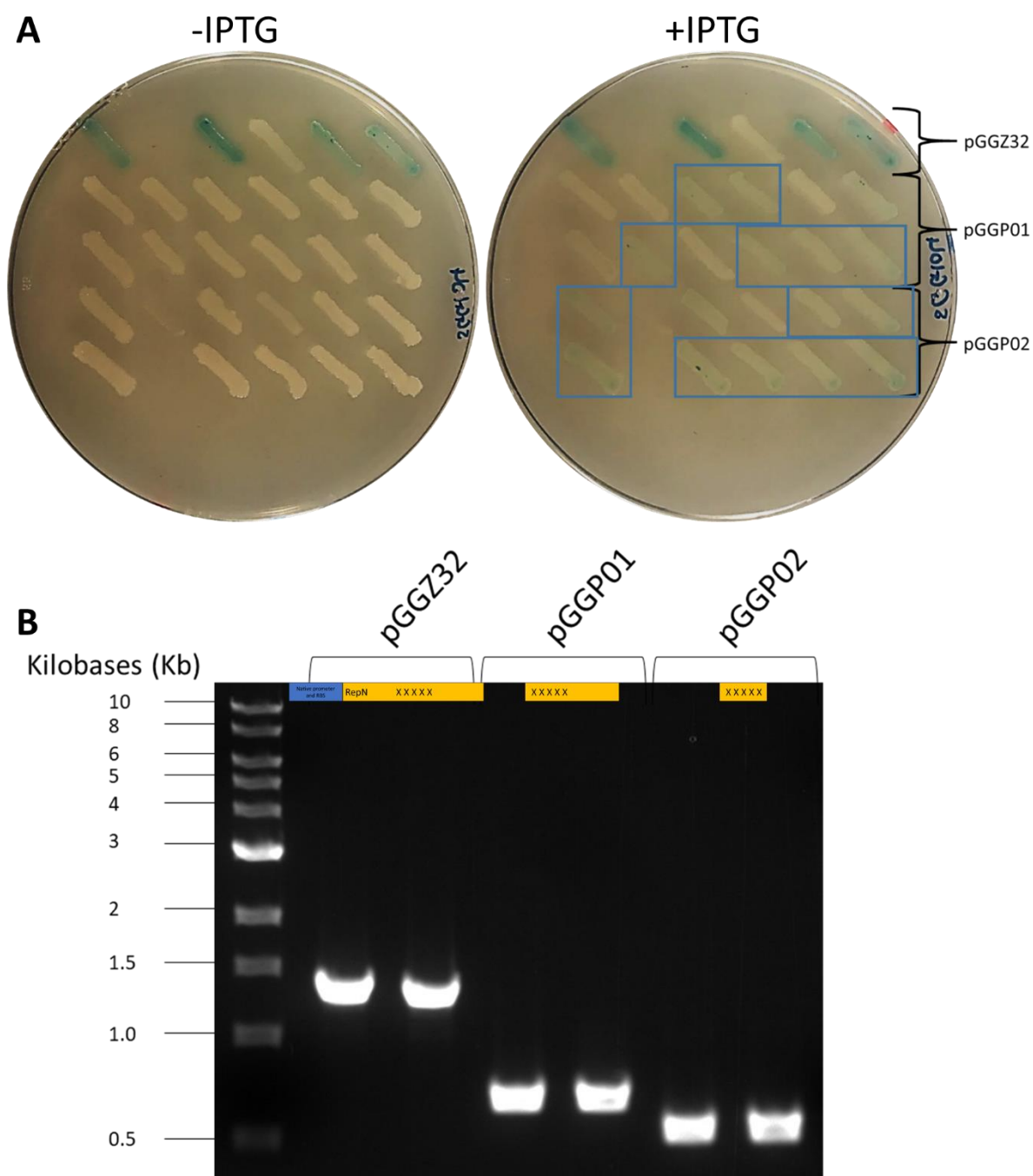


**Figure 4.5. Testing colonies for colorimetric changes with X-gal and different induction conditions. A)** Transformation colonies were patched onto nutrient agar plates supplemented with 1  $\mu$ g/ml erythromycin, 40  $\mu$ g/ml X-gal and with or without 1 mM IPTG. After inoculation, the plates were incubated overnight at 37°C and images with a FujiFilm FinePix camera. **B)** Colonies with the expected phenotype observed on plates were lysed to extract plasmid DNA using a Qiagen miniprep kit with added lysozyme. The extracted DNA were used for PCR with primers binding specifically to the plasmid backbone outside of the origin region. DNA was detected using SYBR gold staining on a 0.8% agarose gel in 1x TAE buffer. Images were taken with Biorad Gel doc.

### 4.2.3 $\beta$ -galactosidase assays to determine functionality

The  $\beta$ -galactosidase activity was analysed in these strains with varying amounts of xylose to increase expression of the RepN protein. If the model worked (see section 4.1.1), then increasing concentrations of xylose were expected to increase plasmid copy number and therefore show greater  $\beta$ -galactosidase activity. However this assay, when performed without IPTG induction, showed little to no expression of  $\beta$ -galactosidase from the iteron only plasmid (pGGP02) which was less expression than that obtained from the pLS20 control (orange bars – fig.4.6A). The pGGZ32 control showed a significant increase in  $\beta$ -galactosidase activity over time (black bars fig.4.6A) which was reciprocated on plates from the previous figure 4.5.

When observing the ‘iteron only’ plasmids (pGGP02; fig.4.4C), it was clear that irrespective of xylose concentration, all strains with the minimal plasmid showed similar levels of  $\beta$ -galactosidase activity which all decreased over time (fig.4.6B). This was suggestive that there was no replicating plasmid present. With the addition of IPTG, there was an increase in  $\beta$ -galactosidase expression over time, as it was not believed that the plasmid was present this potentially suggested that homologous recombination had occurred (fig.4.6D). It was assumed homologous recombination had occurred as IPTG induced *lacZ* expression was still present, and if there were no replicating plasmid then it was likely there was an integration into the chromosome. However, this increase was never as great as that obtained from the control plasmid with induction (pGG08Z – fig.4.6C (plasmid map fig.3.3B)).

IPTG induction resulted in an odd activity profile from pGGZ32 (black bars; illustrated in fig.4.4C) as  $\beta$ -galactosidase activity started greater than the pLS20 control (pGG08Z (plasmid map fig.3.3B) – orange bars) but never reached the same activity units after 4 hours of growth (fig.4.6C). From a plasmid displaying heightened  $\beta$ -galactosidase expression, it was expected that expression would remain greater than the control over time. More interestingly, after 6

hours with pGGZ32 the  $\beta$ -galactosidase decreased to lower levels of activity than time 0 which may have been because of plasmid stability issues (fig.4.6C – black bars).

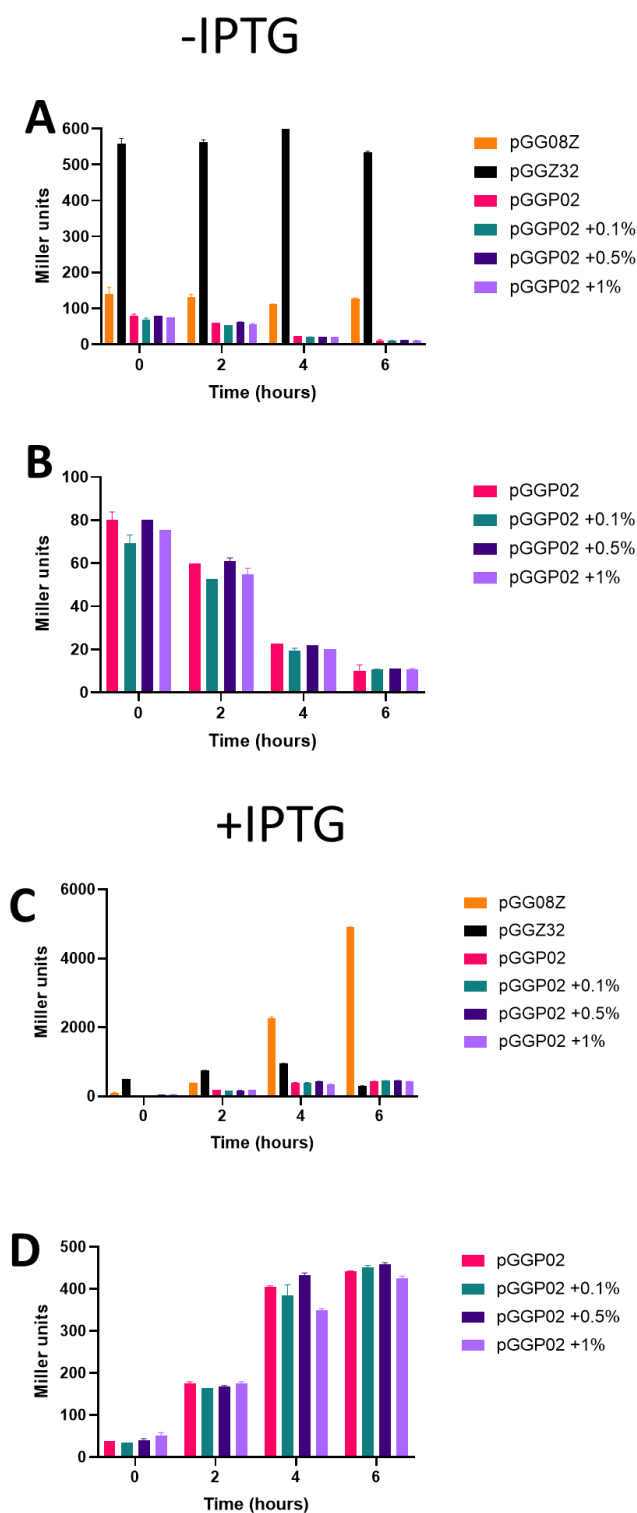


**Figure 4.6.  $\beta$ -galactosidase expression from minimal plasmid strains.** Strains were grown at 30°C in a liquid CDM with plasmid selective antibiotic +/- IPTG induction and varying concentrations of xylose. Aliquots were

taken every 2 hours for 6 hours, pellets lysed before adding ONPG for reactivity testing. Reactions were stopped and absorbance at 420 nm measured to calculate Miller units. **A)** all strains without IPTG induction **B)** strains with pGGP02 only and varying xylose induction (0.1%, 0.5% and 1%) **C)** all strains with IPTG induction **D)** strains with RepN/*oriN* inclusive plasmids only grown in IPTG inducing conditions and increasing xylose concentrations. Miller units were calculated in Microsoft Excel and bar graphs processed in GraphPad prism 9. N=3 technical repeats.

Unfortunately, further testing of extracted pGGP01 and pGGP02 did not yield any transformants when returned to *E. coli*. This suggested that the plasmids were not present or had been mutated, which made them no longer able to replicate in *E. coli*. Overall, this system did not work how the proposed model was hypothesised and segregating RepN from the iteron sequences did not provide a tuneable system (fig.4.1). There seemed to be issues with the plasmid reporter expression from these vectors and a form of instability where the plasmid could no longer be transferred back into *E. coli* DH5 $\alpha$ .

## 4.3 Non-specific plasmid mutagenesis

### 4.3.1 Plasmid stability experiments

To look at plasmid stability of the Portabolomics chassis built by A. Guyet, pAG-P08, methods from fig.4.2 were replicated to ensure stability. The negative control served as a demonstration that erythromycin was effective at inhibiting growth in 168 CA without the plasmid. When sAG1333 was transformed with the plasmid (pAG-P08 – see section 3.2.2 fig.3.3A), growth in liquid with and without erythromycin showed the dilutions on nutrient agar supplemented with and without erythromycin was comparable at high and low temperatures as CFU formed across all dilutions (fig.4.7A and B). On both erythromycin and nutrient agar plates, the plasmid seemed to be stable after 24 hours of growth in liquid media as all spot titres displayed CFU.

After assessing the stability for 24 hours, a longer period of time was then assessed in liquid culture counting CFU/ml per 24 hours in a minicell producing background (sAG1338; fig.4.7C). A single culture for each strain with and without the plasmid was grown for 7 days with back dilution performed every 24 hours, there was no antibiotic selection in liquid. The sAG1338 control strain, with no plasmid, was stable in liquid media as CFU/ml remained at an average of  $5.65 \times 10^8 \pm 7\%$  (fig.4.7C red triangles). When plated onto erythromycin selective media, there was no growth demonstrating erythromycin sensitivity which was not visible on the logarithmic Y-axis as each data point was 0 (fig.4.7C). When the plasmid was present, the strain maintained a steady CFU/ml when spread onto nutrient agar plates (fig.4.7C circles). However, growth on erythromycin selective media showed that the CFU/ml decreased steadily over the time course showing that the plasmid was diluted out. Even after the first 24 hours there was already a reduction in cells producing erythromycin resistance from pGG08Z (day 1 black squares). This demonstrated that, over longer periods of time, the strains were not stable without antibiotic selection. Overall, it is suggested that the plasmid was not maintained

over the 7-day period without antibiotic selection as CFU/ml of the culture shows that the cells are growing well but when the plasmid was selected there was extensive reduction in CFU/ml.

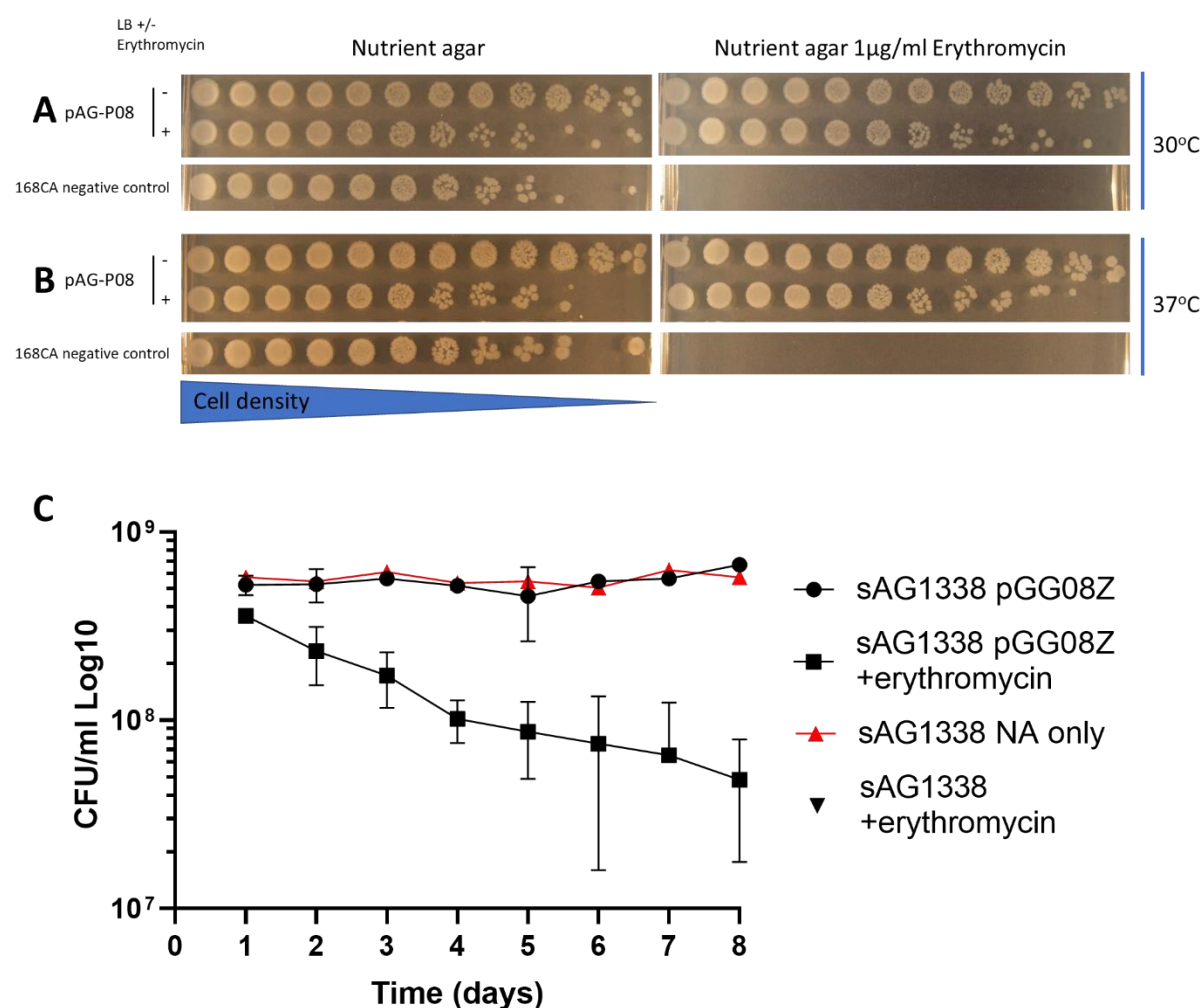


**Figure 4.7. Plasmid stability of pAG-P08 over 24 hour and 7 day periods.** Cultures were grown in a rich media (LB) for 24 hours, after which time they were diluted 4-fold and spotted onto nutrient agar plates and agar supplemented with antibiotic for selection. **A)** strains grown at 30°C **B)** strains grown at 37°C. Images were taken using a FujiFilm FinePix camera. **C)** Strains with pGG08Z and control stains with no plasmid were grown in LB media, cultures were diluted back 1:200 every 24 hours and CFU/ml calculated by spreading dilutions on nutrient agar or agar supplemented with erythromycin. Stability was monitored over ~60 generations. Results shown are the average value of 2 repeat experiments. Error bars show standard deviations.

### 4.3.2 Error prone PCR mutagenesis and selection of mutants

As methods to create a modulated copy number system (section 4.2) and whole plasmid mutagenesis with XL1-red (appendices 6) had both failed, a more direct approach to alter pLS20 was used via error-prone PCR. This method was used to randomly mutate the pLS20 origin with primers specifically designed to amplify, allow for restriction digest and relegation into the vector (fig.4.8B). The  $\beta$ -galactosidase reporter was used to screen for copy number mutants via blue colouration when the X-gal substrate in solid media was cleaved. Blue colouration observed in the absence of IPTG induction suggested high copy number mutants were present as LacI was outcompeted by the number of plasmids. The product from error-prone PCR was a band of ~900 bp corresponding with the size of the product expected by DNA electrophoresis, this band was used to ligate into the vector and replace the WT *oriLS20* (fig.4.8A & B). Plasmids transformed into *E. coli* were pooled for subsequent transformation into sAG1338, random colonies picked were patched on plates with X-gal in the presence and absence of IPTG (fig.4.8C & D). The non-mutated control, circled in black, displayed the expected phenotype of white without IPTG and blue with *lacZ* induction. In the absence of IPTG (fig.4.8C) there was 12 mutants in total which displayed blue colouration (gated with a red outline), potentially indicating an increase in copy number as LacI was outcompeted. In the presence of IPTG, some mutants seemed to lose the colourimetric change (fig.4.8D). This was assumed to be from potential cloning issues where star activity may have allowed damage to the promoter and/or gene, but these mutants were not sequenced as they were not of particular interest.



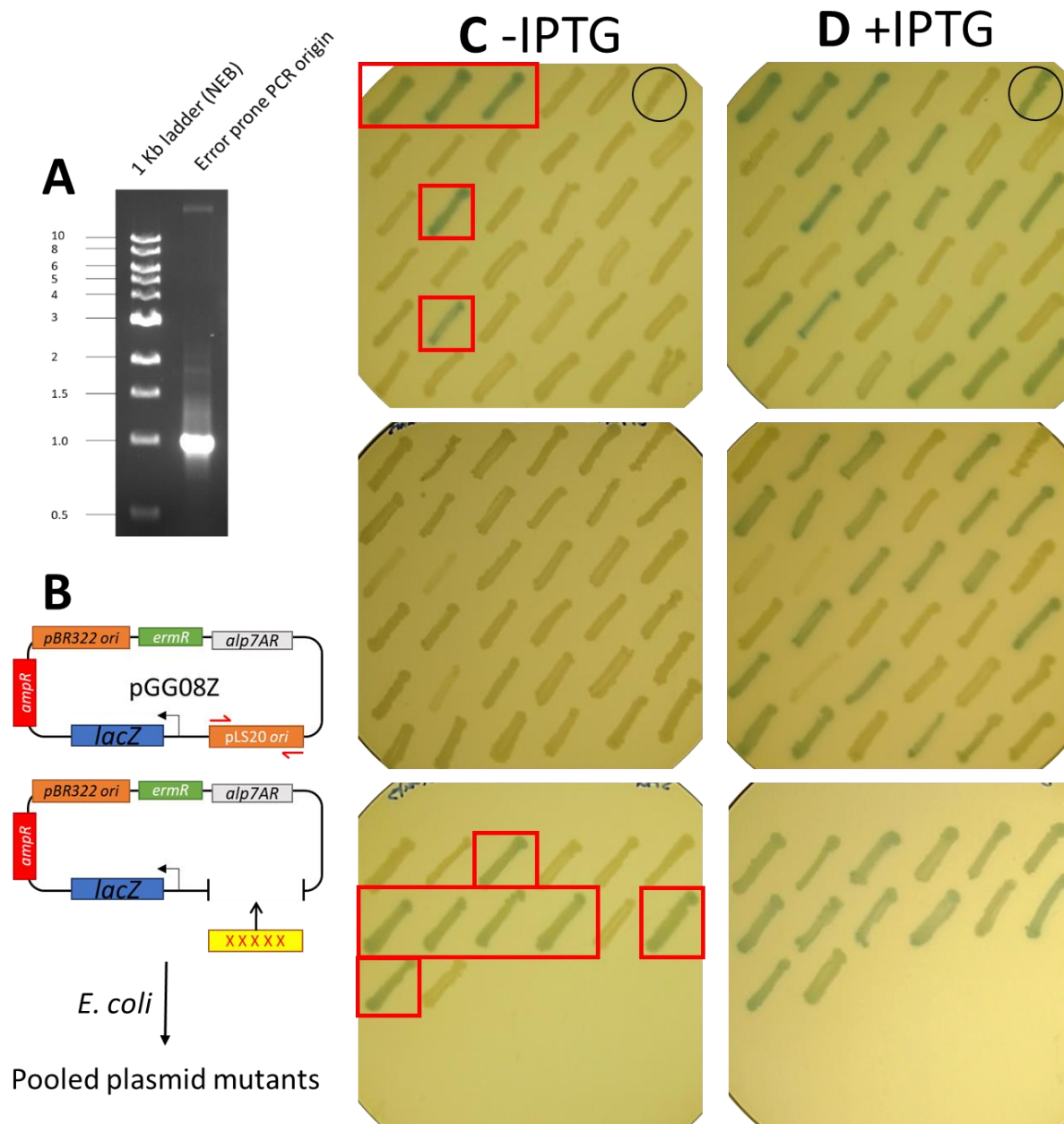


**Figure 4.8. Error-prone PCR pLS20 origin and selection of mutants.** **A)** Using the GeneMorph II error-prone polymerase (Agilent technologies) the PCR was performed in 30 cycles according to the manufacturer instructions. After running on a 0.8% agarose gel, the fragment was cleaned using PCR purification kit (Qiagen) **B)** Diagram of the pLS20 origin amplification, mutant *ori* was digested and ligated into recipient vector which was transformed into *E. coli*, where a pooled plasmid extraction was performed. **C&D)** The pooled mutant plasmids were transformed into sAG1338. When patched onto selective media with X-gal in **B)** the absence and **C)** presence of IPTG. Black circled colony indicates the pGG08Z non-mutated control. Red boxes indicate colonies with potential increased copy number chosen for further analyses.

To ensure the plasmid was the source of the colorimetric change, the plasmids were extracted, passed through *E. coli* and transformed back into sAG1338 indicator strain. Transformants were patched onto selective media with X-gal (fig.4.9A), which showed that the blue colouration in the absence of IPTG was maintained and therefore confirmed the phenotype was consistent. The patches circled in black were the unmutated control transformation which exhibited a white colouration without IPTG, as expected (fig.4.9A). The plasmid origins were subsequently sequenced for each mutant and they were compared to the parent. Alignments showed that there were 2 clusters of mutations found (fig.4.9B). Most of the plasmids had the same base changes with both cluster 1 and 2 mutations present simultaneously. However, 1 mutant, highlighted with the red Asterisk\*, showed the same phenotype as all the others but only had cluster 1 base changes. This may indicate that the cluster 1 mutation was responsible for the copy number change causing blue colouration without IPTG.



**Figure 4.9. Mutants with sustained phenotype.** A) 12 mutant plasmids were extracted from *B. subtilis* strain, the plasmids were transformed into *E. coli* and returned to the same *B. subtilis* species (sAG1338). Transformants

were then patched onto X-gal supplemented media with and without IPTG to see if there was an increased colourimetric change which may indicate copy number increase. The single patch highlighted by a black circle is the non-mutated plasmid. Plates were incubated overnight at 37°C. **B)** Those with a blue phenotype were purified and reintroduced into the strain to ensure the same result was given. When sequenced there were different mutations found which are indicated in the graphic. (Map provided by SnapGene – insightful science)

To further understand the role of the base substitutions identified and how they affect copy number, single clusters and single base changes were constructed. The pLS20 origin consists of 891 bp, cluster 1 base changes were introduced in full (pGG013). Then the first base and last bases of the cluster were mutated singularly (pGG014 and 015 respectively), as well as in combination with one another (pGG016). Cluster 2 base substitutions consisted of 3 base changes, 2 variants of the pLS20 origin were made to include either the whole cluster 2 substitutions (pGGC21) or the first and last bases (pGGC22).

\*all points outlined are single base substitutions

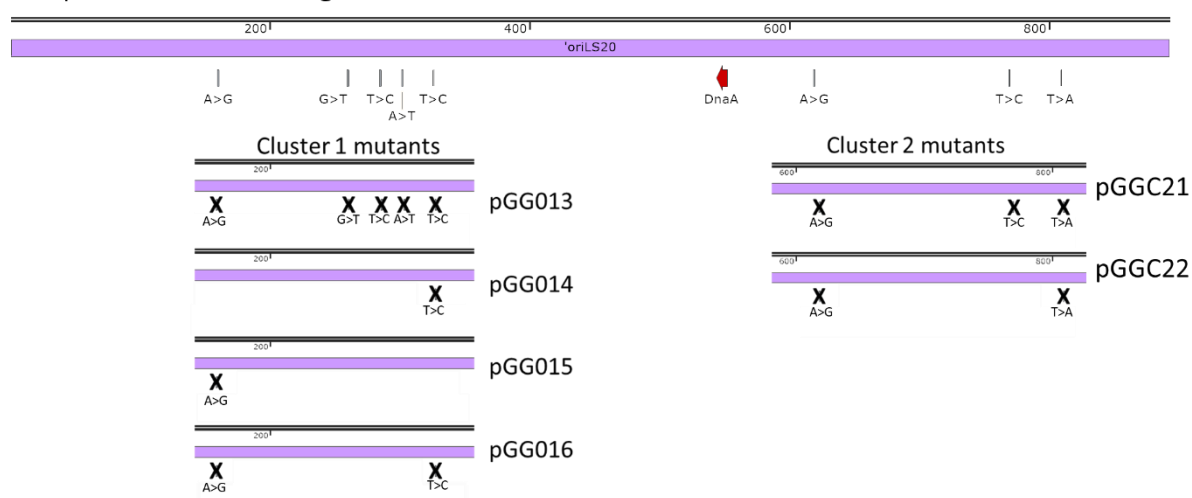


**Figure 4.10. pLS20 copy number mutant intermediates.** The variance is shown between the single base substitutions which have been incorporated into the pLS20 origin. NEB HiFi cloning was used to integrate the base changes into a parental plasmid, the plasmids picked were then sequenced (Dundee sequencing services) to see where the changes were integrated. Black X denotes where base substitutions are for each plasmid labelled.

The array of cluster 1 mutants described in fig.4.10, were transformed into sAG1338 to determine how each base substitution/combination of substitutions altered  $\beta$ -galactosidase expression. The plasmid with the whole cluster 1 mutation (fig.4.11B) exhibited a colourimetric change in the absence of IPTG showing that cluster 1 on its own had a key role in the phenotype. The last base change of cluster 1 (fig.4.11C) did show some colouration which was not as intense compared to the mutant with whole cluster 1 base changes (fig.4.11B). In contrast, the first base substitution of cluster 1 on its own (pGG015Z; fig.4.11D) did not show a colour change and was comparative to that of the non-mutated control (fig.4.11H). This may indicate that this base does not have an effect on copy number singularly. However, first and last base substitutions in cluster 1 present simultaneously (fig.4.11E) did show a slight colouration without IPTG induction, assumed to be a result of the last base affecting copy number but it cannot be ruled out that both have an effect concurrently.

Neither of the cluster 2 mutants showed a colour change in the absence of IPTG (fig.4.11F & G), suggesting that these mutations alone are not playing a role within determining copy number of the pLS20 replicon. Overall, cluster 1 mutants are the main focus to bring forward as they showed promising colourimetric changes in a minicell producing background.

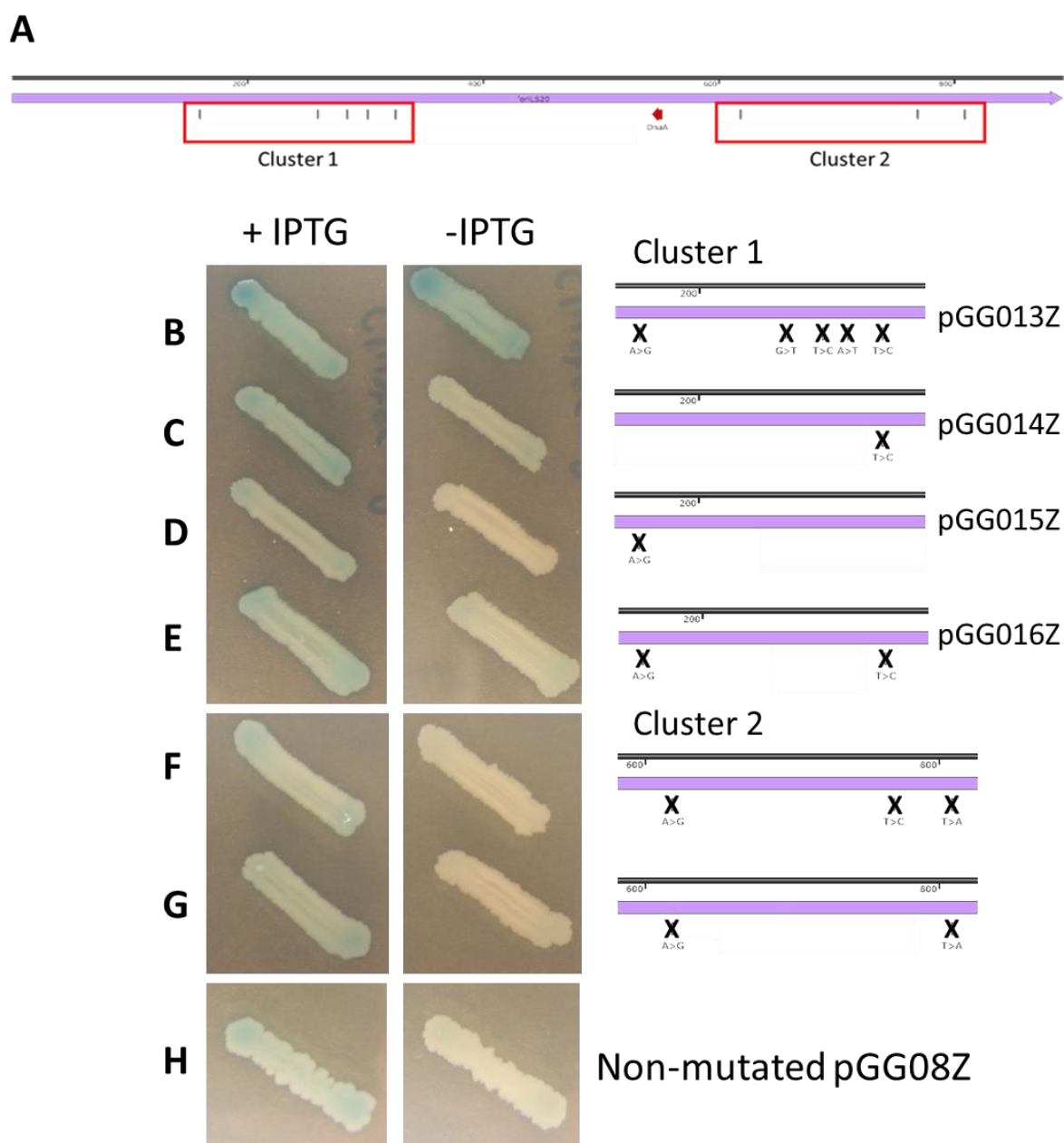


**Figure 4.11. Colourimetric changes between pLS20 mutants.** Plasmids were mutated using error-prone PCR to identify any potential high copy number mutants. Once mutations were identified they were cloned into a non-mutated plasmid and sequenced. Different mutants were transformed into sAG1338 and patched onto plates +/- IPTG induction. In the absence of IPTG colonies differ as copy number may also be affected. Plates (40 µg/ml X-gal, 1 µg/ml Erythromycin, 1 mM IPTG) were incubated overnight at 37°C before images were taken. **A**) demonstration of where cluster 1 and 2 mutants are situated in the 890 bp sequence of pLS20 origin. **B**) whole cluster 1 mutant **C**) last base cluster 1 **D**) first base cluster 1 **E**) first and last base cluster 1, simultaneously. Cluster 2 mutants were displayed in **F**) whole cluster 2 base substitutions and **G**) first and last base substitutions only **H**) non-mutated pLS20 control.

As there was a known high copy number variant of pLS20 from Wilfried Meijer (unpublished), a comparison of the base substitutions was made with the mutants discovered in this study. The pLS100\* mutant had a very high copy number, was found to show a sick phenotype with the Alp7AR operon and could not be repressed easily with a chromosomal copy of LacI expression (Aur lie Guyet, unpublished). This mutant was sequenced, and the mutations aligned (orange shading) with those of the cluster 1 mutants identified by the error-prone PCR in this study (grey shading). The base substitution of cluster 1 circled in red (fig.4.12) showed an overlap with the base mutations of pLS100\*, this single base substitution also showed a colourimetric change on X-gal plates indicating it is singularly effecting copy number (fig.4.11C). This confirmed that this area was important for control of copy number within pLS20. The first base of cluster 1, substituted in pGG015 and pGG015Z, was not included in this diagram as it did not overlap any of the mutants which were identified in pLS100\*.



Figure 4.12. Computational comparison of the "cluster 1" mutants and pLS100\* known to be higher copy number variant of pLS20 replicon. Images were made using SnapGene and sequencing of pLS100\* origin was performed by Aur lie Guyet. The 90 bases were aligned side by side to see what mutations were changed and how they coincide with mutations identified in pLS100\*. Orange/red are pLS100\* identified mutations. Grey shaded bases are mutations identified by this study.

## 4.4 Characterisation of the pLS20 mutants in minicell and maxicell backgrounds of *Bacillus subtilis*

### 4.4.1 $\beta$ -galactosidase assays to quantify the difference between mutants

In the previous chapter, it was shown that the  $\beta$ -galactosidase assay was able to quantitatively represent expression from the whole culture populations with and without mini- or maxicell production. Using  $\beta$ -galactosidase assays, the pLS20 mutants identified were analysed to see how much the potential copy number increased and how this altered the expression from strains with and without anucleated cell populations. This was then correlated with single cell analyses using GFP reporters.

Analysis of the growth of sGG113 parental background, showed that copy number alterations did not significantly affect growth between the strain with different plasmids (fig.4.13A). The  $\beta$ -galactosidase activity assay was used with a non-mutated control (pGG08Z – magenta bars) to set a benchmark for expression, this helped analyse changes to activity which indicated copy number variation.  $\beta$ -galactosidase activity from sGG113 with pGG08Z was low, also shown in chapter 3 where it was discussed that, although expression was leaky, repression was adequate. It was immediately obvious that when the mutations of cluster 1 were present (pGG013Z), there was a significant increase in  $\beta$ -galactosidase activity (ANOVA -  $P=0.0001$ ) (green bars fig.4.13B). This demonstrated that the cluster 1 mutant was likely to be changing copy number so greatly that the LacI repressor was not abundant enough to repress expression. To determine which base substitutions had the greatest effect, the intermediate cluster 1 mutants described in fig.4.11C-E were also analysed. It was shown that plasmids pGG014Z, 015Z and 016Z were not as dramatically increased compared to pGG013Z (fig.4.13C-E). There was a more significant increase from mutants pGG014Z (3-fold;  $P=0.0001$ ) and pGG016Z (3.8-fold;  $P=0.0001$ ) compared to the pGG08Z control (fig.4.13C & E respectively). pGG015 did show

a small increase in  $\beta$ -galactosidase activity, however, this was not as great as the other mutants which may indicate that single base mutation did not affect copy number significantly (fig.4.13D). Overall, it was clear that there was increased activity from most mutants isolated, strongly suggesting copy number was increased.

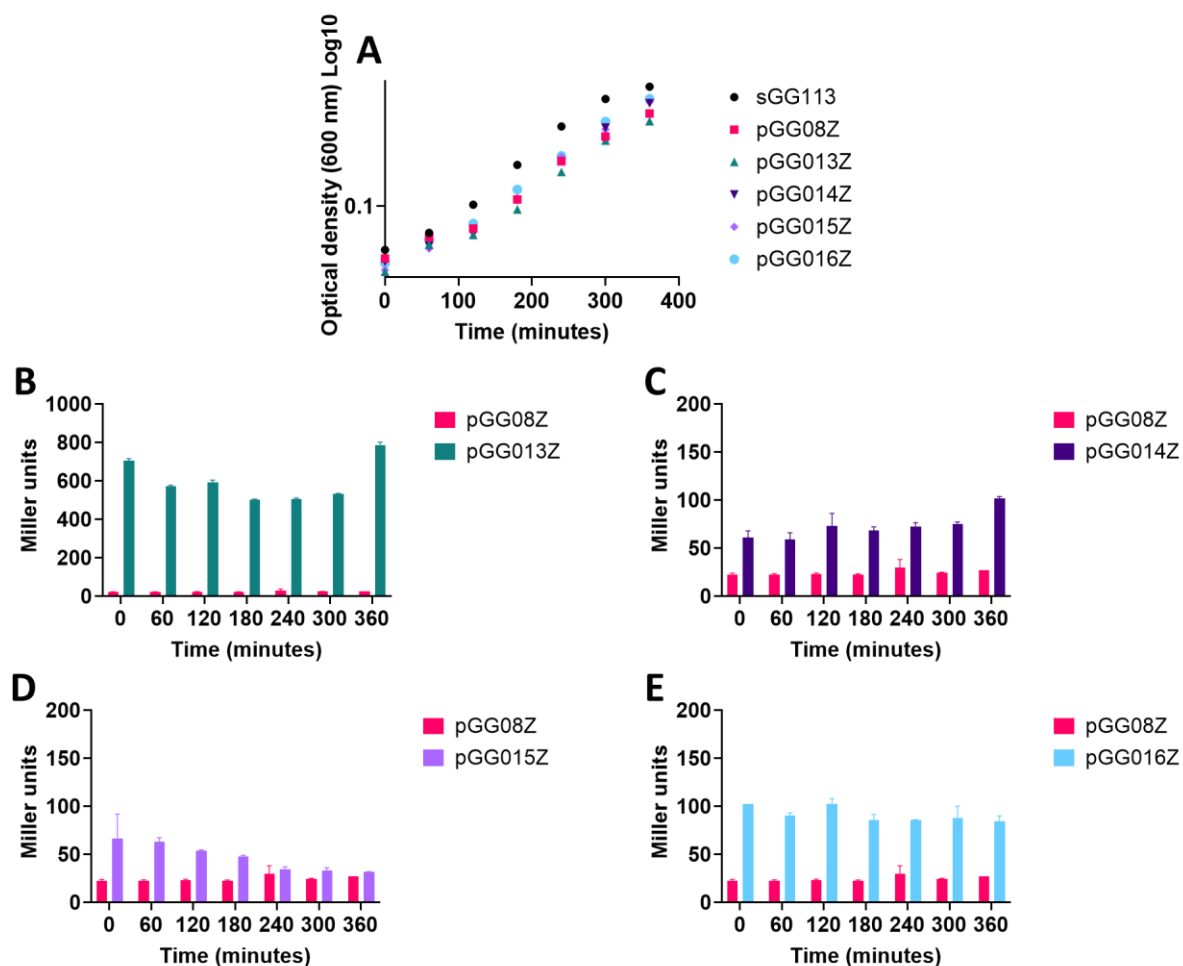


**Figure 4.13. pLS20 copy number mutants  $\beta$ -galactosidase expression in sGG113.** Strains were grown in CDM at 30°C with shaking. Aliquots were taken each hour for lysis and  $\beta$ -galactosidase activity assays using ONPG. **A)** growth curves of sGG113 strains with various plasmids over 6 hours **B)** pGG08Z control and pGG013Z mutant comparison **C)** pGG08Z control and pGG014Z mutant comparison **D)** pGG08Z control and pGG015Z mutant comparison **E)** pGG08Z control and pGG016Z mutant comparison. Error bars are representative of 3 technical repeats for each time point. Statistic included T-testing and analysis of variance (ANOVA).



Introduction of these plasmids into the minicell producing strain (sAG1338) would show if there were increases in expression from the whole population, this in turn indicated if minicell expression was increased. There was no significant changes in growth, as growth curves for all strains were equal to one another, this demonstrated that the plasmids were not burdening (fig.4.14A). Expression of  $\beta$ -galactosidase was varied across all the mutants, but it was obvious that 3 of the 4 mutants had greater significant increases in  $\beta$ -galactosidase activity, indicating copy number may have increased. Similar to what was seen with sGG113 transformants (fig.4.13B), the pGG013Z mutant exhibited a very high  $\beta$ -galactosidase activity (fig.4.14B green bars). Compared to the non-mutated control, averaged across all time points, this corresponded to an ~8-fold increase ( $P=0.0001$ ). When comparing  $\beta$ -galactosidase activity mutants pGG014Z and pGG016Z to the non-mutated control, this was significantly increased in expression of 4-fold and 6-fold respectively ( $P<0.001$  for both compared to the non-mutated control; fig.4.14C & E, purple and blue bars). As this was not as large as the whole cluster 1 mutation, it indicated that these mutants did not affect copy number as greatly as when the whole cluster was mutated. Mutant pGG015Z did not exhibit any significant changes compared to the non-mutated control (fig.4.14D), this showed that the single base mutation was not enough to stimulate a change in copy number. However, in combination with the last base mutation it may be having an effect upon copy number.

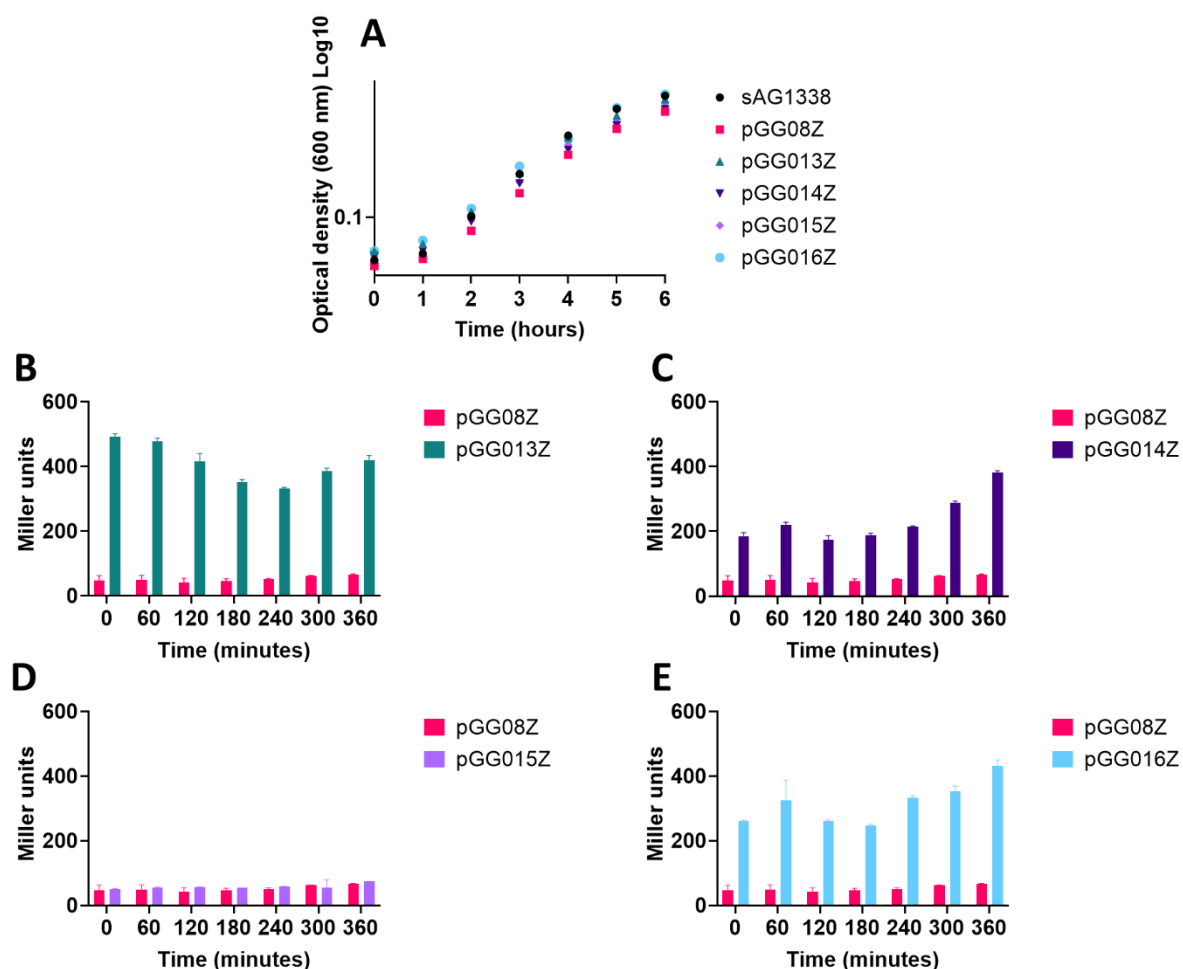


Figure 4.14.  $\beta$ -galactosidase activity from expression of copy number mutants in minicell producing strain sAG1338. Growth in liquid media was a minimal competence media incubated at 30°C with shaking. Aliquots of culture were taken at 1-hour intervals and used for  $\beta$ -galactosidase activity after lysis and ONPG addition.  $\beta$ -galactosidase activity was measured in miller units. A) growth curves of sAG1338 with various plasmids over 6 hours B) pGG08Z control and pGG013Z mutant comparison C) pGG08Z control and pGG014Z mutant comparison D) pGG08Z control and pGG015Z mutant comparison E) pGG08Z control and pGG016Z mutant comparison. Error bars are representative of 3 technical repeats for each time point. Statistic included T-testing and analysis of variance (ANOVA).

A more dramatic outcome was revealed from sGG115, where maxicells were present. Firstly, the strain with mutant pGG013Z (all cluster 1 mutations) showed a greater growth rate across the 6-hour time points (fig.4.15A - green triangles). Secondly, the  $\beta$ -galactosidase assay with pGG013Z showed a significant reduction in activity which was even lower than the pGG08Z control (fig.4.15B). This was contrasting to the previous 2 strains which showed a significant increase in  $\beta$ -galactosidase activity compared to pGG08Z. The other pLS20 mutants all showed similar patterns to the minicell producing strain, where pGG014Z and pGG016Z (fig.4.15C & E) had an overwhelming increase in  $\beta$ -galactosidase expression by comparison to the non-mutated control (magenta bars). pGG014Z with 1 base substitution showed a 4-fold increase over the pGG08Z control, averaged across the whole-time course ( $P < 0.0001$ ). Similarly, pGG016Z displayed a 5-fold increase in expression compared to the non-mutated control plasmid, pGG08Z ( $P < 0.0001$ ). Mutant pGG015Z replicated a similar trend to the minicell producing strain (sAG1338) as there was no significant difference between the mutant and the control strain, suggesting the first base of cluster 1 had no effect on copy number singularly.

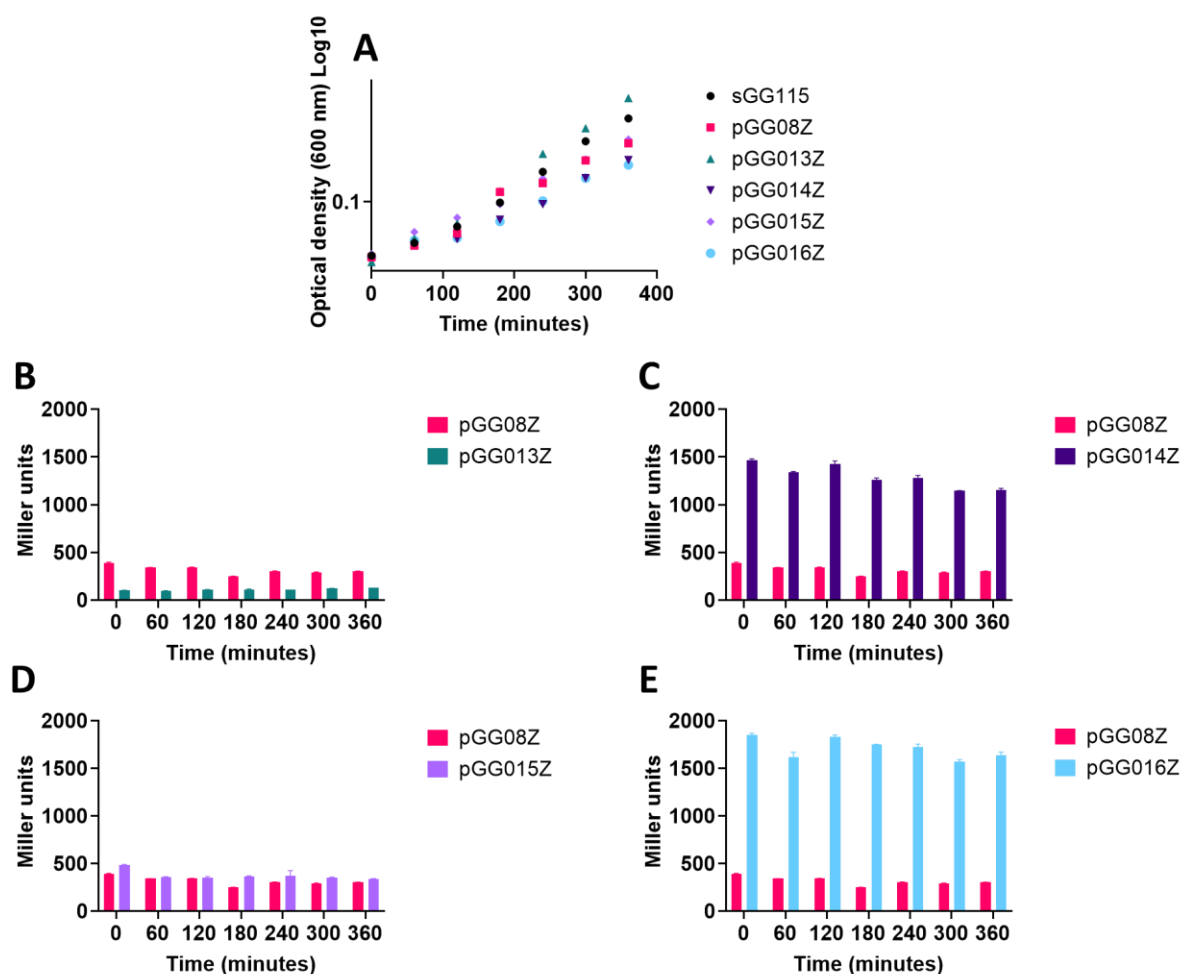


**Figure 4.15. Plasmid expression from maxicell producing strain sGG115.** Maxicell producing strains grown in a minimal liquid media were sampled every hour for 6 hours, growth with shaking was incubated at 30°C. Aliquots of culture were lysed and ONPG added to detect  $\beta$ -galactosidase activity. Sodium carbonate was added to stop the reactions before reading absorbance at 420 nm. **A)** growth curves of sGG115 with various plasmid mutants over 6 hours **B)** pGG08Z control and pGG013Z mutant comparison **C)** pGG08Z control and pGG014Z mutant comparison **D)** pGG08Z control and pGG015Z mutant comparison **E)** pGG08Z control and pGG016Z mutant comparison. Error bars are representative of 3 technical repeats for each time point.

To understand why the maxicell producing strain with pGG013Z did not act as expected, the *P<sub>spac</sub>-lacZ* sequence, mutated pLS20 origin within the plasmid and chromosomal DnaA locus were all sequenced to see if there were any mutations. The plasmid was found to have no extra mutations other than the cluster 1 base substitutions expected. However, the DnaA locus did have a single base mutation which changed amino acid 265 from a phenylalanine to leucine in addition to the *dnaAG152S* mutation which formed maxicells. Therefore, it was concluded that a new strain had been isolated which corrected the reduction in chromosome replication initiation by a single amino acid substitution (sGG137). It was assumed that the mutation was associated with the high copy number variant of pLS20. The growth of controls, sGG113 and sGG113 pGG013Z, on plates and in liquid media exhibited a normal phenotype where all cells were nucleated and density on plates was comparable (fig.4.16A, B, E & F). In contrast, sGG115 and sGG137 showed a phenotypic difference of density on plates (fig.4.16C & D). Where sGG115 showed slower growth, as expected, the sGG137 mutant displayed denser growth which indicated that the maxicell phenotype was no longer present. Subsequent microscopy analyses, from liquid cultures, confirmed that sGG137 did not have any anucleated cells present (fig.4.16H). Whereas the sGG115 parental strain did show anucleated cells, indicated by white arrows of fig.4.16G. Therefore, evidence was provided that the addition of the secondary DnaA mutation at amino acid 265 was suppressing reduced chromosome replication initiation phenotype which was stimulated by the high copy number variant of pLS20 (pGG013Z). Consequently, sGG137 was comparable to the control with no DnaA mutations (sGG113; fig.4.16E). sGG113 with pGG013Z, did not seem to show any changes in phenotype (fig.4.16F) leading to the belief that the mutation was specific to the sGG115 background.

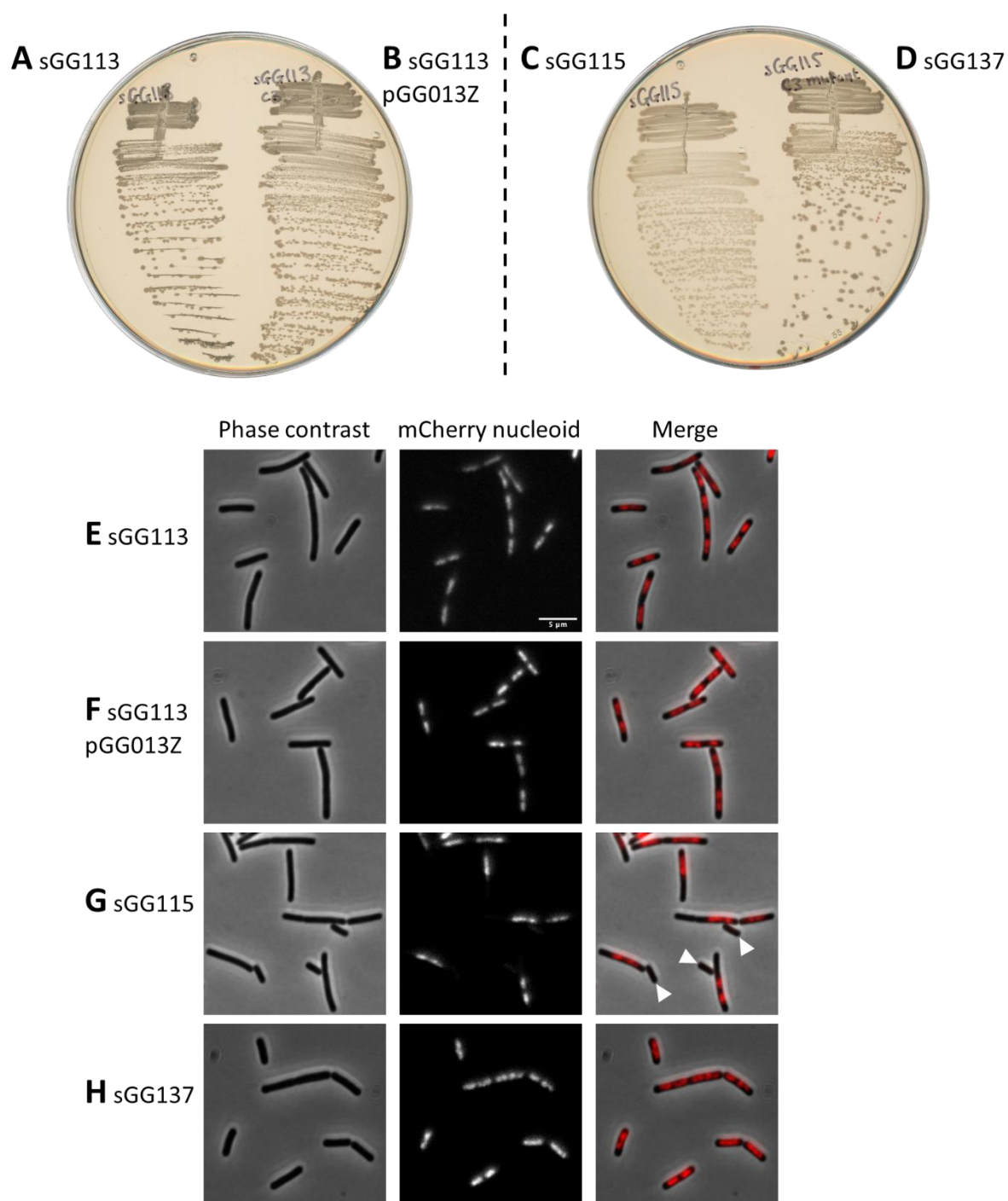
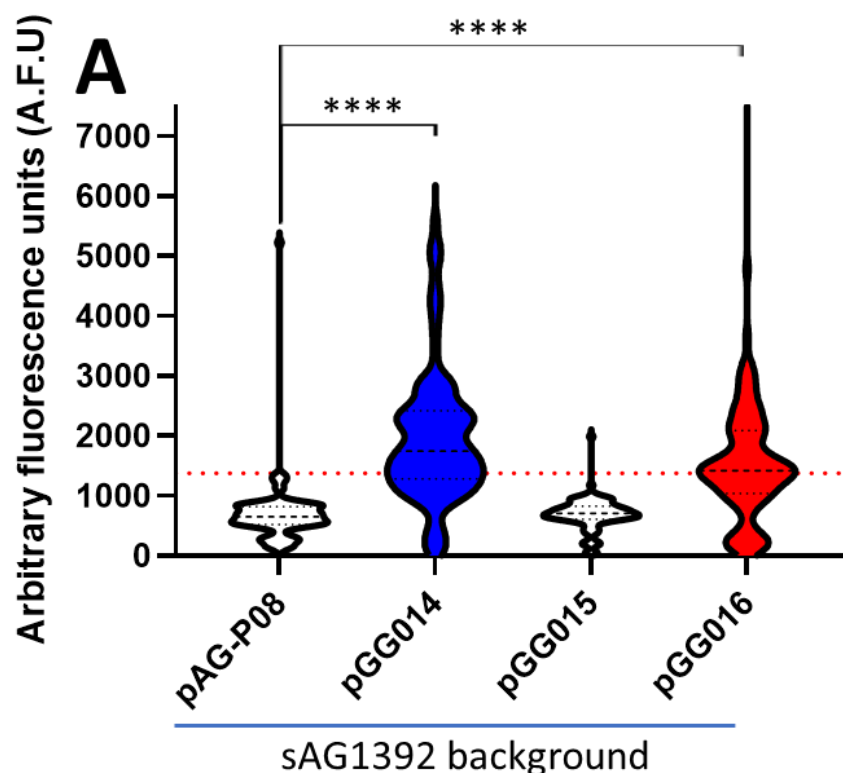


**Figure 4.16. Investigating the reverted maxicell mutant.** Single colonies were streaked onto nutrient agar plates and grown at 37°C for 24 hours **A**) sGG113 parental control **B**) sGG113 after transformation with pGG013Z **C**) sGG115, parental maxicell strain **D**) sGG137 mutant after sGG115 pGG013Z transformation. Images were taken using an Epson scanner with positive film. Microscopy images were taken of cells grown in LB media for 4 hours. Cells were mounted onto a 1.2% agarose pad and imaged with a Nikon Ti eclipse with cool LED white light source and Photometrics prime camera. **E**) sGG113 control **F**) sGG113 after transformation with pGG013Z **G**) parental maxicell strain sGG115 **H**) sGG137 mutant produced after sGG115 transformation with pGG013Z.

#### 4.4.2 Microscopy analysis of pLS20 mutants

To allow single cell and population analyses of specific cell types. The *oriLS20* mutant plasmids analysed using  $\beta$ -galactosidase expression were altered to change the reporter to GFP. As a result of its very high expression rates and causing mutagenesis in the maxicell producing strain, pGG013 was not used any longer for these analyses.

Using nucleated only populations was important to understand how plasmids of varying copy numbers affected the LacI repression. This was central to understanding if LacI was being outcompeted or if further mutations, such as  $\Delta minC$  or *dnaG152S*, were causing disruptions to the system. In the sAG1392 background, pGG015 showed no significant difference to the non-mutated control pAG-P08, consistent with  $\beta$ -galactosidase activity assays in sAG1338 and sGG115. The proportion of cells expressing GFP increased vastly with mutants pGG014 and pGG016, where the population over the threshold increased to 69% and 57%, respectively. This indicated that the two mutants had increased copy number high enough to outcompete LacI repression, therefore, allowed GFP expression from the plasmid. Both pGG014 and pGG016 have a base substitution in common, this is interesting as there is a significant increase in both plasmids compared to the non-mutated control ( $p < 0.0001$ ). Therefore, it was hypothesised that the specific base substitution was significant to increasing copy number (shown in fig.4.11C & E).

**B**

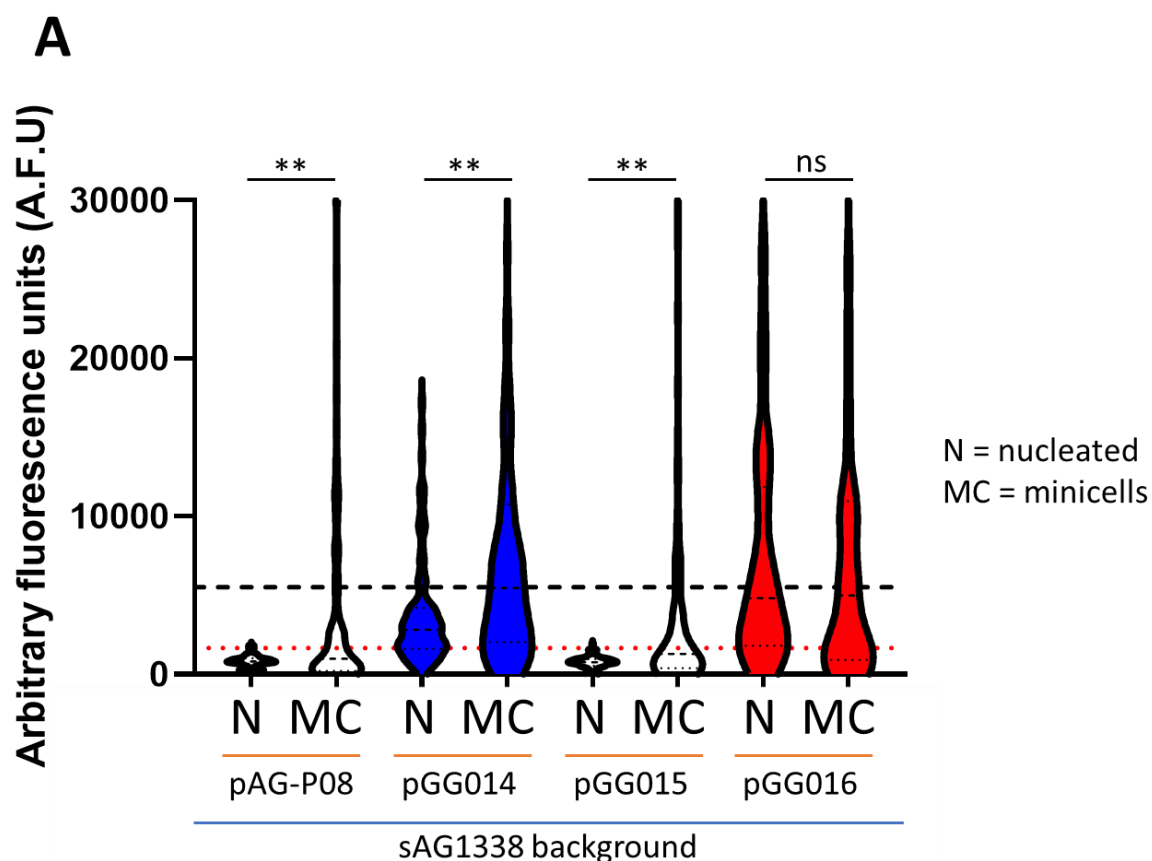
| % population GFP fluorescent (over threshold) |        |        |        |
|-----------------------------------------------|--------|--------|--------|
| pAG-P08                                       | pGG014 | pGG015 | pGG016 |
| 1%                                            | 69%    | 1%     | 57%    |
| Mean fluorescence of sAG1392 nucleated cells  |        |        |        |
| pAG-P08                                       | pGG014 | pGG015 | pGG016 |
| 693                                           | 1968   | 693    | 1588   |

\*sAG1392 control, all cells nucleated.

**Figure 4.17. Fluorescence analyses of pLS20 copy number mutants in sAG1392.** All strains were grown in CDM at 30°C with erythromycin selection. During mid-exponential phase, cells were mounted onto a 1.2% agarose pad before imaging with Nikon Ti eclipse microscope with cool LED light source and Photometrics primer camera. The cells were counted using Fiji software and plotted into violin plots using GraphPad prism 9. **A)** Violin plots representing the populations GFP fluorescence **B)** percentage of cells over the red dotted threshold as calculated on Microsoft excel. N=100 cells per population repeated 3 times. \*\*\*\* represents significant T-test ( $P < 0.0001$ ).



As there was a clear increase in GFP expression in nucleated only populations, it was hypothesised that the number of minicells expressing GFP should also increase. This was hypothesised as a greater abundance of plasmids was assumed to increase the likelihood that a minicell gained a plasmid. The minicell producing strain (sAG1338) was analysed for populations of nucleated rods and minicells separately, this would reveal if more minicells were gaining a plasmid and if LacI was able to actively repress expression in nucleated cells. When pLS20 mutants pGG014 and pGG016 were transformed into sAG1338, it was clear that there was a large increase in all populations GFP expression (fig.4.18A & B). Surprisingly, with pGG014 there was less nucleated cells expressing GFP compared to pGG016. It was also revealed that pGG014 increased minicells fluorescence means significantly compared to nucleated cells (fig.4.18A- blue plots;  $P=0.0001$ ). This was encouraging as it indicated that pGG014 was compartmentalised into minicells. Despite minicell fluorescence significant increase with pGG014, there was only 5% more minicells over the threshold compared to nucleated cells (fig.4.18- blue). Therefore, it was possible that a large majority of minicells were carrying cytosolic GFP from nucleated cells, making it hard to determine if minicells were independently expressing from the plasmid. Consequently, a new threshold was made to see how many minicells were brighter than the end of the normal distribution of nucleated cell fluorescence (fig.4.18A- black dotted line). Although 50% of minicells were counted as GFP positive, there was still a large proportion of nucleated cells with high GFP intensity (19%; fig.4.18C). In contrast, with pGG016 the fluorescence increased so much that there was no regulation and no significant difference between nucleated cells and minicells (fig.4.18A- red plots;  $P=0.46$ ). Overall, with pGG016, it was clear that modulating the copy number did not increase minicell independent expression as nucleated cells also showed an upregulation of GFP. Overall, it was found hard to conclude how many minicells were independently expressing GFP, as nucleated cell populations had increased so highly.

**B**

| % population GFP fluorescent (over threshold) |           |           |           |           |           |           |           |
|-----------------------------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| pAG-P08                                       |           | pGG014    |           | pGG015    |           | pGG016    |           |
| nucleated                                     | minicells | nucleated | minicells | nucleated | minicells | nucleated | minicells |
| 4%                                            | 43%       | 75%       | 80%       | 1%        | 47%       | 82%       | 67%       |
| Mean fluorescence of sAG1338 nucleated cells  |           |           |           |           |           |           |           |
| nucleated                                     | minicells | nucleated | minicells | nucleated | minicells | nucleated | minicells |
| 832.3917                                      | 4394.749  | 3685.09   | 10183.87  | 753.8485  | 6682.824  | 8173.646  | 9395.799  |

**C**

| pGG014    |           |
|-----------|-----------|
| nucleated | minicells |
| 19%       | 50%       |

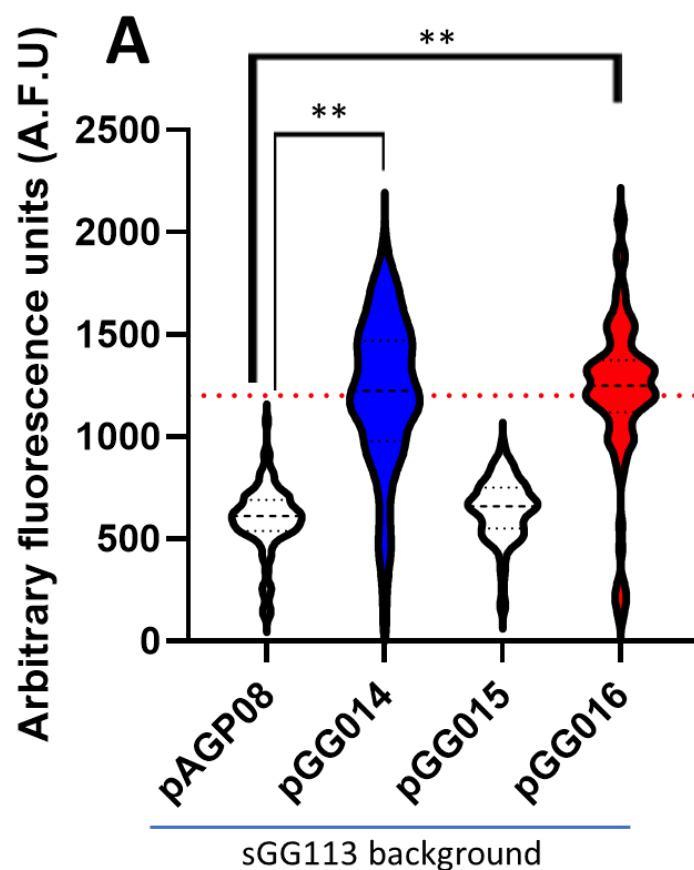
**Figure 4.18. Fluorescence of pLS20 mutants in minicell producing strains.** Cultures with different plasmids were grown in a liquid CDM with antibiotic selection for the plasmid. After 4 hours of growth, during mid-exponential phase, 1  $\mu$ l of cell culture was mounted onto a 1.2% agarose pad. Imaging took place on the Nikon

Ti eclipse microscope with cool LED light source. **A)** Violin plots representing the populations GFP fluorescence **B)** percentage of cells over the red dotted threshold as calculated on Microsoft excel. **C)** number of cell populations with pGG014 over the black dotted threshold. N=100 cells per population. T-tests with Welch's corrects were performed using GraphPad prism 9.

**Maxicells fluorescence with pLS20 mutants.** As maxicells are much larger compartments, it was hypothesised they were more likely to gain a plasmid by comparison to a minicell. However, as chromosome replication is hindered it was likely that LacI expression decreased, potentially leading to much greater expression.

To show how nucleated populations would respond to *oriLS20* mutants and determine how repression was affected, sGG113 was used as a parental control. The parental control would determine how expression was influenced in cells with normal chromosome replication. The threshold line was set in accordance to double the mean fluorescence from cells with pAG-P08. Any cells with fluorescence plotted higher than this threshold was counted as GFP positive as they were expressing more than the baseline from the non-mutated control. This parental strain showed that there were not many bright outliers from groups with pAG-P08 and pGG015 as neither went beyond the threshold line (fig.4.19A- white plots). Both populations showed relatively tight grouping which displayed homogeneity within populations fluorescence intensities. Although both were under the threshold, there was an increase in the mean fluorescence intensity from cells with pGG015 consistently across biological repeats. This indicated that there could be a small alteration in copy number from the single base mutation compared to the non-mutated control. Expression from pGG014 and pGG016 was significantly increased (fig.4.19A;  $p < 0.0001$ ), which suggested LacI was outcompeted in nucleated populations. GFP expression with pGG014 was much more heterogeneous, as violin plots were not as closely grouped. On the other hand, GFP expression from pGG016 was

grouped closer together and indicated that there was greater homogeneity although there were still outliers.



**B**

| % population GFP fluorescent (over threshold) |        |        |        |
|-----------------------------------------------|--------|--------|--------|
| pAG-P08                                       | pGG014 | pGG015 | pGG016 |
| 0%                                            | 54%    | 0%     | 61%    |
| Mean fluorescence of sGG113 nucleated cells   |        |        |        |
| pAG-P08                                       | pGG014 | pGG015 | pGG016 |
| 601                                           | 1205   | 646    | 1225   |

\*sGG113 control, all cells nucleated.

**Figure 4.19. Maxicell parental strain analyses of pLS20 mutants in sGG113.** *B. subtilis* LacI strains were transformed with plasmids including those with pLS20 mutations. Cultures were grown in CDM with antibiotic to select for the plasmid and continuous shaking. Cell culture was applied directly to a 1.2% agarose pad for imaging. GFP fluorescence analysis was counted using Fiji ImageJ and violin plots using graphpad prism 9.

Statistical tests carried out were comparisons of the mean, T-test with Welch's correction. **A)** Violin plots representing GFP intensity. **B)** % of populations over the threshold (dotted line) and the means for each population's fluorescence intensity. N=100 cells per population.

In the sGG115 background, there was a more positive outlook for maxicell expression and nucleated cell repression. Regardless of which plasmid was present, the fluorescence intensity means for all maxicell populations were significantly greater than that of cognate nucleated cells ( $P < 0.001$  for each). When the copy number was increased in mutants pGG016 and pGG014, GFP expression increased significantly in both anucleate maxicell populations and fluorescence intensity appeared to be more heterogeneous (fig.4.20; 88-95%, respectively). Although there was a large increase in nucleated cells over the control threshold (red dashes), there was evidence of greater repression than what was seen in the minicell strain (fig.4.18A). When the threshold was moved to the end of the normal distribution for pGG014 and pGG016 nucleated cells (fig.4.20A black dotted line), 70-75% of maxicells were over the new threshold (fig.4.20C). The values in nucleated cells were grouped together tighter which showed that there was a more homogenous population and greater repression than what was seen in nucleated cells of sAG1338 (fig.4.18A). This was an encouraging result as it showed modulation of *oriLS20* can have an effect in anucleated cells and indicated that more maxicells were gaining a plasmid. This demonstrated that the increase in plasmid copy number could increase maxicells gaining a plasmid from ~50% to ~70%. Interestingly, there was no significant difference between GFP fluorescence in nucleated cells with pAG-P08 and pGG015 as there was with the sGG113 control ( $p=0.5$ ). This indicated that any copy number alterations in pGG015 did not have an effect in the sGG115 background. Overall, the cost did not seem as drastic for nucleated cell repression as it did in sAG1338 and there was a more positive reflection that more maxicells were gaining a plasmid.

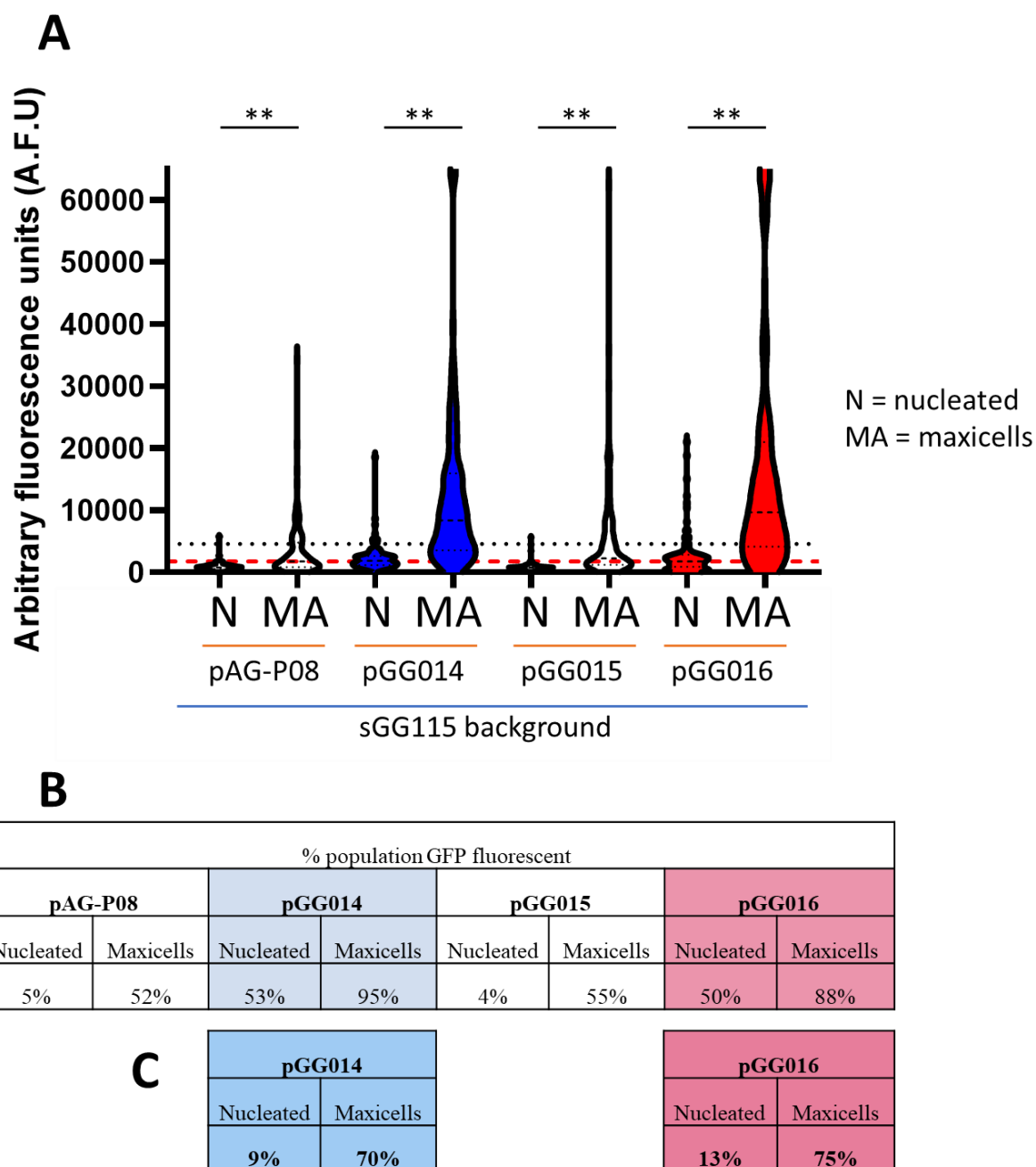


**Figure 4.20. pLS20 mutants in the maxicell chassis fluorescence analyses.** sGG115 strains with various pLS20 mutants were grown in CDM with erythromycin to select for mutants with the plasmid. The culture was spotted directly onto agarose pads before imaging with Nikon Ti eclipse and cool LED light source. **A)** violin plots of cell populations and their fluorescence **B)** percentages of populations over the red dotted line threshold set calculated via excel. Fluorescence values were counted using Fiji. Statistical tests carried out were comparisons of the mean, T-test with Welch's correction. **C)** number of cell populations over the black dotted threshold applied to show that many maxicells are over the normal distribution of nucleated cells. N= 100 cells per population.

#### 4.4.3 Copy number determination for *oriLS20* mutants

As the effects of the *oriLS20* mutants had been revealed through  $\beta$ -galactosidase and fluorescence intensity quantification, determining copy number was required to understand the effects of each base substitution. As it was hypothesised that each cell has one genome, plasmids were quantified in relation to the chromosome terminus. Therefore, plasmid copy number was relative to genome number. Primers were used to amplify the chromosome terminus or *lacZ* gene specific to the plasmid. Standard curves shown in appendices 7 were used to define Ct values which were then used for quantification. The samples were taken across different time points to show copy number during early exponential, mid exponential and stationary phase. After each 2-hour interval the plasmid number either remained the same or increased. The most notable difference in copy number was when the whole cluster 1 mutations were integrated (pGG013Z) where the copy number increased 10-fold compared to the non-mutated control consistently across each time point. This may explain why the maxicell producing strain mutated because the mutant DnaA protein was not able to act upon the higher copy number plasmids demand. The mutants with milder phenotypes (pGG014Z and pGG016Z) also revealed a significant increase in copy number, however, the greater increase was shown in pGG016Z. This was interesting as, the single base mutant of pGG015Z showed a small increase in copy number compared to the non-mutated control and no significant phenotype change in sAG1338 and sGG115. However, in combination with the last base mutation the copy number increases significantly again suggesting that these two mutations are working in tandem to increase the copy number. The last base mutation on its own (pGG014Z), was ~3-fold greater than the non-mutated control. The first base on its own (pGG015Z) showed a 1.5-fold increase and when both first and last bases were substituted the plasmid copy increased 4 to 5-fold, respective of time. Another interesting observation was that the mutant

plasmids increased copy number steadily over the growth phases (fig.4.21A-C) whereas pGG08Z control remained stable.

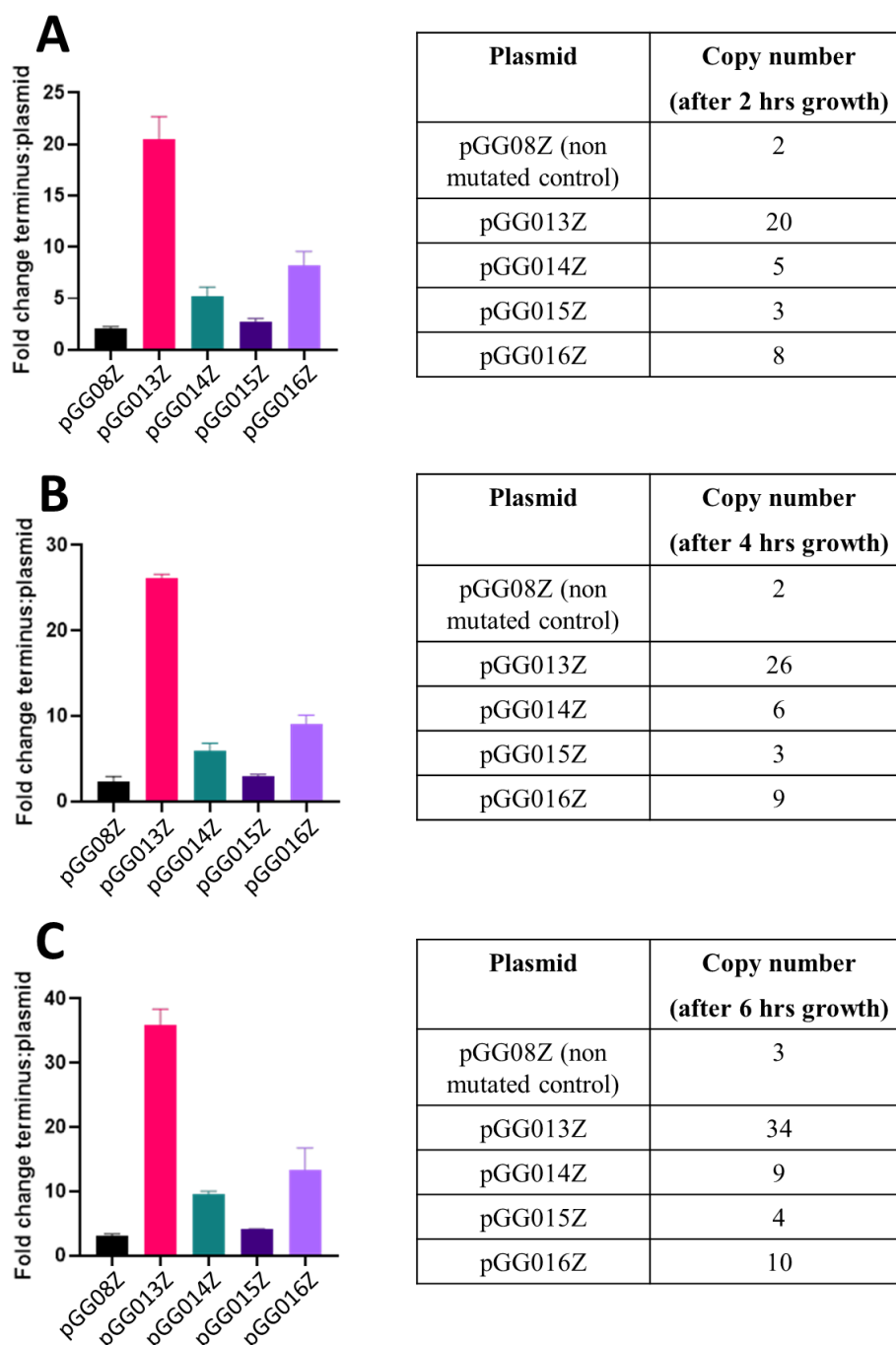


**Figure 4.21. qPCR analyses of the pLS20 mutants found by error-prone PCR.** sGG113 with control and mutant *oriLS20* plasmids were grown in CDM at 30°C before taking 5 ml samples every 2 hours after time 0. The cells were harvested via centrifugation, lysed in lysozyme based lysis buffer before sonicating in 10 second pulses 10x. The resulting lysates were centrifuged and the supernatant transferred to clean tubes. The DNA was diluted



1000x before pipetting by the QIAgility robot into NEB Luna reactions, data was obtained and analysed from RotorGene-Q (Qiagen) software. Plasmid copy number was calculated in Microsoft Excel. **A)** plasmid copy number after 2 hours growth **B)** plasmid copy number after 4 hours growth **C)** plasmid copy number after 6 hours growth. N=2 biological replicates. Error bars are representative of biological replicated standard deviation.

## 4.5 Conclusions and discussion

This chapter has demonstrated the effect of altering the copy number of the pLS20 replicon in *B. subtilis*. Random mutagenesis techniques revealed mutations that increased copy number and further analyses gave insight into how this altered segregation efficiency into anucleate compartments. The results obtained indicated that moderate increases affected regulation in sAG1338 significantly, whereas, in the maxicell model there was 70-75% of anucleate compartments expressing GFP with better regulation in nucleated cells observed.

### 4.5.1 RepN iteron mutant approach.

An approach was attempted where the pLS32 replicon was separated into the identified iteron sequences on the plasmid and a mutated RepN protein expressed from the plasmid with  $P_{xyl}$  promoter (Tanaka *et al.*, 1998). This tuneable system would have been a good tool for looking at optimal copy numbers for adequate nucleated cell repression and anucleate expression. With the initiator protein on the chromosome under  $P_{xyl}$  control, the amount of induction would have determined the amount the plasmid replicated. Therefore, the system could have been optimised to yield the best number of compartments with a plasmid, while repression in nucleated cells was retained to a high level. This study did show that mutating the RepN iteron sequences did allow compatibility with a plasmid harbouring *repN/oriN*, as sGG104 was able to be transformed with pGGZ32. However, unfortunately the system did not work as it is believed the plasmid had integrated into the chromosome through homologous recombination events. A diagnostic PCR showed that the plasmid was present as oligonucleotides were able to amplify plasmid specific sequence (see section 4.2.2). However, the inability to transform

plasmid DNA extracted back into *E. coli* was suggestive that the plasmid was no longer present as an extra chromosomal element (i.e., free plasmid).

Mutations were required as previous data had suggested that RepN expressed from the chromosome and present on a plasmid was incompatible (Tanaka *et al.*, 2005). Though the mutations to remove AT-rich iteron regions from the protein were silent, it isn't known if the protein was functional or how functionality could have been affected by these mutations. There is evidence in the literature which suggested silent mutations can significantly alter structure and function of proteins by affecting translation (Patel *et al.*, 2019; Kimchi-Sarfaty *et al.*, 2007). Therefore, it may be possible that the mutant RepN protein was incapable of binding to iteron sequences to initiate plasmid replication.

Future work into this system would be to determine if the plasmid was present or if *P<sub>spac</sub>-lacZ* and erythromycin cassette was integrated into the chromosome. This might simply be done by determining the stability of the antibiotic resistance marker. If it were found that there was antibiotic sensitivity, it would suggest there was a free plasmid present and perhaps the inability to transform *E. coli* was mutagenesis of *oriBR322*. If there was 100% stability, it would be highly suggestive that homologous recombination had occurred. However, other issues with this system would still be prevalent in terms of Portabolomics project requirements since *oriN/repN* does not work in *E. coli* (see section 1.6.2). In addition, *S. elongatus* compatibility was required hence other replicon systems were investigated (see section 5.2).

#### **4.5.2 pLS20 stability**

One major factor which was considered for industrial application was plasmid stability, this is an important factor as cells without the required genetic circuits are not useful. Therefore, the plasmid stability of pLS20 in the Portabolomics vector was analysed on solid and liquid media. It was seen over 24 hours the integration of the Alp7AR operon did increase the stability of the

pLS20 origin, described by Derman *et al.* 2012. Growth in liquid media, without erythromycin selection, showed the plasmid was not stable over 24 hour cycles as the CFU/ml decreased each day (see section 4.3.1). The addition of erythromycin, to select for the plasmid, could be costly if large batches were required for industrial fermentation. Future work would focus on optimising stability, to ensure each cell had a plasmid, by exploring different partitioning systems for both stability and compartmentalisation into anucleate cells.

The solution used in this project was the Alp7AR operon which increased stability of the low copy number plasmid significantly (see section 4.1.2), however, this system requires strict control as work by Derman *et al.* 2012 showed that depletion of the Alp7A regulator (Alp7R) destabilises the plasmid and eventually causes a lethal phenotype. This was further shown in the pLS100\* high copy number mutant gifted by Wilfried Meijer, when Alp7AR was integrated the plasmid became unstable and exhibited a sick phenotype (A. Guyet, unpublished). This is why future work would explore the use of different partitioning systems as, although this system was successful, it could cause issues in plasmid variants with high copy numbers.

### **4.5.3 pLS20 random mutagenesis**

Various routes for mutagenesis were explored, the most successful was error-prone PCR generating 8 single base mutations to investigate. Further analyses identified 5 candidates controlling plasmid copy number singularly or in combination. Taking the approach of looking for high copy number mutants which outcompeted LacI was beneficial as the mutations found randomly could be dissected to understand the drive for copy number control. Attempts to mutagenize the plasmid with XL1-red mutagenesis were not successful (see appendices 6). Some mutants were identified, however, most did not retain their blue phenotype in the absence of IPTG whereas others had no significant origin mutations detected (Appendix 6).

Firstly, error prone PCR did provide mutants which were able to out-compete the LacI repressor clearly on solid media which retained their phenotype when passed through *E. coli* (see section 4.3.2). Although there were 2 clusters of base substitutions found, cluster 2 did not have any phenotype and therefore was not studied further. The sum of mutations in cluster 1 resulted in the reversion of the maxicell strain (section 4.4.1). The single DNA base mutation of DnaA caused the phenylalanine amino acid at position 265 to change to leucine. This amino acid was positioned next to an identified arginine finger (264A) which is essential for oligomerisation of the DnaA molecule and deletion is lethal (Scholefield *et al.*, 2012). This was suggestive that DnaA was essential for replication of pLS20 as increased demand for DnaA, by increasing copy number, forced a mutation selecting for DnaA with greater functionality. The amino acid 265 mutation seemed to correct the *dnaAG152S* phenotype, therefore it was hypothesised that DnaA efficacy was increased as no anucleate cells were produced. While both amino acids are hydrophobic, phenylalanine has an aromatic ring which has been known to aid in protein structure and protein-DNA complexes (Anjana *et al.*, 2012). Aromatic rings are not present in leucine, potentially altering the structure around the arginine finger. As the  $\beta$ -galactosidase activity had decreased to lower levels than the control, it was also hypothesised that the mutant may be over replicating the chromosome and therefore increasing the expression of LacI. It is clear from this study that the *dnaAPhe265Leu* mutation can increase DnaA efficacy, however, it is unknown if this mutation is effective on its own or only with the *dnaAG152S* accompaniment as this mutation has not been documented before. Spontaneous DnaA mutagenesis supports the theory proposed in chapter 3 that, although the specific DnaA box was not essential, DnaA has a role in pLS20 replication and there are likely other DnaA recognition motifs within the replicon.

Unpublished transcriptome data by Wilfried Meijer suggests that the pLS20 replicon could open a replication bubble via 2 opposing promoters acting against one another, recruiting RNA

polymerase enzymes which can open the DNA. Two opposing promoters could fit with some of the mutations found. However, as it has been discussed, it is believed that DnaA is essential for the pLS20 replicon and is likely initiating replication. One identified DnaA box was found to be non-essential (see section 3.4.2), yet it is not certain if there are more DnaA boxes present. It was interesting that the area in which mutations were found affecting copy number were in alignment with a mutant Wilfried Meijer had identified as very high copy number (see section 4.3.2) (Meijer, unpublished). This area is clearly of importance and the mutants identified by this study show singular base mutations can affect copy number. Bioinformatic analyses may be beneficial for this area of the replicon and may even identify essential DnaA box(es). Experimentally, this might be addressed using Chromatin immunoprecipitation and DNA sequencing or qPCR (ChIP-seq or ChIP-qPCR, respectively). This would identify where the DnaA protein is bound on the plasmid in a way that is similar to experiments described by Merrikh and Grossman (2011).

The original aim was to find copy number mutants which increase anucleated cells gaining a plasmid, as chapter 3 showed both *B. subtilis* chassis' had ~50% of GFP positive compartments during mid-exponential growth. Firstly, the  $\beta$ -galactosidase assays showed some promising results as sAG1333, was retaining a good level of repression. The mini- and maxicell producing strains then showed much greater expression which was assumed to be a result of more anucleated cells gaining and expressing  $\beta$ -galactosidase from the plasmid. However, microscopy analyses refuted this as it showed there was a large increase in rod cell expression of GFP, particularly in the minicell producing strain, showing the importance of single cell analyses. It seemed from this data that the original pLS20 replicon (pAG-P08) was most consistent between *B. subtilis* chassis as there was a very fine balance between copy number and repression of GFP expression in nucleated cells, when tipped, affected repression in nucleated cells greatly (see section 4.4.2).

In contrast to the minicell system, maxicell producing strains displayed a much more encouraging outcome with altered copy number mutants, although there was an increase in nucleated cell expression there was a large increase in maxicells expressing GFP. Though there was a threshold set in accordance with the control (pAG-P08), when further analyses were performed to change the threshold to the end of the normal distribution of the nucleated cells with pGG014, it showed >70% of maxicells were GFP positive. It is unknown why these copy number mutants work better in the maxicell producing strains, one hypothesis was that the larger compartments were taking more plasmids and therefore reducing burden on nucleated cells as the number of plasmid was reduced. This is plausible as a minicell is estimated to be  $1/10^{\text{th}}$  of the size of the rod cell and therefore is much less likely to gain plasmids (Adler *et al.*, 1967). While increasing the amount of maxicells gaining a plasmid was encouraging, the repression system could still be improved. Firstly, LacI was already abundant within cells as it was constitutively expressed from  $P_{pcn}$ , a penicillinase promoter from *Bacillus licheniformis* (Yansura and Henner, 1984). Therefore, to improve repression, alternate promoters and repression systems could be trialled to see if a more efficient combination is possible.

It is unknown if higher copy number means more maxicells are gaining one or more plasmids or if a maxicell gaining more plasmids expresses GFP quicker during exponential phase. To know for sure how many maxicells have a plasmid and how many plasmids are in maxicells, more experiments would be required to label the plasmid and use microscopy to visualise localisation. Fluorescence *in situ* hybridisation or GFP-LacI/LacO microscopy methods could be very useful for this and could provide a way to look at all cell types in the culture and see what is occurring in nucleated and anucleate populations (Pogliano *et al.*, 2001).

qPCR yielded some interesting results, it was shown the whole of cluster 1 mutants together increased the copy number to 20-30 copies per chromosome. Single base mutations were also able to have a significant effect on copy number, for example, pGG014Z increased copy

number ~3-fold by one base substitution (pGG014Z; see section 4.4.3). Fascinatingly the mutant pGG015Z did not show any phenotypic changes in either  $\beta$ -galactosidase activity or GFP assays in sAG1338 or sGG115, so it was assumed the mutation did not significantly affect copy number. However, qPCR analyses revealed there was a small copy number increase in all time points. This could potentially help to understand the role in copy number control as small alterations could be important for incremental increases in copy number to try balance minicell gain and nucleated cell repression.

In conclusion, from the mutants gained via error-prone PCR there were some *oriLS20* base substitutions of interest which could be used to increase the amount of anucleate cells gaining a plasmid. pGG014 was a promising candidate which indicated increasing copy number to 6 copies per chromosome, 3-fold greater than wild type *oriLS20*, could be useful if repression were to be enhanced. However, increasing copy number did not give a desired effect in the minicell-producing mutant as regulation in this chassis was much more sensitive.

## Chapter 5 - *Escherichia coli* minicell chassis

### 5.1 Introduction

To replicate the minicell system described in *B. subtilis* in a Gram-negative organism, *E. coli* was adopted. A low copy number plasmid was investigated to try reproduce the *B. subtilis* system in an attempt to have the same or higher number of *E. coli* minicells expressing from the plasmid. The pLS20 replicon in *B. subtilis* is a theta-type replication system with a copy number of ~2-4 plasmids per cell (Meijer *et al.*, 1995). For *E. coli*, the SC101 replicon is also a theta-type replicon composed of 770-bp with a 220-bp minimal essential replication region (Kazuo and Mitsuyo, 1984; Sugiura *et al.*, 1993). SC101 requires host replication initiation protein DnaA as it has a *dnaA* box within the essential 220-bp region and has ~5 copies per chromosome (Hashimoto-Gotoh and Sekiguchi, 1977; Sugiura *et al.*, 1993). Within the replicating region, 3 iteron sequences have been identified which are required for initiation of replication, iterons are the target for replication initiator proteins to recognise and melt AT-rich regions (Sugiura *et al.*, 1993; Tanaka, 1998). SC101 is well characterised in *E. coli* and there are many different mutations found which can increase the copy number or temperature sensitive replication mutants (Hashimoto-Gotoh and Sekiguchi, 1977; Peterson and Phillips, 2008).

The second approach for the vector replicon was to use RSF1010, this replicon encodes its own initiation protein for self-replication, not dissimilar to pLS32 described in section 4.1.1. RSF1010 is broad host range in most Gram negative bacteria; it has approximately 10 copies per chromosome in *E. coli* and is extremely mobile via conjugation (Frey *et al.*, 1992; Katashkina *et al.*, 2007). RSF1010 initiates replication independently of host replication factors such as the *dna* genes but relies on proteins encoded within the plasmid replicon. It was of interest to this project as it is known to replicate within *S. elongatus* and some Gram positive



actinomycetes such as *Streptomyces lividans* and *Mycobacterium smegmatis* and could be introduced via conjugation, meaning this vector may work within all hosts without using a dual replicon system (Gormley and Davies, 1991). Utilising a single replicon on the plasmid was an aim of the Portabolomics project, where it was aimed to use one vector between *E. coli* and *B. subtilis* chassis.

## 5.2 Results: microscopy analyses of Portabolomics chassis

Two plasmids were constructed to generate low copy number replicons. These were based on the *B. subtilis* plasmid pAG-P08 which had a pMB1 origin from pBR322 (Bolivar *et al.*, 1977). As pMB1 was ‘medium’ copy number (~20 copies), it was clear this was too high for LacI repression as GFP expression was evident on solid media when colonies were illuminated with blue light (5.1B & D). The *ori* region of pAG-P08 derived from pMB1 was replaced with SC101 origin to give pGGS01 (~5 copies per cell) or the RSF1010 origin to give pGGR01 (~10 copies per cell) (fig.5.1A; Kazuo and Mitsuyo, 1984; Frey *et al.*, 1992).

The first replicon tested was SC101 driven (pGGS01) compared to the parental plasmid (pAG-P08), strains were observed in DH5 $\alpha$  and XL1-blue under blue light illumination to excite GFP and show expression (fig.5.1B-E). The XL1-blue strain was used as it contained the *lacI<sup>q</sup>* gene which enhanced repression and was planned to be used in the minicell producing strain (fig.5.1D & E). It was obvious with the parental plasmid (pAG-P08; fig.5.1D) that *lacI<sup>q</sup>* did not make a difference to expression levels (fig.5.1D), however, there did seem to be a reduction in fluorescence with the SC101 driven plasmid (fig.5.1E) compared to the same vector in DH5 $\alpha$  (fig.5.1C). It was interesting that, in the presence of IPTG, the SC101 driven plasmid did not show a large increase in GFP fluorescence. This indicated that GFP was expressed less in this vector, although, sequencing had confirmed the *P<sub>spac</sub>* promoter and *gfp* were not damaged. However, testing on solid media was not very sensitive, especially as cell populations were in

mixed phases of growth, but this method did reveal that the SC101 vector was reducing “background” levels of GFP expression. Then to get a better idea of the degree of control on expression, single cell analyses was required.

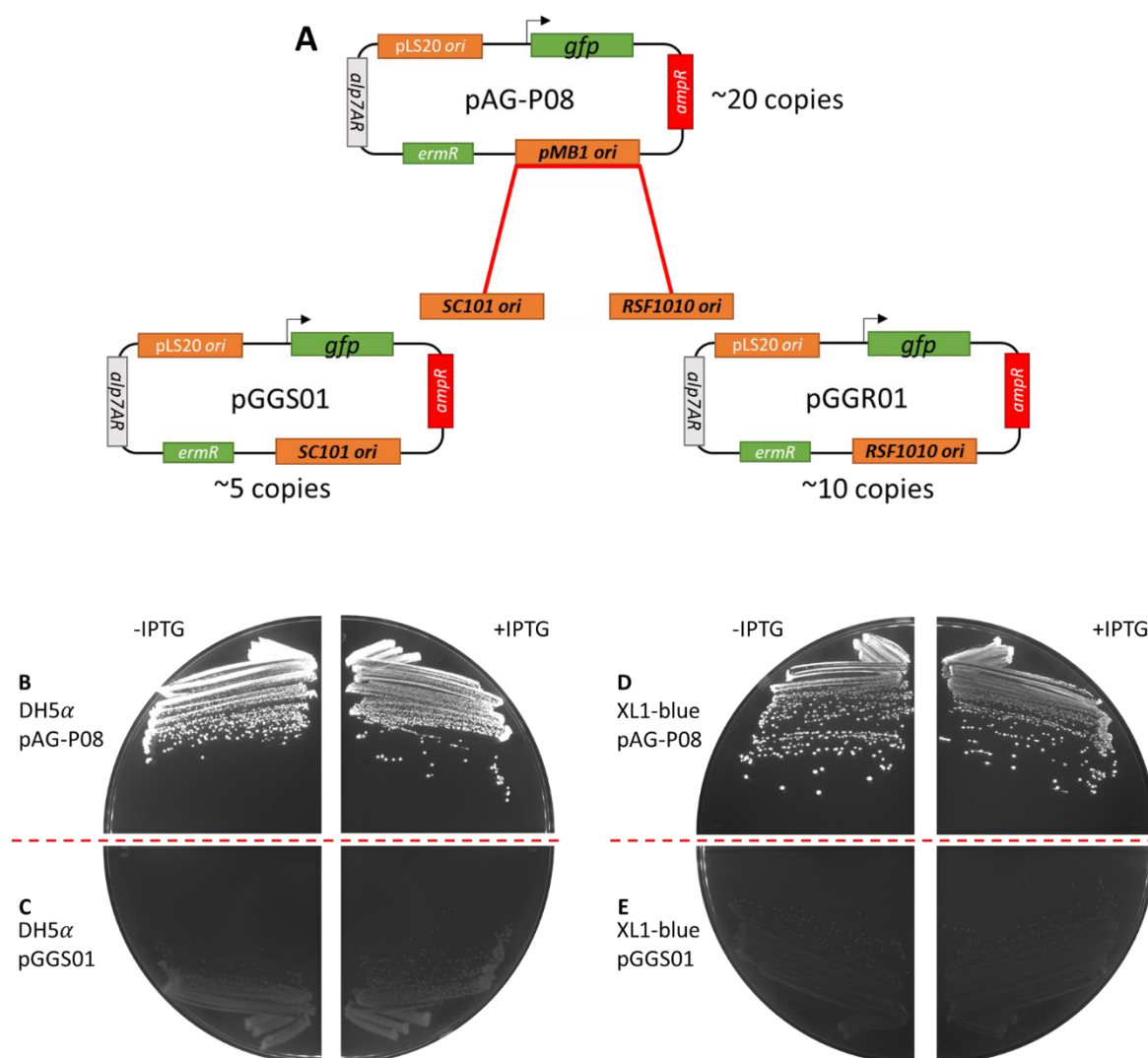


**Figure 5.1. Dual replicon system alterations for regulation.** **A)** The plasmid optimised for *Bacillus subtilis* (pAG-P08) is shown diagrammatically compared to the 2 new plasmids used for *Escherichia coli* chassis. The plasmids were altered to replace this replicon for lower copy number alternatives SC101 and RSF1010. **B-E)** After alteration the plasmid with SC101 replicon was transformed into two different chemically competent strains of *E. coli*, one with *lacI<sup>q</sup>* and one with native LacI only, to see the effects of the GFP expression. Strains were grown on ampicillin selective nutrient agar with different induction conditions (+/- IPTG). The same single colony was used to inoculate each plate with different induction conditions, plates were incubated overnight at 37°C and GFP fluorescence excited by blue light with 500 ms exposure in a Biorad Gel Doc. Unaltered pAG-P08 serves as a control for phenotype before replicon change.

As it was seen in the DH5 $\alpha$  strain that the pGGS01 plasmid had dramatically lower levels of fluorescence (fig.5.1C & E), the BW25113  $\Delta minC$  strain was tested to see if the native LacI could repress the low copy plasmid. Unfortunately, at the single cell level it was obvious that there was expression from the rod cells (fig.5.2A). Although some minicells appeared to be significantly brighter than rod cells (yellow arrows). This was a positive indication that minicells could express GFP after formation. It was also evident that GFP expression from rod cells was heterogeneous, this was interesting as it suggested expression levels from the plasmid varied. Overall, the BW25113 background suggested that there was some compartmentalised expression, however, levels of repression in rods cells from native LacI were not adequate.

To start the construction of the Portabolomics chassis, a consistent background strain was required for testing throughout. As MG1655 had been used in other strands of the Portabolomics project, the same background was adopted. The rationale for this was that *E. coli* MG1655 is a well characterised laboratory strain which had been developed for other Portabolomics strands such as stress load detection (section 1.6.1). To remain consistent, and to enable integration of multiple work strands, a minicell producing strain was constructed introducing the *minC* deletion from BW25113 into the MG1655 background. Microscopy analyses revealed that the wild type (MG1655) consisted of rod shaped cells only, whereas the  $\Delta minC$  mutant presented two cell types as expected (fig.5.2B). Minicells were small and rounded, outline by red arrows, some were free floating whereas others remained attached to rod cells. Overall, a defined *E. coli* background was established to test the plasmids constructed.

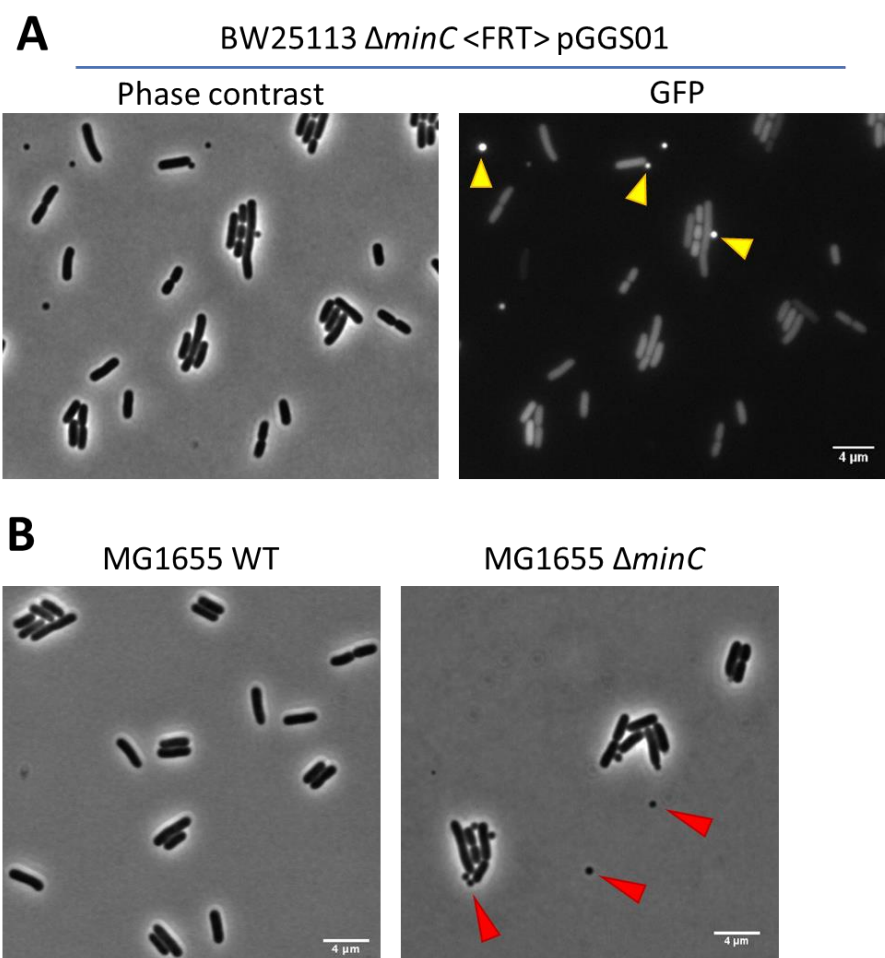


**Figure 5.2. Native LacI repression.** Mutant strains were transformed with the GFP reporter plasmid with low copy number SC101 driven replicon. The cultures were grown in LB and imaged at mid-exponential phase when they were mounted on a 1.2% agarose pad. **A)** BW25113  $\Delta minC$  pGGS01, yellow arrows indicate minicells with high fluorescence intensity comparable to rods and other minicells. Images were taken with 100 ms exposure using a Nikon Ti eclipse. **B)** MG1655 WT and  $\Delta minC$ , red arrows indicate minicells. Scale bars represent 4  $\mu$ m.

### 5.2.1 Construction of a defined *E. coli* chassis

As it was demonstrated in figure 5.2A, *E. coli*'s native LacI was not effective for repression of the low copy plasmid. However, it was suggested in figure 5.1E that *lacI<sup>q</sup>* may have had a positive effect. The *lacI<sup>q</sup>* promoter is stronger than the native gene to allow for greater expression of the transcriptional repressor. Therefore, *lacI<sup>q</sup>* was integrated into the *intS* locus. The fragment integrated consisted of a kanamycin resistance cassette (Neo<sup>R</sup>) which was surrounded by FRT sites which provided the opportunity to remove resistance (fig.5.3A). This fragment was PCR amplified by an addgene strain, HL713, gifted by Han Lim (strain #52931).

Once the strain was constructed (sGG206), both pGGS01 and pGGR01 plasmids were transformed into this background to analyse GFP expression (fig.5.3B). For microscopy analyses, and the overall Portabolomics project, growth temperatures remained consistent to allow for comparison between constructed strains. Therefore, *E. coli* strains were also grown at 30°C for all analyses hereafter. This revealed that *lacI<sup>q</sup>* did greatly affect repression in rod cells, and there was evidence of bright fluorescence from minicells. Nevertheless, it was also noticeable that there was still some background fluorescence in rod cells which suggested the repression system did not work as well as examples in *B. subtilis*.

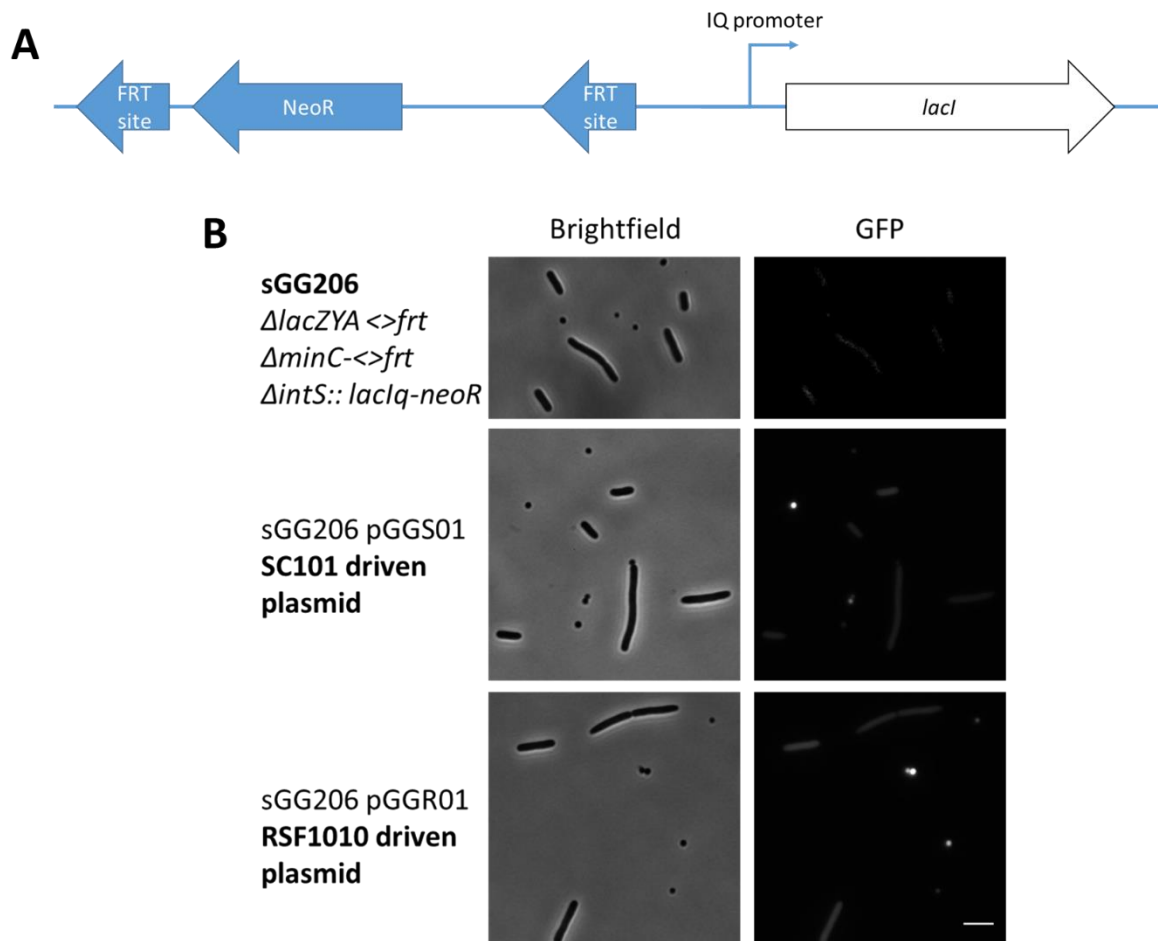


**Figure 5.3. Construction and analysis of the *E. coli* minicell chassis.** **A)** A map of the sequenced site integrated into the *intS* locus of the minicell-producing *E. coli* strain using  $\lambda$ -red recombineering (Datsenko & Wanner, 2000). Addgene strain HL713 (#52931) genomic DNA was used as a template for the fragment to be inserted. **B)** Microscopy of cultures grown in M9 media at 30°C. Cultures were imaged at mid-exponential phase mounted on a 1.2% agarose pad (agarose and water). Scale bar represents 4  $\mu$ m and is applicable to all images. >3 independent repeats carried out for microscopy.

Microscopy images were used to measure the fluorescence of single rod cells and minicell populations for strains carrying different plasmid replicons to determine which repressed better while providing greater minicell expression. Violin plots were used to demonstrate the fluorescence intensity for each population of rods and minicells with each plasmid replicon (fig.5.4A). Cell populations with the SC101 based plasmid pGGS01 were comparable as the fluorescence intensity were similar, though minicells did present more outliers (fig.5.4A - purple plots). To quantify the amount of minicells expressing GFP, a threshold was set at the end of the normal fluorescence distribution of the nucleated cells (fig.5.4A red dotted line). Any minicells over this threshold, were counted as GFP positive. These analyses showed that, with pGGS01, there was only 17% of minicells counted as GFP positive. In contrast, the minicell population with pGGR01 exhibited higher GFP expression in comparison to the rod cells and appeared to have two significant populations of intermediately fluorescent minicells (fig.5.4A – red plots). The secondary population of minicells that was over the threshold and accounted for 34% of GFP positive minicells (fig.5.4B), indicating that the higher copy number vector was better for minicells gaining a plasmid. Repeats (n=5) have reproducibly shown that ~30% of minicells were GFP positive during mid-exponential phase with pGGR01. However, the rod cells with pGGR01 were brighter than those with the pGGS01. Statistical tests were carried out and a 2-sample T-test (with Welch's correction) showed the difference was significant between the populations of rods and minicells with pGGR01. There was no statistical significance between the two populations harbouring pGGS01. The replicons, differing in copy number, demonstrated which was best for minicells to efficiently gain a plasmid while repressing rod cells expression. Overall, it was found that pGGR01 was most efficient at segregating into minicells for independent expression.

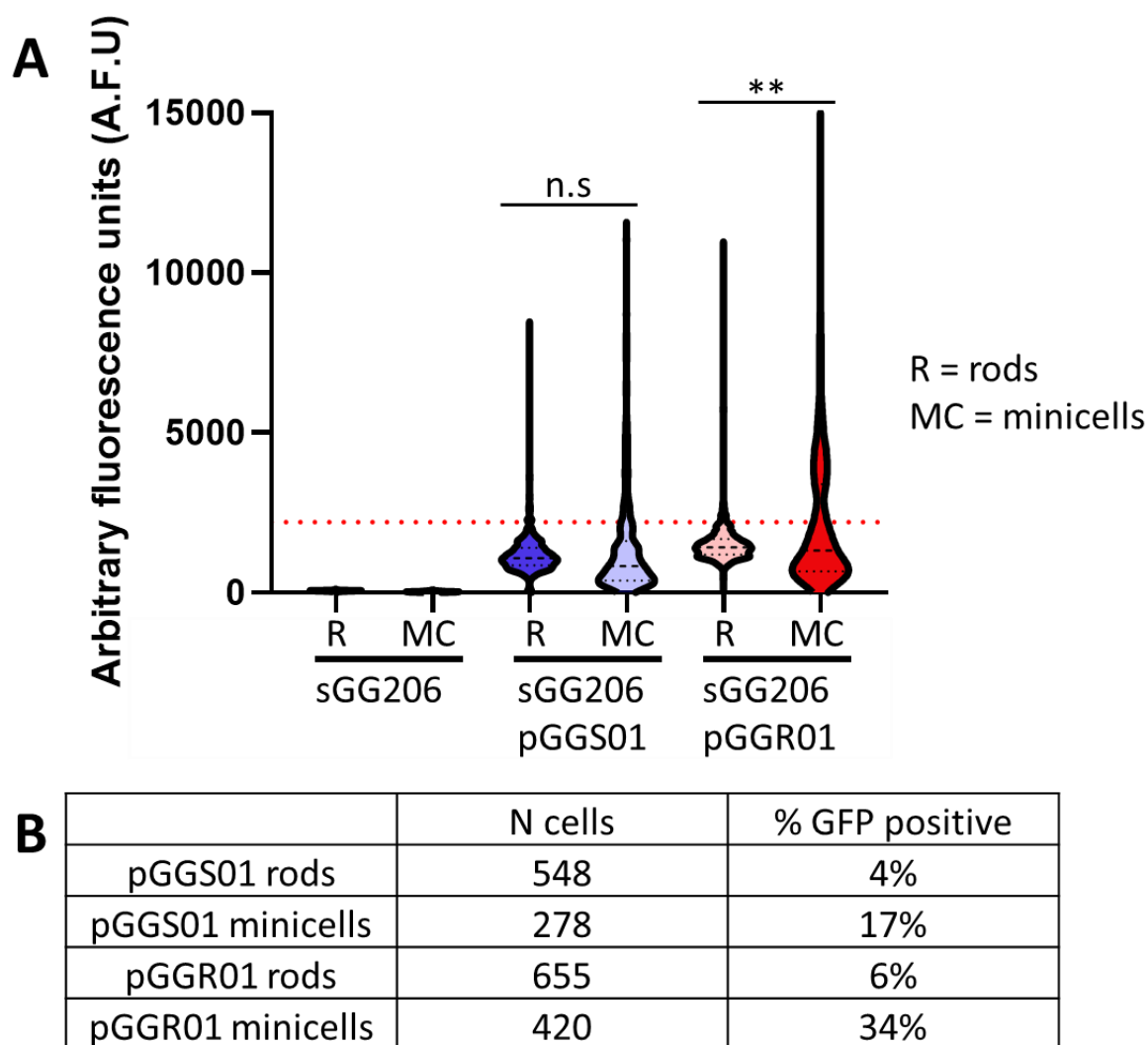


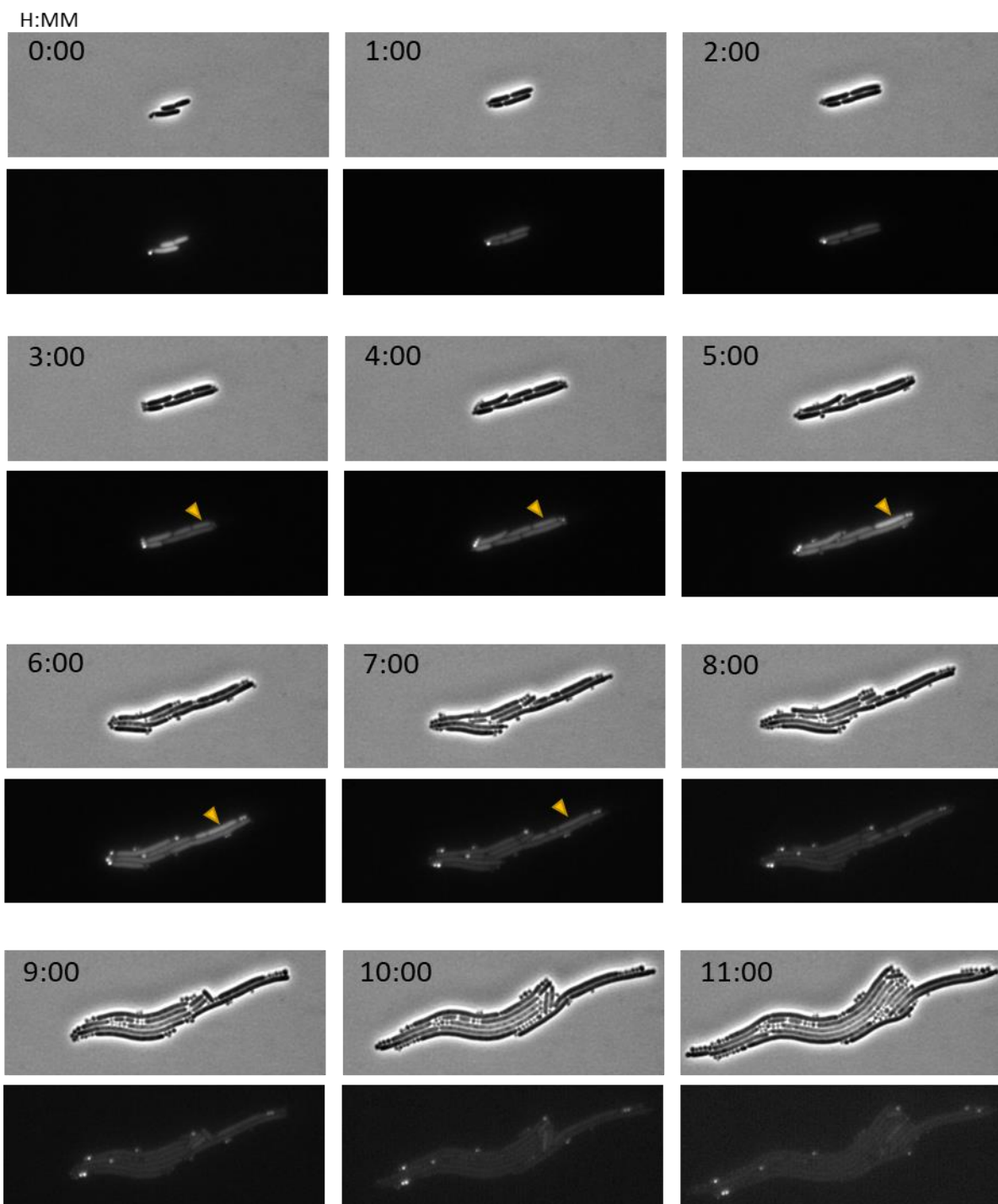
**Figure 5.4. Populations of GFP expression.** **A)** sGG206 cultures were grown in M9 media before imaging at mid-exponential phase. Counting was done using Fiji software and fluorescence means plotted into a violin plot using GraphPad Prism 8. Fluorescence values were plotted as arbitrary units (a.u.). pGGS01 no significant difference ( $p > 0.05$ ) pGGR01 was significant  $**p < 0.0001$  both using T-Test accounting for Welch's corrections. Fluorescence intensity values were analysed using Fiji software. Cell populations for each were ~500 cells counted  $\pm 150$ . Reference line represents 2200 fluorescence (a.u) on the Y axis. Graphs were made using GraphPad prism 8 **B)** Cell populations were counted using Microsoft excel, determining a reference line to distinguish the cut off for GFP positive at 2200 fluorescence (a.u). >3 biological repeats have been performed.



### 5.2.2 Time lapse microscopy of strains with different plasmid replicons

As single time points had been analysed, it was then questioned how the minicell populations performed over time. Therefore, time-lapse microscopy was used to observe the time taken for GFP fluorescence to occur after minicell formation. When sGG206 with pGGS01 was analysed, rod cells showed changeable fluorescence intensities over time (indicated by the yellow arrow – fig.5.5A). There were minicells obviously expressing GFP brighter than rod cells but not found frequently in these cells (fig.5.5A). Previous analyses (fig.5.4) and cell counting during mid-exponential phase showed there was only ~17% of minicells with pGGS01, this was consistent with the time lapse where there was a small number of minicells which were GFP positive (fig.5.4). Three cells from each rod and minicell populations were tracked over time, where the fluorescence was measured at 6-minute intervals and plotted. Over time, the rod cells fluorescence fluctuated (fig.5.5B). At the beginning of imaging, it was found that rod cells showed an initial dip in fluorescence within the first 100 minutes which gradually peaked after a further 100 minutes. Rod 3 showed a greater fluctuation later in the time course after ~300 minutes, as a sharp peak was observed (fig.5.5B transparent red triangles). Minicell fluorescence was not too dissimilar to the rod cells, with exception to minicell one (fig.5.5B - grey squares). When compared, there was a clear symmetry with minicells 1 and 3 with the rod cells. Whereas minicell 2 had significantly greater expression than rod cells (fig.5.5B open squares). This showed that the minicells fluorescence remained similar to that of rod cells and only increased when the plasmid bound GFP reporter was compartmentalised.

## A – pGGS01



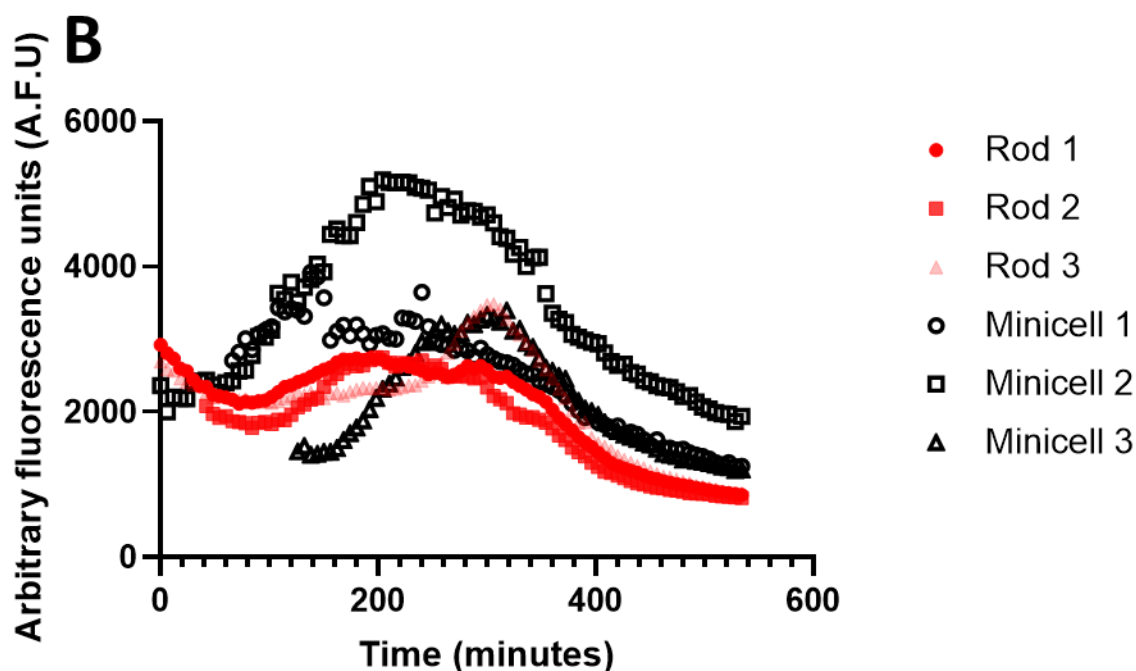


**Figure 5.5. Independent minicell expression of GFP reporter.** After overnight back dilution, sGG206 pGGS01 cultures were grown at 30°C for 2 hours until and OD600 of 0.1. 1  $\mu$ l of the cell suspension was transferred onto a 1.2% M9 media agarose pad before imaging using Nikon Ti with LED excitation lamps. The time-lapse was captured every 6 minutes for 10 hours (until focus was lost). Scale bar is representative of 1  $\mu$ m and is applicable to all images. Graphs were produced using GraphPad prism 9 software. **A)** Hourly images of the growing culture with phase contrast and GFP excitation (*E. coli* cells with SC101 driven plasmid) **B)** Fluorescence of single cells over the time course with each measurement captured after every 6-minute image. N=2.

Replicating the same analytical method used for the pGGS01 derivative, a clear parallel was seen for the rod cells in a strain carrying pGGR01 as there was an initial decrease in fluorescence which then increased greater than the starting fluorescence. However, the increase in fluorescence with pGGR01 occurred later than that of pGGS01 as fluorescence peaked between 300-400 minutes after imaging commenced. When the fluorescence of 3 rod cells was measured throughout the time lapse, it was revealed that the fluorescence intensity was homogenous over time (fig.5.6B red markers). The fluorescence of minicell 1 and 3 peaked after ~200 minutes from the first recording of each cell, although the minicells were formed at different times it showed that there seemed to be homogeneity in expression (fig.5.6B black markers). When fluorescence intensity of rod cells and minicells were compared over time, it was clear that minicells 1 and 3 were significantly brighter. However, it was demonstrated in the time lapse that minicells gradually increased fluorescence after an ~80-minute lag. Comparatively, minicell 2 did not increase fluorescence, indicating that the cell probably did not gain a plasmid (fig.5.6B; grey/open squares). Minicell 2's fluorescence remained the same as the rod cells through the rest of the growth phase, this was encouraging as it showed that those with increased fluorescence were likely to have gained a plasmid and expressed the reporter independently (fig.5.6B). Overall, the data suggested that minicells had a lag before expressing independently from the plasmid, which may have been a result of LacI degradation or plasmid replication occurred resulting in escape from repression.

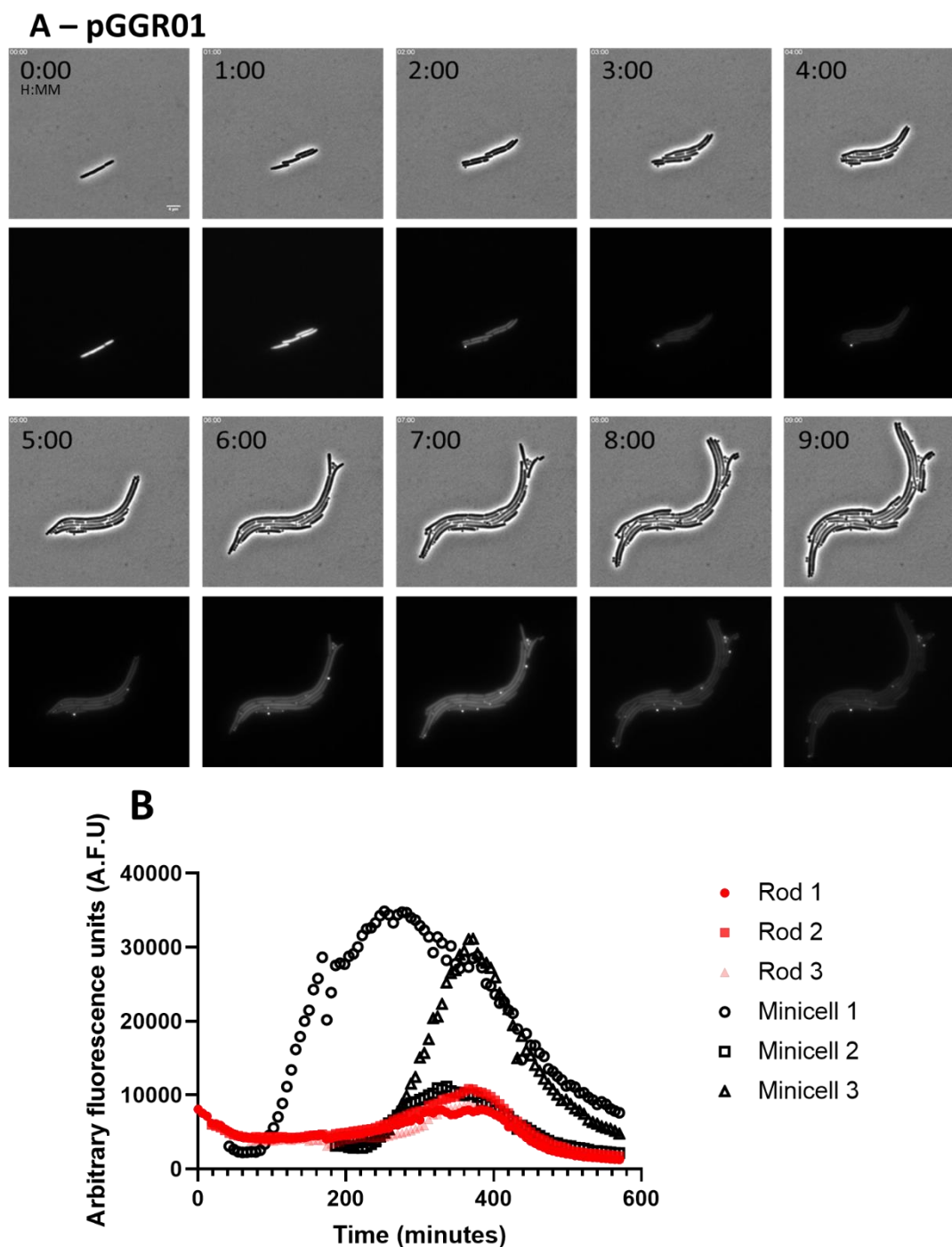


**Figure 5.6. RSF1010 driven plasmid GFP expression time lapse. A)** Hourly images of sGG206 with RSF1010 reporter plasmid (pGGR01) **B)** fluorescence intensity plotted of rod or minicells over time. fluorescence was measured for each cell at every 6-minute image. For time lapse microscopy, overnight cultures were back diluted in fresh M9 media and grown for 2 hours at 30°C. At an OD600 of 0.1, 1  $\mu$ l of culture was applied to a 1.2% M9 media agarose pad before imaging with Nikon Ti with LED excitation lamps. Time lapses were captured every 6 minutes for 10 hours. Scale bar is representative of 4  $\mu$ m and is applicable to all images. Plots were produced with GraphPad Prism 9. H:MM describes hours:minutes. n=2

### 5.2.3 Analyses of the *E. coli* $\Delta$ minC ultrastructure

From initial microscopy experiments and publications, it was clear that *E. coli* minicells were significantly different to those of *B. subtilis*. To confirm this, transmission electron microscopy (TEM) was used. Thin sections were imaged to show finer details and ultrastructures within cells. Imaging showed that the *E. coli* rod cells looked short, which may have been a result of cultures being close to stationary phase where cells stop growing (fig.5.7A). However, the culture was still actively dividing as stages of division were presented clearly as invaginations formed at the mid-cell position (fig.5.7B & C). The constriction generated by invaginations was distinct where 2 new daughter cells were being formed and the new poles of the cells were becoming prominent. The TEM data also presented an image of *E. coli* cells which had recently finished dividing and was clear that two daughter cells of equal size had been produced (fig.5.7D).

In contrast to the wild type strain, sGG206 showed invaginations at the cell poles, which was expected. The invaginations at the pole of the cell seemed to form minicells smaller than the diameter of the cell. Small minicells were perhaps expected because the mechanisms of division, where constriction might reduce cell volume in contrast to *B. subtilis* septum formation. After identifying that minicells were smaller in size, it was hypothesised that minicells of *E. coli* would be easier to purify by filtration. The first stages of minicell division were evident when the invagination first formed at the cell pole (fig.5.7E) this invagination continuously constricted as the divisome worked to close the Z-ring (fig.5.7F). Once this event had finished, a whole rounded minicell was seen attached to the rod cell which would detach later in growth (fig.5.7G & H). Individual minicells could not be accurately measured as the presence of rods turned upright caused confusion, thus minicells remained attached to rods for reliable imaging.

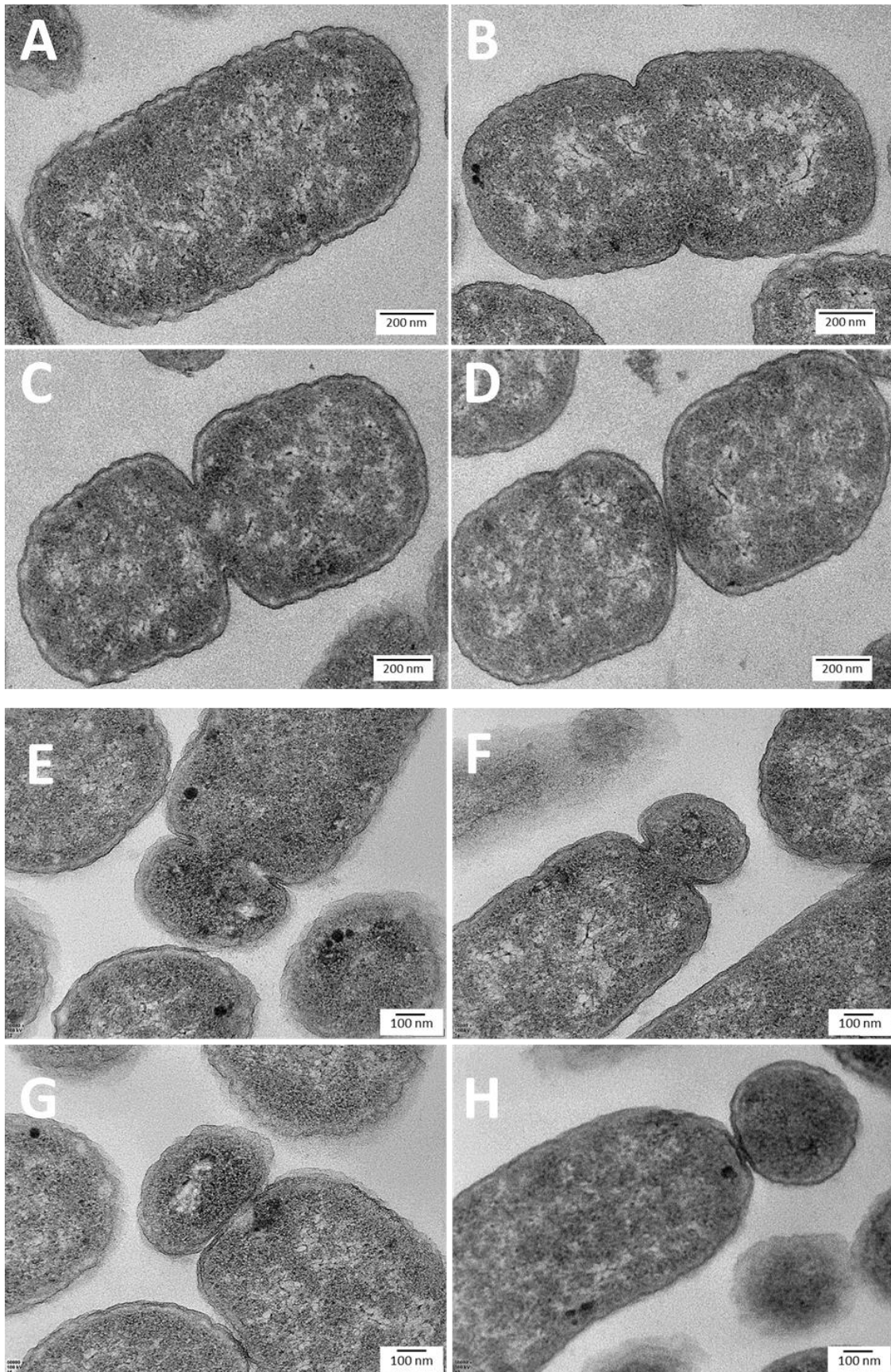


**Figure 5.7. Electron microscopy imaging of *E. coli* minicells to investigate ultrastructures.** Initial overnight growth in LB was incubated at 30°C, next day cultures were diluted back to the same OD600 in fresh LB media

and grown with shaking at 37°C. Cells were harvested during late-exponential phase after 4 hours of growth and suspended in glutaraldehyde for fixation. Sections of resin containing cells were stained using heavy metal salts. Electron microscopy images were acquired using Hitachi HT7800 120kV transmission electron microscope (TEM). **A-D)** Wild-type *E. coli* TB28 parental strain. **E-H)**  $\Delta minC$  knockout strain in a TB28 background.

## 5.3 Population analyses of *E. coli* Portabolomics chassis

### 5.3.1 Whole culture population analyses using flow cytometry

Following from single cell analysis, to get a more complete idea of the population and diversity flow cytometry was used. Here cell populations were determined using forward and side scatter as a way to obtain cell size and granularity in combination with fluorescence analysis to indicate the level of GFP expressed in individual cells. To determine how to calibrate the system, a wild type control was first characterised to understand how culture populations changed. From a sample of exponentially growing cells, 30,000 events were obtained and graphically plotted by side vs forward scatter (fig.5.8). In HL713, population 1 (P1) showed a dense grouping of cells, accounting for 93.5% of the whole population (fig.5.8A). A second population (P2) was gated where events with lower granularity and size were detected, in the wild-type strain this accounted for 2.74% of the population. Plots were shown as heat maps with the greatest density of events being in the red zone, gradually fading out to yellow, green and blue to indicate less events recorded in the area respectively. sGG206 displayed a dramatic shift in both P1 and P2 as 27% of the population had shifted into P2 where cells were smaller and less granular, assumed to be minicells (fig.5.8B). The rod cell population (P1) showed that size and granularity were not as tightly grouped which indicated heterogeneity. This was seen with microscopy analyses as heterogeneous populations were observed as both filaments and short rods.

The introduction of plasmids into sGG206 did not significantly change the population detected by scatter as they remained at ~70% rod cells to ~25% small particles (fig.5.8C & D). This



showed that addition of plasmids did not change the phenotype of the rod or minicell populations. Interestingly, approximately a quarter of the sGG206 population consisted of small, less granular cells indicating the accumulation of free minicells was significant.

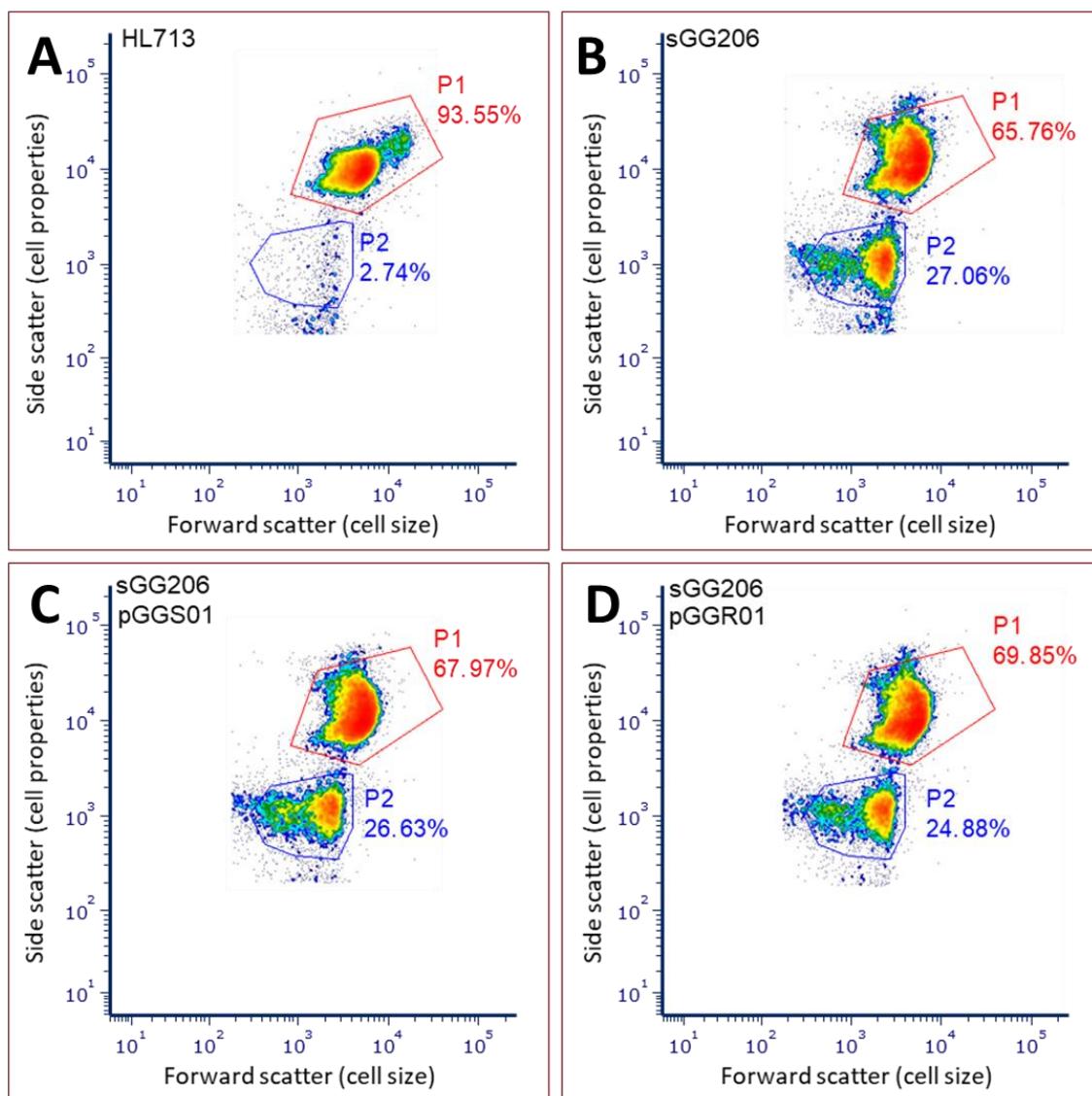


**Figure 5.8. Analysing individual populations of wild type and minicell producing strains.** *E. coli* cultures were grown in M9 media at 30°C until mid-exponential phase, 25  $\mu$ l of cells were mixed with 500  $\mu$ l filter sterile phosphate buffered saline (PBS) sheath fluid and 30,000 events analysed by BD Symphony. Data was analysed using FCS express version 6. **A-B)** top panels do not have a plasmid and indicate WT and minicell producing strain (sGG206). **C-D)** bottom panels represent populations of minicell producing strains with 2 different plasmids for analyses, **C)** those with SC101 driven plasmid (pGGS01) and **D)** those with RSF1010 driven plasmid (pGGR01).

Fluorescence excitation filters of the flow cytometer were used to measure populations of cells and plot in accordance with fluorescence intensity. The sGG206 negative control was used to define non-fluorescent populations, therefore, counts outside these zones would represent fluorescently positive cells (fig.5.9A). As a result of this, 2 gates were produced: the first (Gate 1) was to define negative fluorescence and the second gated region was for cells with GFP fluorescence (Gate 2). Measurement of cultures with pGGS01 or pGGR01 both showed a shift in intensity within Gate 2 (fig.5.9B & C, respectively). Presence of pGGS01 was responsible for 73% of the population's green fluorescence, whereas, pGGR01 increased populations with GFP fluorescence to 78% of the whole population (fig.5.9B & C). The majority of both populations showed GFP fluorescence that was similar to what was observed in microscopy data, outlined in figure 5.4B. Heterogeneity of fluorescence expression was clear with the presence of both plasmids as GFP negative cells were clearly detected which either represented cells repressing plasmid expression or the absence of the plasmid. Although not informative about minicells, this data did provide an insight into comparing pGGS01 and pGGR01 (SC101 and RSF1010 driven replicons respectively). In summary, the cells with pGGR01 were marginally brighter than those with pGGS01 indicating that the rods contained slightly more GFP through "leaky" regulation, presumably a reflection of increased copy number.

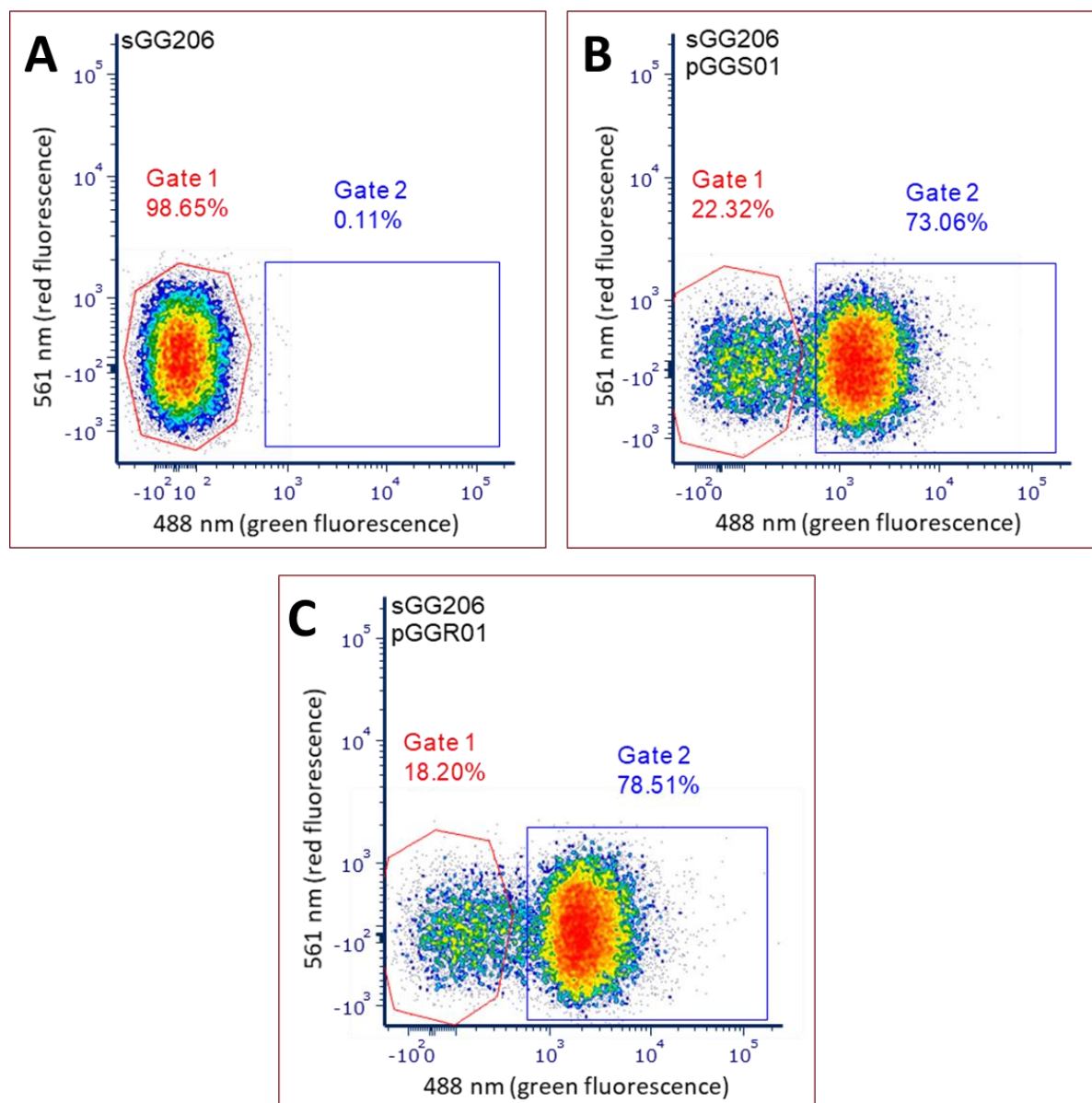


**Figure 5.9. Fluorescence analyses of minicell producing strains.** *E. coli* cultures grown in M9 media at 30°C until mid-exponential phase, 25  $\mu$ l of cells were mixed with 500  $\mu$ l filter sterile phosphate buffered saline (PBS) sheath fluid and 30,000 events analysed by BD Symphony. Lasers were applied to excite red or green fluorescence at 561 and 488 nm respectively. Data was analysed using FCS express version 6. **A)** minicell producing strain with no plasmid **B)** minicell producing strain with SC101 driven pGGS01 **C)** minicell producing strain with RSF1010 driven pGGR01.

### 5.3.2 Minicell purification and analyses using flow cytometry

Enrichment of minicells was desirable for the ability to analyse larger populations as well as for potential industrial applications, examples had been developed in *B. subtilis* by A. Guyet (unpublished). After performing filtration, microscopy and flow cytometry were used to view and quantify concentrated minicells. Using the same enrichment methods developed for *B. subtilis*, minicells of *E. coli* were found to be more concentrated in microscopy analyses which demonstrated that minicells passed through the filter efficiently and potentially showed a higher number of free minicells (not attached to parent cells). However, it was evident that small rod cells had also been able to pass through the filter as confirmed by figure 5.10A & B. Flow cytometry with the same gating as figure 5.8 of populations 1 and 2 (P1 and P2) were used to identify and count the same sized populations shown prior. Before enrichment, sGG206 displayed ~70% rod cells and at least 25% minicells (fig. 5.8B). Comparatively, after filtration there was a large increase in the small cell population (increasing from ~25% to 71%). The small cell population was not as tightly grouped within the forward scatter scale which indicated that there was a range of sizes present within this population (fig. 5.10C). Within P1, gating large more granular rod cells, the population had reduced dramatically but there was still 20% of the counts represented. The remaining rod cells were grouped close together, which suggested homogeneity in size, therefore it was clear that small rod cells probably passed through the filter. Overall, this demonstrated that although enrichment was successful, there was a scope for improvement.

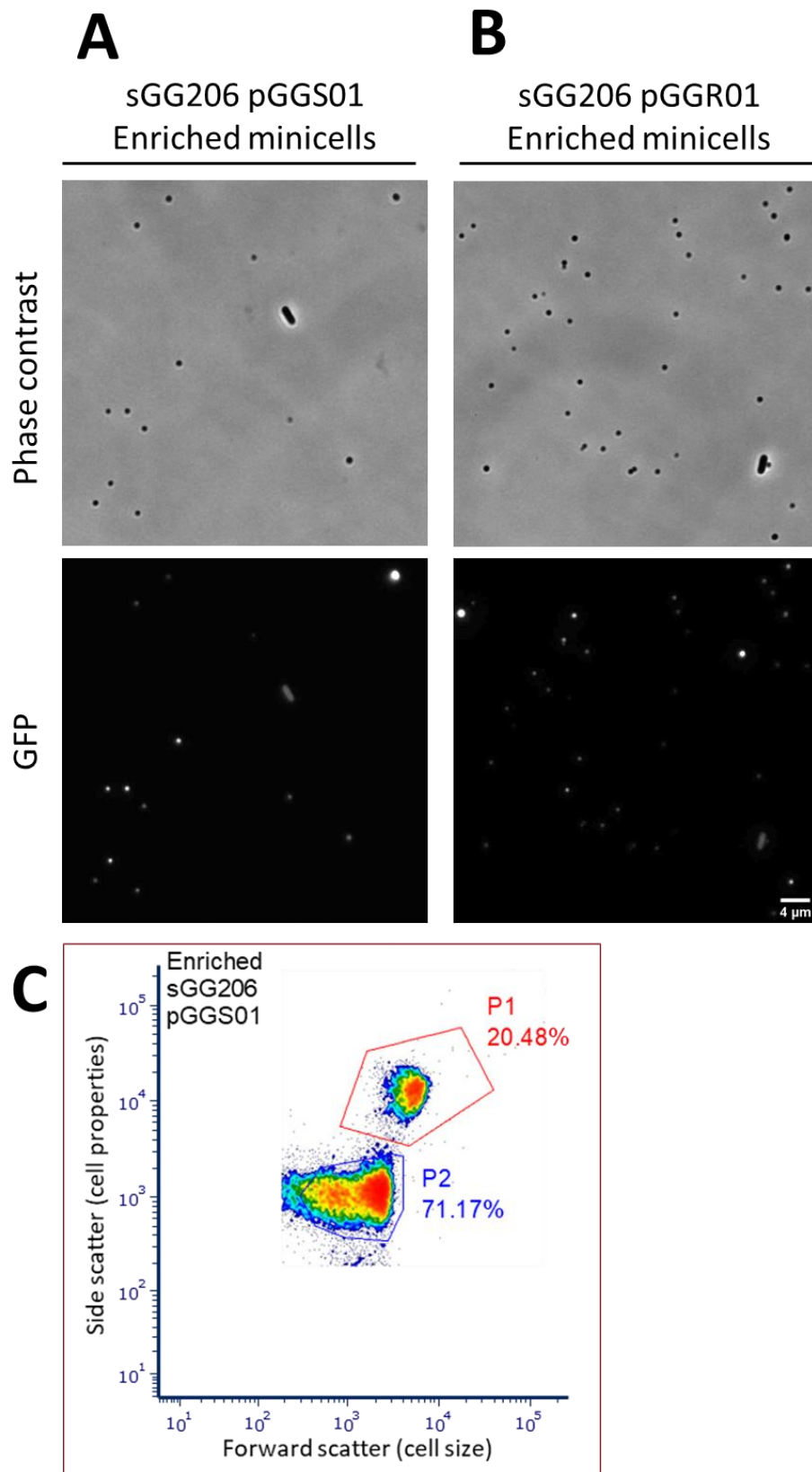


**Figure 5.10. *Escherichia coli* enrichment microscopy.** *E. coli* cultures were grown in M9 media at 30°C until mid-exponential phase, enriched cells were gained by filtering the whole culture through a 1.2  $\mu\text{m}$  filter, the flow through was then centrifuged at 3,750 RPM followed by supernatant discarding. The pellet was resuspended and

centrifuged again before removing supernatant and resuspending in fresh media. **A + B**) microscopy analyses after mounting 1 µl of enriched cells onto a 1.2% agarose pad. Data was analysed using Fiji software. **A**) minicells from the population harbouring SC101 driven plasmid and **B**) minicells with RSF1010 driven plasmid. Scalebar is representative of 4 µm and applies to all images. **C**) 25 µl of enriched minicells were mixed with 500 µl filter sterile phosphate buffered saline (PBS) sheath fluid and 30,000 events analysed by BD Symphony.

As the flow cytometry software (FCS Express 7) was able to analyse gated populations abstracted from the entire sample, fluorescence from enriched small cell populations was performed to analyse how many were expressing from the plasmid. The P2 gate of enriched minicells from fig.5.10C was analysed for both strains carrying pGGS01 and pGGR01. Once again, the negative control was used for gating which did not have any fluorescent markers and therefore helped visualise what populations were GFP positive. The flow cytometry data revealed that 35% of minicells from the population with pGGS01 had shifted to GFP fluorescent (fig.5.11B). Whereas those with pGGR01 showed 45% of minicells were GFP positive (fig.5.11C). This result deviated from microscopy data, but both methods suggested that pGGR01 was more frequently located in minicells (fig.5.4). Suggesting more minicells were gaining pGGR01, assumed to be because of a greater copy number. Microscopy analyses were not in agreement about the number of minicells expressing GFP, however, this could be because of the time passing between the enrichment process and flow cytometry allowing more minicells to overcome the repressor and independently express. By comparison, microscopy analyses were more rapid as cells were imaged immediately after mounting onto agarose pads, rather than being processed for flow cytometry.

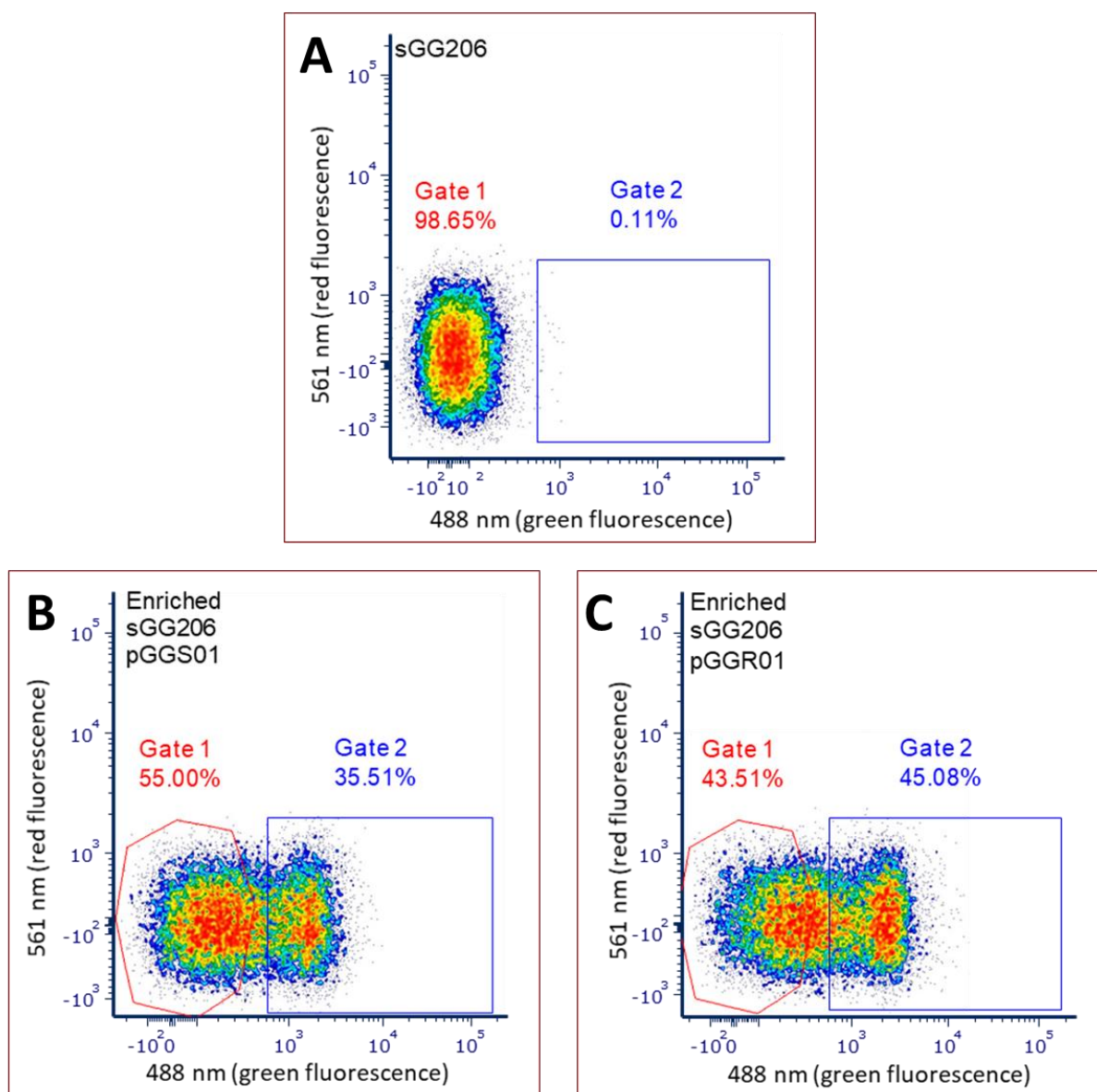


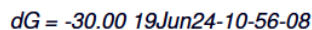
**Figure 5.11. Flow cytometry analysis of *E. coli* host populations fluorescence.** *E. coli* cultures were grown in M9 media at 30°C until mid-exponential phase, 25  $\mu$ l of cells were mixed with 500  $\mu$ l filter sterile phosphate buffered saline (PBS) sheath fluid and 30,000 events analysed by BD Symphony. Enriched cells were gained by filtration methods described previously. Data was analysed using FCS express version 6. **A)** negative control of cells with no fluorescence reporter **B)** enriched minicells fluorescence analyses from a population with pGGS01 and **C)** pGGR01.

## 5.4 Increasing rod cell repression of the reporter

### 5.4.1 antisense RNA construction to improve rod repression

From the initial analysis, it was clear that regulation of GFP was a common issue with various constructs in *E. coli* and less so in *B. subtilis*. To try and resolve this issue, attempts to increase LacI in *E. coli* had limited success so an alternate approach was investigated. Antisense RNA's (asRNA) was a possible solution. These were designed to hybridise to *gfp* mRNA. Subsequently, double stranded RNA was susceptible to degradation. From the literature, a basic format was determined incorporating the segment of sequence complementary to the mRNA sequence of *gfp* and hairpin loops to permit Hfq association. The aim being to optimise hybridisation with the target. The first 20 bp from the 5' end carried the *gfp* mRNA target homology. This structure was then modelled using mFold to predict the folding of the asRNA *in vivo* at 37°C (fig. 5.12) (Zuker, 2003). The asRNA was placed under a  $P_{tet}$  promoter with no repressor expressed to allow constitutive expression in *E. coli* from a high copy number plasmid or chromosome (pUC19 – NEB with Spec<sup>R</sup>). Plasmids were co-transformed with either pGGS01 or pGGR01 to see the effect on fluorescence intensity.





The antisense RNA expression cassette was then integrated into the chromosome of sGG206 to aid in repression and reduce fluorescence in nucleated rod cells. After integration into the *arsB* locus, the subsequent strain with asRNA (sGG223) was transformed with GFP reporter plasmids (pGGS01 or pGGR01). Growth curves showed there was no significant difference with the addition of asRNA expression compared to strains without (sGG206, sGG209, sGG210; fig.5.13A). Fluorescence analyses was used to determine if the asRNA had the desired repressive effect on GFP expression. However, this indicated that there was no significant difference between rod cells mean fluorescence intensities of strains with and without asRNA integration (fig.5.13B). Minicell fluorescence between both populations harbouring pGGS01 or pGGR01 looked very similar with the presence of asRNA to those without. This potentially indicated that the asRNA was either not abundant enough or did not bind efficiently to the mRNA as expected.

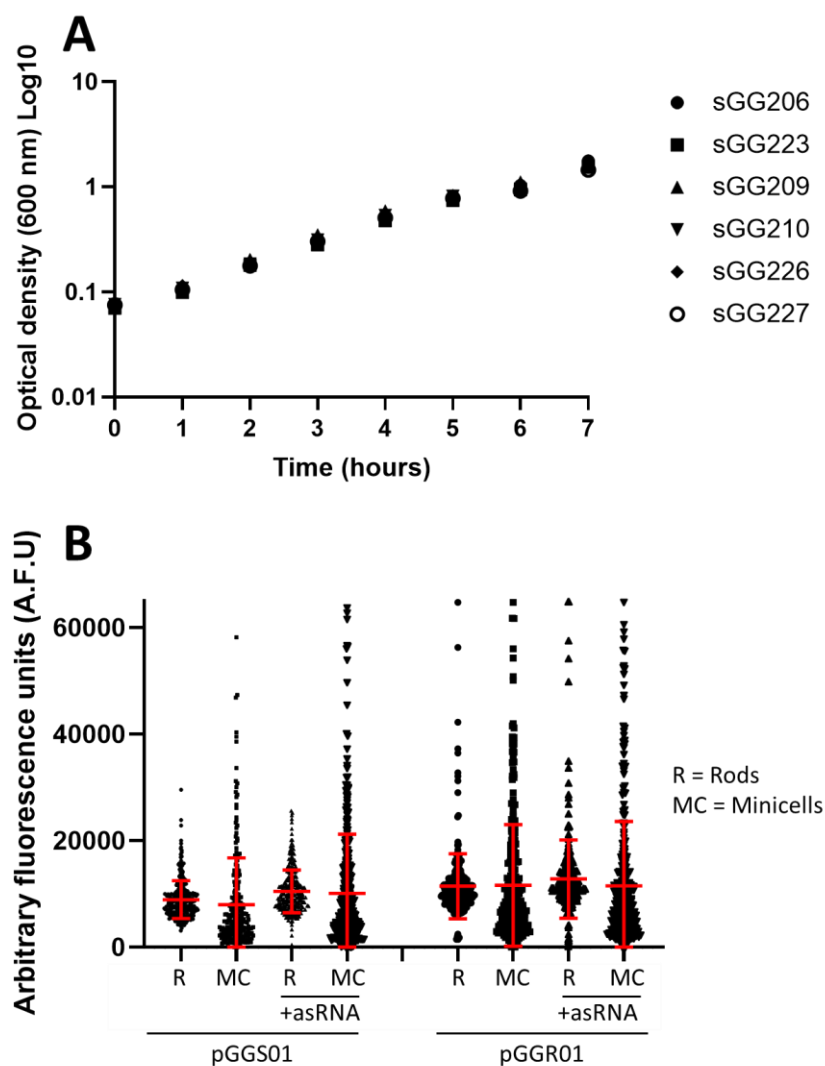


**Figure 5.13. Chromosomal expressed asRNA for reduction of GFP background.** Strains were grown overnight in M9 media before back dilution the following day in fresh M9 media with 30°C incubation and shaking. Cells were mounted onto a 1.2% agarose pad for imaging. **A)** hourly optical density read at 600 nm **B)** dot plots of arbitrary fluorescence values after fluorescence intensity were calculated using Fiji software. Means and standard deviations are shown in red for each population. N= 100 cells per population.

To resolve the question of asRNA expression level, the asRNA cassette was cloned into a high copy number plasmid which was co-transformed into sGG206 with pGGS01 or pGGR01. As these plasmids were high copy number, there was no regulation and these plasmids would be present in minicells via diffusion from the cytoplasm. There were no obvious phenotypic changes from the microscopy images and no clear change in the fluorescence by eye (fig.5.14A & B). However, single cell mean fluorescence showed that pGGS01 co-transformed with the asRNA plasmid produced a secondary population of dimmer rod cells (fig.5.14C – red arrow). When comparing means between rod cells with pGGS01 only and with the addition of the asRNA construct, there was a significant difference ( $P=0.0176$ ) reflected in figure 5.14C. There was a more profound difference in the minicell population which reduced fluorescence significantly ( $P=0.002$ ), this was expected as the high copy number plasmids with asRNA expression were also likely to be present in minicells. The control with pGGS01 and the pUC19 empty vector seemed to increase the fluorescence intensity, when comparing averages there was a significant difference ( $P=0.004$ ). The effect from the addition of the pUC19 empty vector was striking and indicated that there could be stress or other transcriptional changes in the cell. Since the asRNA construct had the opposite effect, it indicated that the asRNA was potentially repressing expression.

Similar results were obtained with pGGR01, except in this case there was a high range of fluorescence values. When the asRNA expression vector was co-transformed with pGGR01, there was no significant change in rod fluorescence compared to rod cells with pGGR01 only ( $P=0.172$ ) (fig.5.14D). There was evidence of repression with asRNA constructs as a significant decrease in minicell fluorescence was observed ( $P=0.044$ ). However, the pUC19 empty vector had an opposite effect with pGGR01 as fluorescence increased significantly ( $P<0.0001$ ). Therefore, it seemed logical that high expression of asRNA was functional, but

the pUC19 vector it was carried on was inducing high expression of GFP. Subsequently, a poor level of repression was detected which may in fact be significant.

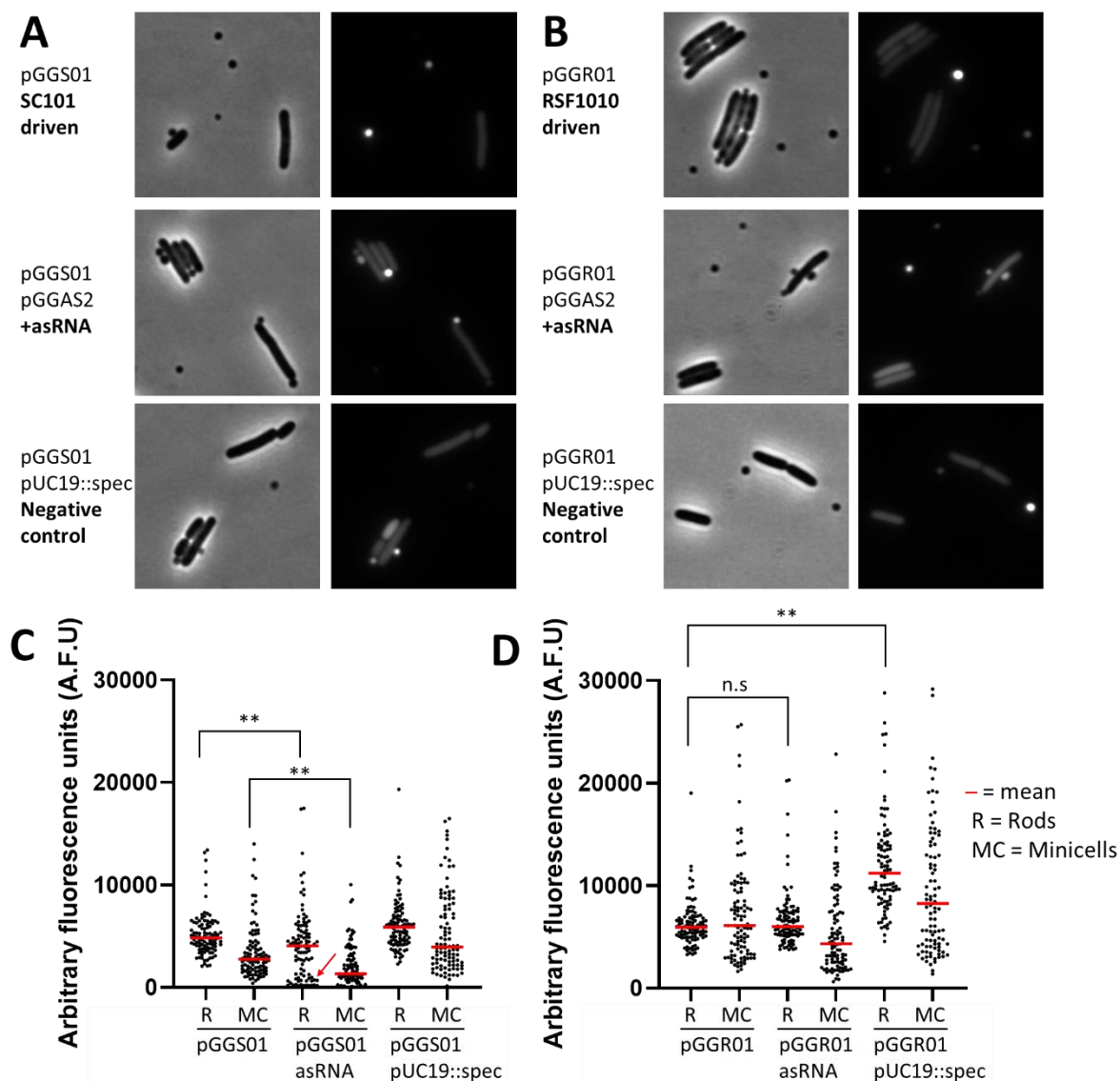


**Figure 5.14. Quantification of fluorescence and asRNA efficiency.** sGG206 strains were grown overnight in M9 media before back dilution the following day in fresh M9 media with 30°C incubation and shaking. Selective antibiotics for the plasmid were present by adding 2 antibiotics for selection of the 2 plasmids (Ampicillin 100 µg/ml, Spectinomycin 50 µg/ml). Cells were mounted onto a 1.2% agarose pad for imaging. After imaging fluorescence intensity was calculated using Fiji software. **A)** microscopy images from strains with pGGS01 +/- asRNA **B)** microscopy images from strains with pGGR01 +/- asRNA **C)** pGGS01 was present in all strains where asRNA and pUC19::spec shows additional plasmids co-transformed. **D)** pGGR01 denotes the plasmid carrying

the GFP reporter, where asRNA and pUC19::spec shows additional plasmids co-transformed. N=100 cells per population counted. N=3.

## 5.5 Expression of an industrially relevant product

To see if minicells of *E. coli* could be used for expression of heterologous proteins, secretion or decoration with antigens, a plasmid was made which replaced the GFP reporter for protein A. The RSF1010 driven vector was chosen as it had shown better minicell independent expression, although there was leaky expression in rod cells. This would serve as a proof of principle for industrial applications of minicells from this chassis.

### 5.5.1 Expression of protein A construct in *Escherichia coli*

Strains sGG206 and HL713 were used for comparison of culture expression with and without minicell production, respectively. As there was leaky expression in rod cells, it was important to compare both strains to see how the level of rod cell expression potentially decorated cells. Western blot analyses revealed that protein A was detected (fig.5.15 black arrow), although it appeared there was some cross reactivity with *E. coli* as small non-specific bands were present. Lower bands at around ~30 kDa were consistent across all samples irregardless of IPTG induction or the strain. This is likely a result of antibodies reacting to proteins from *E. coli* which contaminated the protein A purification used to yield antibodies from a rabbit host. Without IPTG induction there was only one band present which was the appropriate size for protein A, potentially this was cytosolic protein as there was no evidence the signal peptide had been cleaved. With IPTG induction (lanes denoted I), there were multiple bands present. There were two bands close together, which indicated the signal peptide had been cleaved after translocation across the inner membrane. Subsequently, a 3<sup>rd</sup> band was present which could correspond to a degradation product.

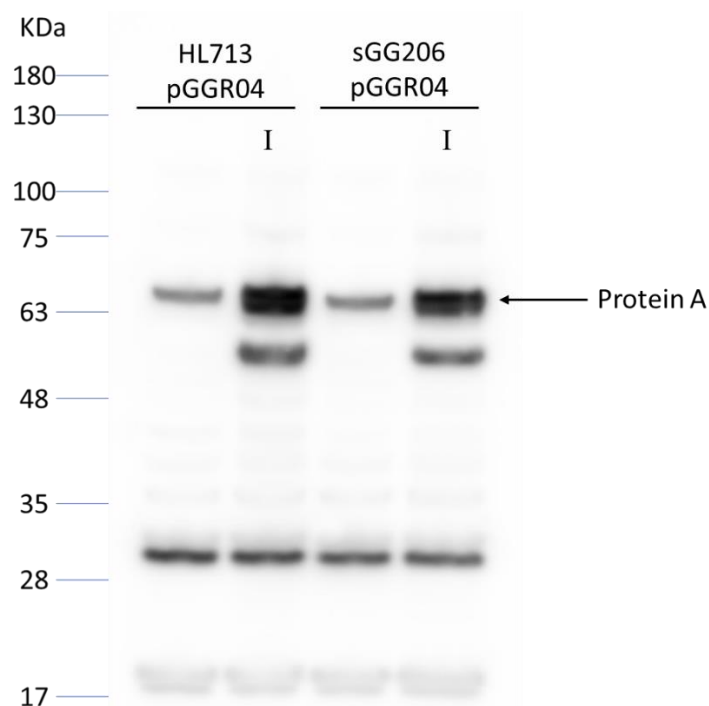


**Figure 5.15. Western blot analyses of protein A expression.** Strains were grown in the absence and presence of IPTG for induction in M9 media. Aliquots were taken during mid-exponential growth and cell lysates were separated on a 4-12% gradient SDS-PAGE before transferring to PVDF membrane.  $\alpha$ -Protein A (1:2000)(Abcam) was used to label the protein with  $\alpha$ -rabbit HRP secondary labelling (1:10,000) (1 minute exposure).

### 5.5.2 Localisation of protein A

Immunofluorescence staining of fixed cells was utilised for the localisation of protein A with and without induction. After following the same protocol used in *B. subtilis*, alterations were made as the fluorescence signal was weak without lysozyme treatment. The sGG221 background showed there was staining from  $\alpha$ -Protein A, in the non-induced strain the FITC staining was not as prominent in the cells and was mostly found between clusters of cells. This was similar to what was seen with the negative fluorescence control (appendices 4). Although there was leaky expression exhibited in *E. coli* cells, seen using the GFP reporter, it was clear that there was not a vast amount of protein A translocated over the inner cell membrane. With the addition of IPTG, to induce expression, it was evident that staining was much brighter surrounding the cell wall. However, the brightness of the staining appeared to be heterogeneous between cells similar to what was described with the GFP reporter in non-induced strains.

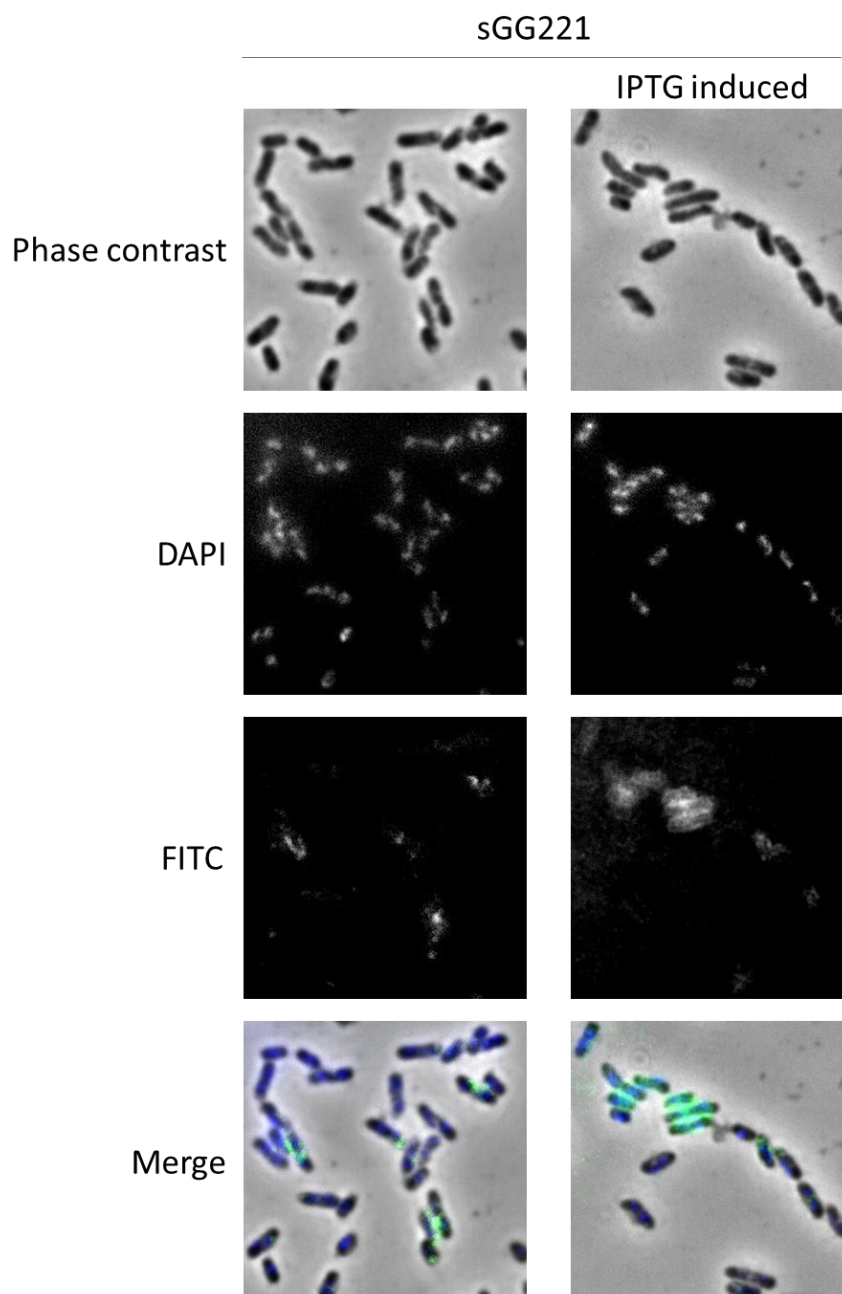


**Figure 5.16. Localisation of protein A in HL713.** Strains were grown in the presence or absence of IPTG, in M9 media. Cells were harvested during mid-exponential growth. Cells were fixed in 1% paraformaldehyde/PBS before applying to 0.01% poly-L-lysine treated slides and permeabilising with lysozyme. Primary antibody was applied ( $\alpha$ -Protein A, 1:250 in BSA) and incubated overnight at 4°C before washing and blocking in BSA. To detect primary antibody, FITC conjugated  $\alpha$ -rabbit were applied (1:250). After washing, mounting medium was applied with 0.2  $\mu$ g/ml DAPI and imaged using a Nikon Eclipse Ti with Cool LED pE-300 white light source (25% blue light intensity - 50 ms exposure). Photometrics Prime camera and Metamorph 6 software. N=3.



To determine if *E. coli* minicells expressed and secreted the protein, immunostaining was performed in the  $\Delta minC$  background. This would show if protein A was expressed and secreted, analysing both minicells and rod cells with and without induction of expression. Previous Western blot analyses, fig.5.15, did show protein A was expressed, however, it was not evident that the signal peptide had been cleaved. Therefore, fixed cells were stained with  $\alpha$ -Protein A for localisation which was expected to show if the protein was cytoplasmic or secreted. Without IPTG induction there was some protein A staining associated with minicells forming a circular shape around the cell, this demonstrated potential secretion and cell wall labelling (fig.5.17 yellow arrows). Although, it was also hypothesised that protein A could be residing within the periplasm as opposed to being attached to the cell wall. When protein A expression was induced, with addition of IPTG, there was strong signalling from rod cells. Staining showed that signals were mainly at the periplasmic/cell wall area with some labelling at the asymmetric septum, cell wall labelling intensity was heterogeneous as some cells labelled much greater than others. It was not confirmed if this was a result of staining efficacy or heterogeneous expression of protein A. Comparison of minicells from uninduced and induced cultures appeared to show that minicells from uninduced strains had greater intensity.

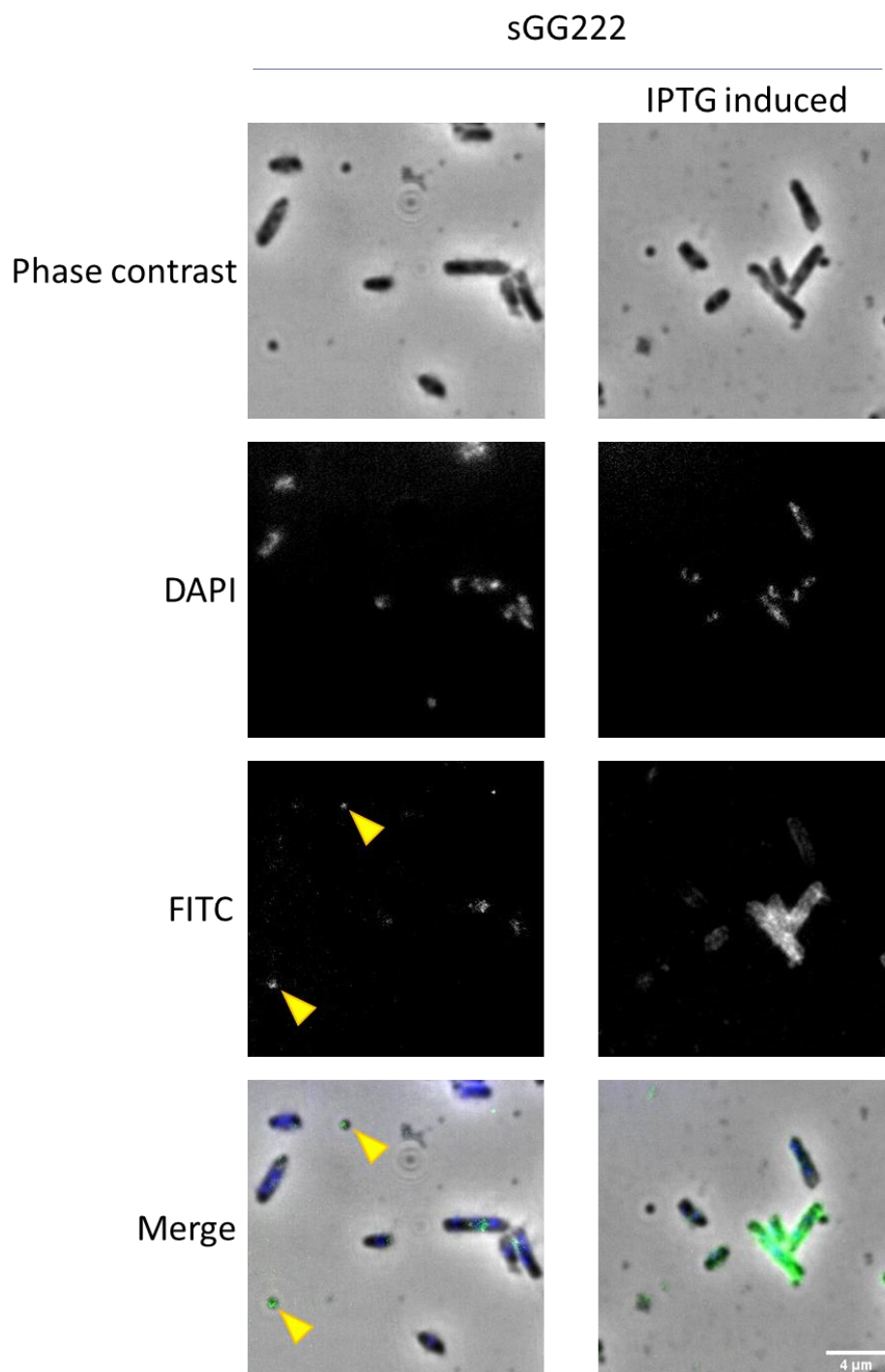


**Figure 5.17. *E. coli* minicells minicell protein A localisation.** *E. coli* minicell producing strains were grown in M9 media with the presence or absence of 1 mM IPTG. Exponentially growing cells were harvested and fixed in paraformaldehyde before applying to poly-L-lysine treated glass slides. Cells were permeabilised with lysozyme and before applying the primary antibody ( $\alpha$ -Protein A, 1:250 in BSA). Primary antibody detection was facilitated with FITC conjugated  $\alpha$ -rabbit were applied (1:250). To image, mounting medium was applied with 0.2  $\mu$ g/ml DAPI and imaged using a Nikon Eclipse Ti with Cool LED pE-300 white light source, Photometrics Prime camera and Metamorph 6 software. 25% blue light intensity - 50 ms exposure. N=3.

Clusters of minicells were found in fixed cells attached to slides and showed more prominent staining of protein A to detect localisation (fig.5.18 – yellow arrows). It was observed that minicells could of been expressing protein A from the plasmid and translocated it over the cytoplasmic membrane. There were minicells which did not have any discernible staining, however, this was consistent with GFP reporter data which showed only ~30% of minicells were expressing from the plasmid. Some minicell signals were distributed evenly around the edges, whereas others showed punctate fluorescence labelling with varied levels of intensity.

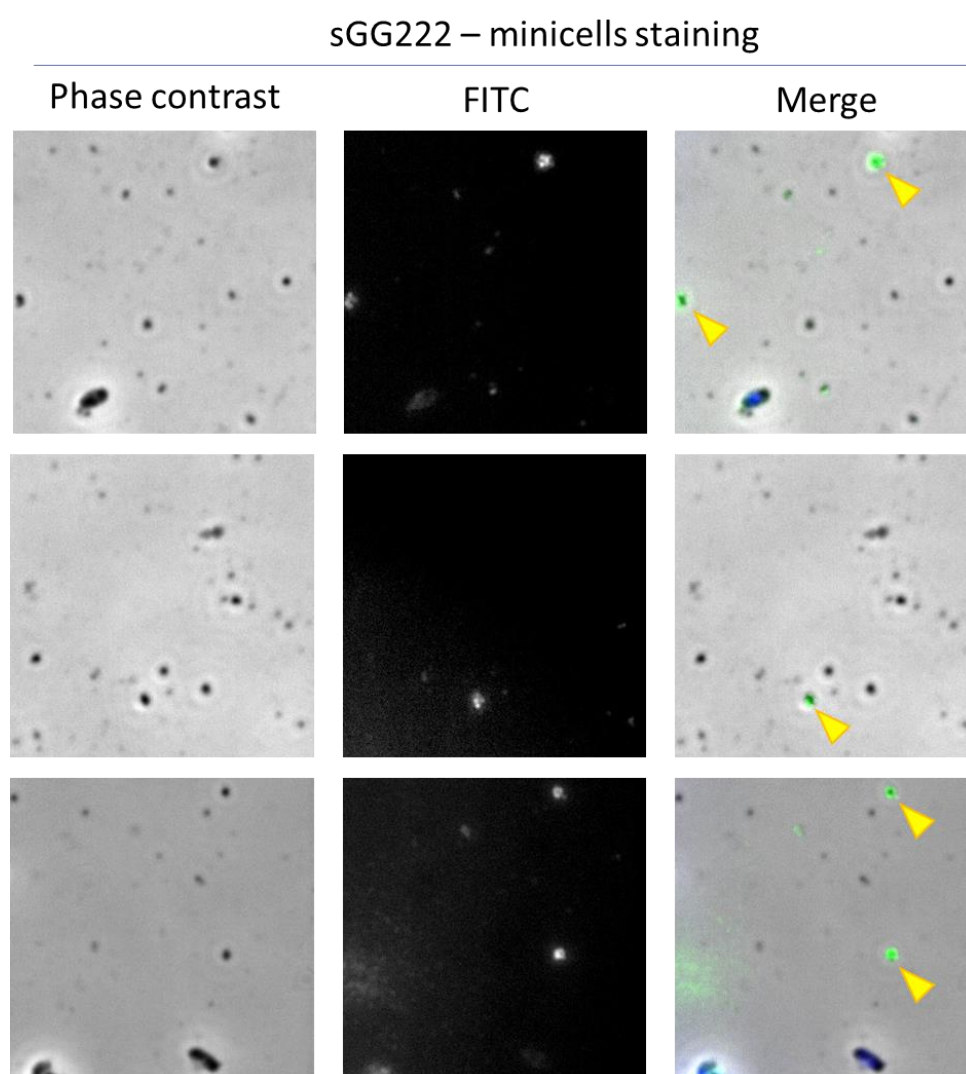


**Figure 5.18. Minicells immunostaining of protein A.** *E. coli* minicell producing strains were grown in M9 media in the absence of IPTG. Harvested cells were fixed in 1% paraformaldehyde/PBS and attached to slides with poly-L-lysine. Cells were permeabilised with 10 mg/ml lysozyme, primary stained with  $\alpha$ -Protein A (1:250 in BSA) and secondarily stained with FITC conjugated  $\alpha$ -rabbit (1:250). To image, mounting medium was applied with

0.2 µg/ml DAPI and imaged using a Nikon Eclipse Ti with Cool LED pE-300 white light source, Photometrics Prime camera and Metamorph 6 software.

## 5.6 Dual replicon system in *B. subtilis* affects pLS20 replication

### 5.6.1 RSF1010 origin effects on GFP expression in minicell production strains

As mentioned in the introduction (section 1.6), for portability the project aimed to use the same vector across all strains. For this reason, the RSF1010/pLS20 driven dual replicon plasmid, pGGR01, was tested in *B. subtilis* chassis (fig.5.1A – pGGR01). From studies already completed in *E. coli*, it was clear there was some leaky expression in rod cells but there were ~30% of minicells expressing from the plasmid independently (fig.5.4). Studies from chapter 3 using a pLS20/pMB1 dual replicon (pAG-P08) showed in *B. subtilis* ~50% of minicells were independently expressing from the plasmid and rod cells repressed expression well. However, pAG-P08 used a medium copy number pMB1 replicon which was not suitable for *E. coli*. This was why it was required to replace pMB1 with the RSF1010 replicon to have a plasmid that was compatible with *B. subtilis*, *E. coli*, and potentially *S. elongatus*.

When pGGR01 was transformed into sAG1392, there was a notable increase in GFP fluorescence and greater bright outliers compared to pAG-P08 under the same conditions (fig.5.19A magenta plot). A threshold was set at the end of the normal distribution of the rod cells fluorescence intensity with pAG-P08. Analyses of fluorescence values revealed that 60% of rod cells with pGGR01 were greater than the set threshold (figure.5.19A & C - red dotted line). This was a significant increase in expression which was hypothesised to be caused by the RSF1010 origin of replication. Transformation into sAG1338 (fig.5.19B) showed a similar effect where fluorescence increased significantly in both rod and minicell populations (Teal and purple plots). Rod cells of sAG1338 with pGGR01 showed a 61% increase in cells over the threshold set by pAG-P08 control, whereas minicells were 69% over the same threshold

(red dotted line – fig.5.19B & D). A new threshold was set in accordance with the end of the normal distribution of fluorescence intensity in rods with pGGR01 (black dotted line fig.5.19B). This was expected to show how many minicells were brighter than rods with this vector and indicate if more minicells were expressing independently. However, it was found that a significant number of rod cells were outliers (20% - fig.5.19E). This demonstrated that the plasmid was not repressed well with an RSF1010 replicon added to the vector (pGGR01).

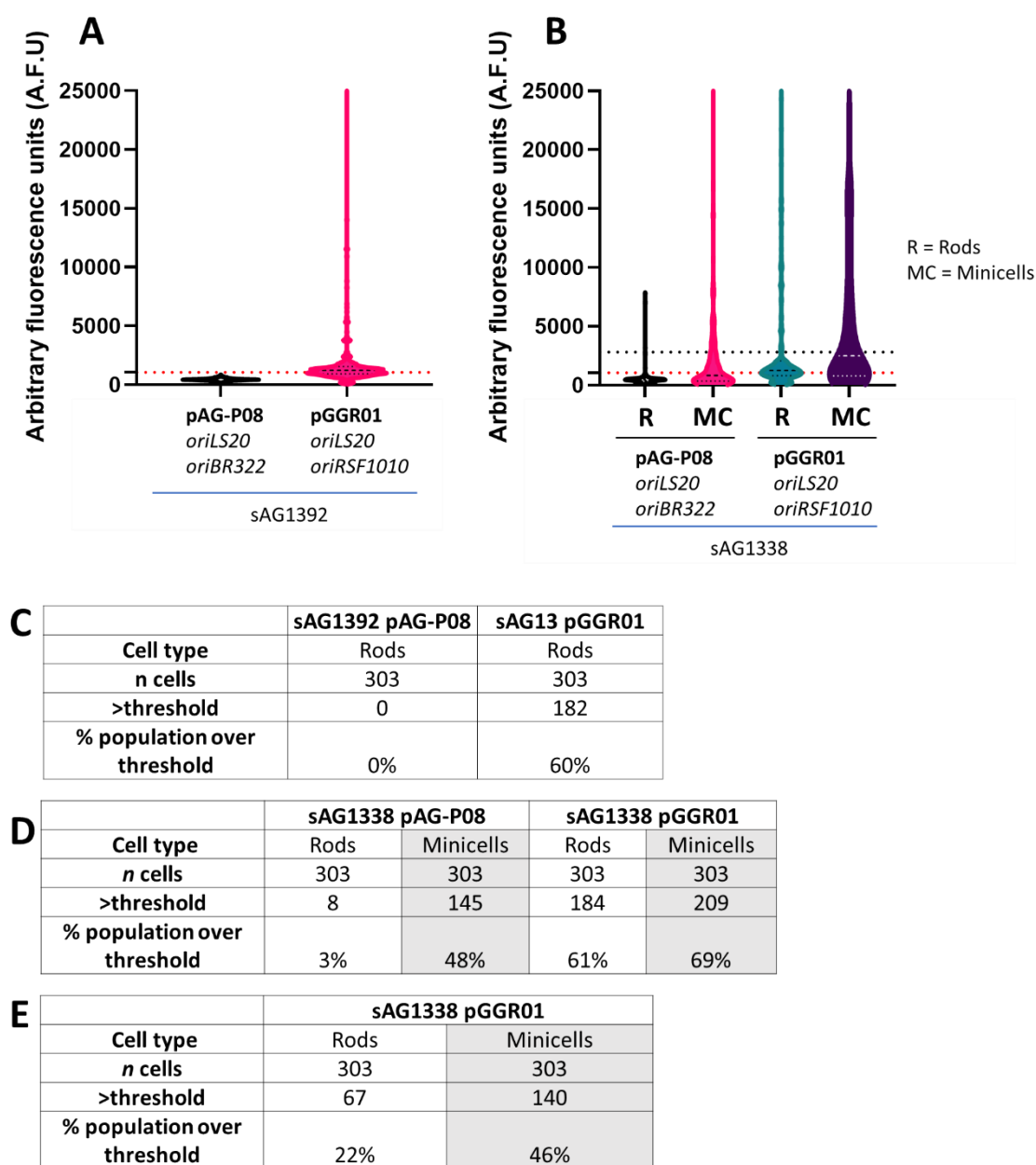


**Figure 5.19. RSF1010 and pLS20 dual replicon in *B. subtilis*.** *B. subtilis* sAG1392 and sAG1338 described in chapter 3 were transformed with pGGR01. Cultures were grown in CDM at 30°C before imaging during mid-

exponential growth. Counting took place using ImageJ software and plots were made in GraphPad Prism 9. **A)** sAG1392 with pAG-P08 control and pGGR01 **B)** sAG1338 fluorescence of rods and minicells R = rods MC = minicells. **C-E)** cell populations counted over thresholds shown on the respective graphs calculated in Microsoft excel.

### 5.6.2 RSF1010 effects on pLS20 driven plasmids copy number

To gain a better understanding of why regulation of GFP expression was affected with pGGR01 in *B. subtilis* chassis, qPCR was used to investigate if plasmid copy number had been altered by the presence of the RSF1010 replicon. If plasmid copy number had been altered, this would show why LacI could not repress the  $P_{spac}$  promoter as efficiently as the control, pAG-P08. Copy number was determined relative to the genome where it was hypothesised each cell had one chromosome terminus. The pAG-P08 controls demonstrated 2 copies per genome, in agreement with previous data (Chapter 3 & 4) and the literature (Meijer *et al.*, 1995). In comparison, the copy number was much greater in cells with pGGR01 over both time points. In sAG1392 pGGR01, there was a significant increase of 3.5-fold compared to pAG-P08 after 4 hours of growth. This intensified after 6 hours of growth as pGGR01 copy number increased 7-fold compared to pAG-P08 ( $P < 0.0001$ ). There was an even more dramatic increase in sAG1338 where, after 4 hours (fig.5.20A), there was a 7-fold increase which was significantly greater ( $P < 0.0001$ ). Similarly, after 6 hours there was an 8-fold increase in copy number which was also significant ( $P < 0.0001$ ). An interesting attribute to the RSF1010 driven plasmid was copy number increased between time points, whereas the pAG-P08 control maintained a stable copy number (comparing fig.5.20A & B).

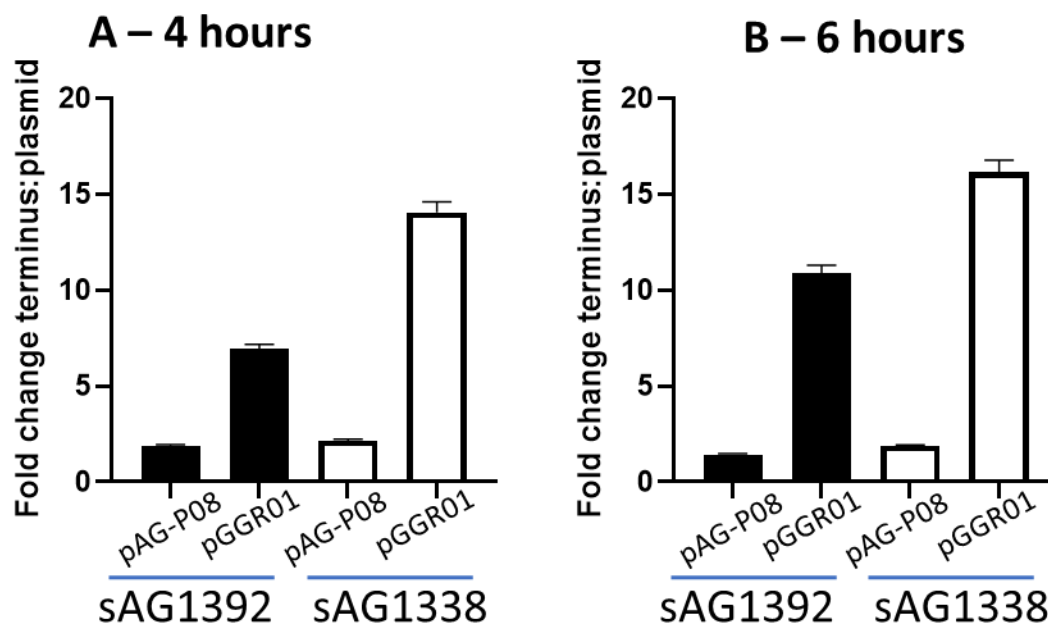


**Figure 5.20. Copy number of RSF1010/pLS20 dual replicon in a *B. subtilis* background.** Strains with plasmids were grown in CDM with erythromycin selection at 30°C, samples were taken after 4 and 6 hours of growth and processed in lysis buffer. The lysates supernatant was collected and diluted 1000x before pipetting into reactions with NEB Luna universal qPCR polymerase and appropriate primers. Reactions were run and analysed using Rotor-Gene Q software. Terminus to plasmid ratios were calculated using Microsoft excel and error bars represent triplicate experiments.

## 5.7 Conclusions and discussion

This chapter explored the use of *E. coli* as a chassis for the Portabolomics minicell compartmentalisation system. It was found in this system that GFP expression was not easily regulated using the same repression system that worked in *B. subtilis* (see Chapter 3). The native LacI system was investigated to see if it could repress the lowest copy number plasmid, however, high expression was observed. Therefore, the LacI repressor was integrated into this chassis as a single mutation within the -35 promoter sequence increased promoter strength significantly and was 50-100 times stronger (Calos and Miller, 1981). In brief, this discussion will consider the plasmid replicons, repression systems and products expressed from this chassis in detail as well as considering dual replicon plasmids in *E. coli* and *B. subtilis*.

### 5.7.1 *E. coli* chassis optimal vector

As part of the characterisation of the *E. coli* chassis, two replicons were analysed to determine which was optimal for minicell expression. The SC101 replicon is native to *Salmonella panama* but the literature showed this replicon was well studied in *E. coli* and had been reduced to the minimal essential components that are: the *par* region for segregation and stability, the origin for unidirectional replication and *repA* plasmid specific replication protein/gene (Kües and Stahl, 1989; Manen and Caro, 1991). It was documented that the SC101 replicon requires host DnaA to initiate replication, this was demonstrated in a temperature sensitive DnaA mutant where SC101 replication was arrested (Hansunuma & Sekiguchi, 1977). As mentioned in section 5.1, it was known from work by Hansunuma & Sekiguchi that the SC101 replicon was ~5 copies per cell. This potentially made it ideal for this study as work in *B. subtilis* showed that a low copy number was best in terms of regulated expression (see chapter 4). A low copy number plasmid was required but not ideal as the minicell is ~1/10<sup>th</sup> the size of the rod cell (Adler *et al.*, 1967), meaning it would reduce the likelihood of plasmids moving into minicell compartments by diffusion. This was reflected in the data given as it was shown with pGGS01 that 17% of minicells were counted as GFP positive, this was interesting as the *B. subtilis* minicell chassis carrying a plasmid with a copy number as low as 2 copies per chromosome (*oriLS20*) was able to occupy ~50% of minicells. However, it had been hypothesised in the *B. subtilis* chassis that the plasmid could be replicating within minicells, which was how it out-competed LacI repression. If SC101 was heavily dependent upon DnaA for replication initiation, then potentially, there may have been issues with pGGS01 replication within minicells and therefore the plasmids may remain repressed for a greater amount of time. There was also uncertainty surrounding the use of the *alp7AR* operon as it was unknown if the segregation system was working within this model (See section 4.1.2 for stability work in *B. subtilis*). Future direction would include identifying if this partition system worked within *E.*



*coli*. Then the segregation system could be enhanced to see if minicells gaining a plasmid could be increased.

As the probability of minicells obtaining a plasmid increased with higher copy number in *B. subtilis* (see section 4.4.2), the RSF1010 replicon was also trialled as it was reported to be ~10-12 copies per chromosome and therefore double pGGS01 (Frey *et al.*, 1992, Kües and Stahl, 1989). The RSF1010 replicon was also favourable as it encoded its own replication initiation proteins and therefore did not rely on host DnaA. In examples shown in this study, it was found that pGGR01 showed a greater percentage of cells over the threshold set with all repeats considered ~30% of minicells independently expressing GFP. However, pGGR01 did show a higher average in fluorescence intensity in rod cells compared to pGGS01, this demonstrated that there was an effect on LacI repression where regulation was not as efficient when copy number was increased (see section 5.2.1). Nevertheless, pGGR01 was still considered a better candidate after time-lapse analyses. Here it was seen over time, there were clearly more minicells expressing GFP from the plasmids and fluorescence intensity tracked over time revealed there was greater expression from minicells harbouring pGGR01 (section 5.2.2). Using rod cells as the base line, it was obvious that minicells with pGGR01 were able to express from the plasmid ~80 minutes after formation. Whereas a minicell that presumably did not gain a plasmid had fluorescence signals that remained similar to that of rod cells.

Further analysis of time-lapse data also indicated that the rod cells homogenously increased leaky GFP expression over time, it was hypothesised that this was a phenomenon associated with flat pad growth for microscopy as single time point images from cell suspensions showed heterogeneity. It was possible that fluorescence fluctuations could be caused by stress related transcriptional changes which could affect LacI expression. However, it appeared as if cells had a surge of expression before elongating and dividing which diluted cytosolic GFP, once the cells divided it did not occur again but the time-lapse lost focus shortly after making it

problematic to confirm the phenomenon. Near the end of the time-lapse it was obvious that fluorescence increased, this could be associated with cellular stressors affecting plasmid replication or LacI expression via transcriptional changes. *E. coli* is known to change genome wide expression with the use of  $\sigma^S$  which controls the general stress response, this tends to be activated in times of starvation alongside other non-optimal growth conditions (Weber *et al.*, 2005).

As mentioned previously (see section 5.2.1), it was evident *E. coli* rod cells exhibited leaky expression that could potentially mask the amount of minicells which were independently expressing GFP from the plasmid. Therefore, a secondary repression system targeting the mRNA of *gfp* was designed but unfortunately did not work as anticipated as expression of asRNA from the chromosome did not show a significant reduction in rod cell GFP. Whereas, expression from a high copy number plasmid affected transcription but also appeared to impose stress. There was evidence that suggested the asRNA was reducing GFP intensity in some cells when co-transformed with pGGS01 (see section 5.4.2), however, this was only seen when asRNA was expressed from a high copy number plasmid and did not affect the whole population homogenously. Potentially indicating that the asRNA was not abundant or stable enough to show an effect when expressed from the chromosome. The asRNA in this work was designed in accordance with the rules set out by Hoynes-O'Connor & Moon with MicF M7.4 Hfq binding sites described to be most efficient (Hoynes-O'Connor & Moon, 2016). The mRNA annealing sequence was designed to bind within mRNA encoding *gfp* to prime it for degradation, however, as the system did not work it may be worth targeting different areas of the mRNA such as the RBS to block translation. An alternate possibility is that temperature was also playing a major role in the efficiency of this system, the mFold structure of the asRNA (fig.5.12) was designed to anneal at 37°C, with the expectation that as the strains were grown

at 30°C it should be more effective. However, this may have led to misfolding of the Hfq hairpin loops and have instability of the RNA.

### **5.7.2 Population analyses of minicell producing *E. coli***

Analyses by flow cytometry revealed that the minicell producing strain of *E. coli* had a ‘small cell’ population of ~26%, these were assumed to be free minicells and was comparable to what was seen in *B. subtilis* minicells (A. Guyet, unpublished). There was slight deviation with the addition of plasmids which was not considered to be a result of the plasmid but more likely a result of growth phase where more minicells remained attached to rod cells. Slight elongation of rod cells was expected to be seen as this was reported in the literature by Zhou and Lutkenhaus in 2005. Interestingly, in sGG206, rod cell characteristics were much more heterogeneous as the populations side scatter (cell properties) were not grouped as tightly. When the whole population were screened for fluorescence it was obvious that most of the population had shifted into the ‘GFP positive gate’, this was expected from microscopy data as leaky expression had been identified by microscopy.

Enrichment techniques developed by Aurèlie Guyet (unpublished) based on published methods by MacDiarmid *et al.* 2007, using filtration, were found to be successful but did require more optimisation to be practically used. Using these methods, the minicell population was easily enriched and concentrated minicells to ~71% of the population, though, ~20% of the rod population remained. The enriched minicells populations were analysed for GFP fluorescence using flow cytometry, interestingly, there was only 10% difference between minicells with pGGS01 and pGGR01 vectors for GFP positive minicells. However, this method was not able to show if cells were independently expressing GFP or if they were overlapping with cytosolic GFP gained from rod cells.

To improve the enrichment of minicells, methods could be considered to accompany filtration that would aid in removal of rod cells. As TEM data displayed minicells in *E. coli* were smaller than the cylindrical body of the rod cells (see section 5.2.3), then it was postulated that a smaller pore size could be used for the filtration techniques. Alternatively, the same filtration method could be repeated 1 or 2 more times to filter the 20% rod cells out, however, this may become problematic as the final volume was not large and further dilution would be required to carry out steps again. Jivrajani *et al.*, 2013, outlined another minicell enrichment method where the addition of penicillin was used to remove dividing rod cells. The antibiotic used would need to be considered for industrial applications, as it was known historically that minicell expression could be affected by rifampicin and therefore would affect yields (Crooks *et al.*, 1983). The most common method for minicell enrichment within the literature was to use sucrose gradients with up to 3 successions to remove rod cells. These enriched minicells could be stored at -70°C or within liquid nitrogen (Reeve, 1979). Lastly, MacDiarmid *et al.* used all three density centrifugation, cellular stress to elongate rod cells and filtration in order to enrich minicells, a method verified on agar plates where no colony forming units were observed (MacDiarmid *et al.*, 2007).

### **5.7.3 Expressing an industrially relevant compound**

To represent applications for minicells of *E. coli*, protein A was used as a reporter on the RSF1010 driven plasmid. *E. coli* minicells had been reported to express protein A, however, it was not clear if minicells were expressing full length protein as there was conflicting information from the authors Western blot and pulse-labelled polypeptide analyses (Duggleby & Jones, 1983). This study revealed that minicells were able to express protein A and had evidence to suggest they were able to secrete the protein across the inner membrane into the periplasmic space.

Furthermore, it was hypothesised that Protein A was trapped within the periplasm as a result of requiring lysozyme for detection, the immunostaining did show outer wall like staining of minicells which potentially indicated it had crossed the inner membrane but not peptidoglycan. If trapping in the periplasmic space or efficiency of minicell labelling was an issue, further work could be performed to use autotransporters. Investigations by Jain *et al.*, 2006 demonstrated how some autotransporters localised to the cell poles of *E. coli*. It was further shown by Yu *et al.*, 2021 that these transporters could be enriched in minicells (Yu *et al.*, 2021). Therefore, autotransporters could be used to enhance secretion of heterologous proteins that would decorate *E. coli* minicells. This would be beneficial for industrial application as minicells could secrete products directly into the media, or be decorated for vaccination purposes.

#### **5.7.4 Integration of the dual replicon into *B. subtilis* chassis**

The role of the Portabolomics project was to have portability within the chassis for ease of changing species for expression of products. Therefore, the RSF1010/pLS20 dual replicon plasmid pGGR01 was used in *B. subtilis* to see if it would be compatible for use in both species. Although RSF1010 had been documented to replicate within some Gram-positive organisms (Gormley and Davies, 1991), a vector constructed by removing *oriLS20* from pGGR01 was produced but did not yield any transformants when positive controls did (Appendix 8). Therefore, it was assumed since RSF1010 did not work in the form used here in *B. subtilis*, the dual replicon with two replicative origins for *E. coli* and *B. subtilis* could be used between both systems.

Even with the assumption that the RSF1010 replicon did not work in *B. subtilis* to support independent plasmid replication, it was clear that RSF1010 had an effect within *B. subtilis* (section 5.6). GFP expression showed that 60% of rod cells had greater mean fluorescence than obtained with the pAG-P08 control, without the RSF1010 origin. Effects in the minicell

producing strain were similar to sAG1392, with the pAG-P08 threshold there was 61% of rods over the threshold and 69% of minicells (red dotted line fig.5.19B). This was a significant increase and demonstrated that there was a high increase in expression. This suggested that there was an increase in plasmid copy number, confirmed by qPCR. Copy number analyses revealed there was a 3.5-7-fold increase depending upon growth phase.

There are no published indication that RSF1010 replication or mobility proteins interacting or affecting other plasmid replicons. However, replication proteins of RSF1010 used for initiation, priming and unwinding of DNA could all alter the functionality of the pLS20 origin as little is known about how replication is initiated in this replicon (Miao *et al.*, 1995; Meijer *et al.*, 1995). The mobility system, *mob/oriT*, of RSF1010 were present in the replicon used for this study. It was hypothesised that these factors could be affecting the plasmid in *B. subtilis* as mobility proteins nick *oriT*, initiating single stranded transfer to the next host (Willetts and Wilkins, 1984). Therefore, single stranded intermediates could have effected expression and copy number detected by qPCR. The upregulation of GFP expression may have been because of mobility elements inducing an SOS stress response that, in turn, effected the transcriptional profile (Baharoglu *et al.*, 2010). However, the functionality of *mob/oriT* in *B. subtilis* is questionable and therefore further investigations would be required to understand the effect RSF1010 had on the plasmid. Further study would include using a minimal replicon which removed all mobility proteins. This would leave only the essential replication proteins for RSF1010 as shown by Scherzinger *et al.*, 1991 and used by the Standard European Vector Architecture database (SEVA-DB) (Silva-Roche *et al.*, 2013). If there were still issues with the removal of mobility proteins, then the replication proteins could be individually deleted to see what affect they may be having on the pLS20 origin in *B. subtilis*.

### 5.7.5 Conclusions and future work

To conclude this chapter, a minicell chassis was constructed in *E. coli* in accordance to the model (see chapter 1) and an optimal vector was determined, which was RSF1010 driven at ~10 copies per cell (Frey *et al.*, 1992). Overall *E. coli* minicells were a promising candidate for compartmentalised gene expression as minicells were found to be able independently express GFP from the plasmid. Interestingly, flow cytometry analyses showed *E. coli* and *B. subtilis* produced a similar amount of free minicells (~26%). Furthermore, minicells of *E. coli* demonstrated potential industrial applications by expressing heterologous proteins and translocated protein A over the inner membrane.

Considering the bigger picture, areas that require more work to produce a successful *E. coli* model would be to start with improving regulation. To do this, it could be better to deviate from the aims of Portabolomics and use a specific repression system for each species to ensure the best regulation is possible in terms of repression in rods and expression in minicells. Furthermore it was outlined that segregation systems, which increased plasmid stability in *B. subtilis* (see chapter 4), were not studied in this work with *E. coli*. Therefore, it was unknown if the segregation system was functional or if it could be utilised to increase minicells gaining a plasmid. As the maxicell system of *B. subtilis* was a promising compartmentalisation candidate, this study would look to repeat this in the *E. coli* strain, anucleated cells have been described frequently in the literature by different mutations including H-NS, SMC complex mutations to *oriC* mispositioning (Kaidow *et al.*, 1995; Mäkelä *et al.*, 2021; Niki *et al.*, 2000, respectively).

## Chapter 6 - Cyanobacterial minicell producing chassis

### 6.1 Introduction

The cyanobacteria family are known for their ability to produce energy by photosynthesis in environments with low nutrient availability and studied extensively for their circadian rhythm cycles, which regulate gene expression in 12-hour sequences. Cyanobacteria do not need large quantities of nutrients added to the media, only requiring trace metals and salts in water. Photosynthetic properties mean that cyanobacteria rely on carbon dioxide (CO<sub>2</sub>), water (H<sub>2</sub>O) and light for metabolism. This was a useful feature as cells could directly produce carbon-based compounds, creating an opportune chassis for biofuels (Steuer *et al.*, 2012). Cyanobacteria are often isolated from freshwater or marine environments, interestingly marine cyanobacteria have been found to produce numerous secondary metabolites which may be useful for therapeutic applications because of anti-proliferative, cancer cell cytotoxic, antibiotic and anti-inflammatory activities (Nunnery *et al.*, 2010).

The host organism used in this study, *Synechococcus elongatus* PCC 7942, is a Gram-negative rod-shaped bacterium with specialised thylakoid membranes to facilitate energy production and house photosynthetic apparatus. Not dissimilar to chloroplasts, the thylakoid membranes of cyanobacteria facilitate light-driven photosynthetic electron transport where proton-motive forces drive ATP synthesis. These membranes also emit autofluorescence under far red excitation at 561 nm (Heinz *et al.*, 2016). As mentioned in chapter 1, *S. elongatus* also contains carboxysomes where the carbon reduction cycle is contained to prevent the accumulation of toxic biproducts from RuBisCO oxygenation (Rae *et al.*, 2013).

The aims of this part of the study are to construct minicell producing strains of *S. elongatus* as a potential chassis for the Portabolomics project. Minicell producing strains were to be analysed in further detail to characterise the production of Cyanobacterial minicells and



determine if the strain would be industrially useful. The intention was to try and create a minicell producing chassis analogous to those described in *B. subtilis* and *E. coli*.

Questions this chapter aimed to elucidate was, because of specialised thylakoid and carboxysome elements, if organelle-like structures were able to segregate naturally or randomly into minicells. It was also questioned if multiple chromosomes and native plasmids would affect minicells, or if identified partitioning systems were able to move chromosomes away from asymmetric septa.

## 6.2 Results

### 6.2.1 Construction of a minicell producing mutant

To construct a minicell producing strain, a plasmid which did not replicate within *S. elongatus* was created using the pUC19 backbone. To disrupt *minC*, and for selection, the *aadA* antibiotic resistance cassette was inserted (*Spec<sup>R</sup>*; fig.6.1A). To stimulate homologous recombination at the correct locus for efficient *minC* gene disruption, 300 bp of homology from around the *minC* gene were also cloned. Instructions were followed according to Golden *et al.*, 1987. The plasmid was assembled to make pGGCA1, the 3 fragments which constructed the vector for  $\Delta minC$  were clearly visualised in fig.6.1B, a 1 pot reaction was used to assemble all fragments with homologous arms. The assembled plasmid (pGGCA1) was then used for subsequent transformation into naturally competent *S. elongatus*.

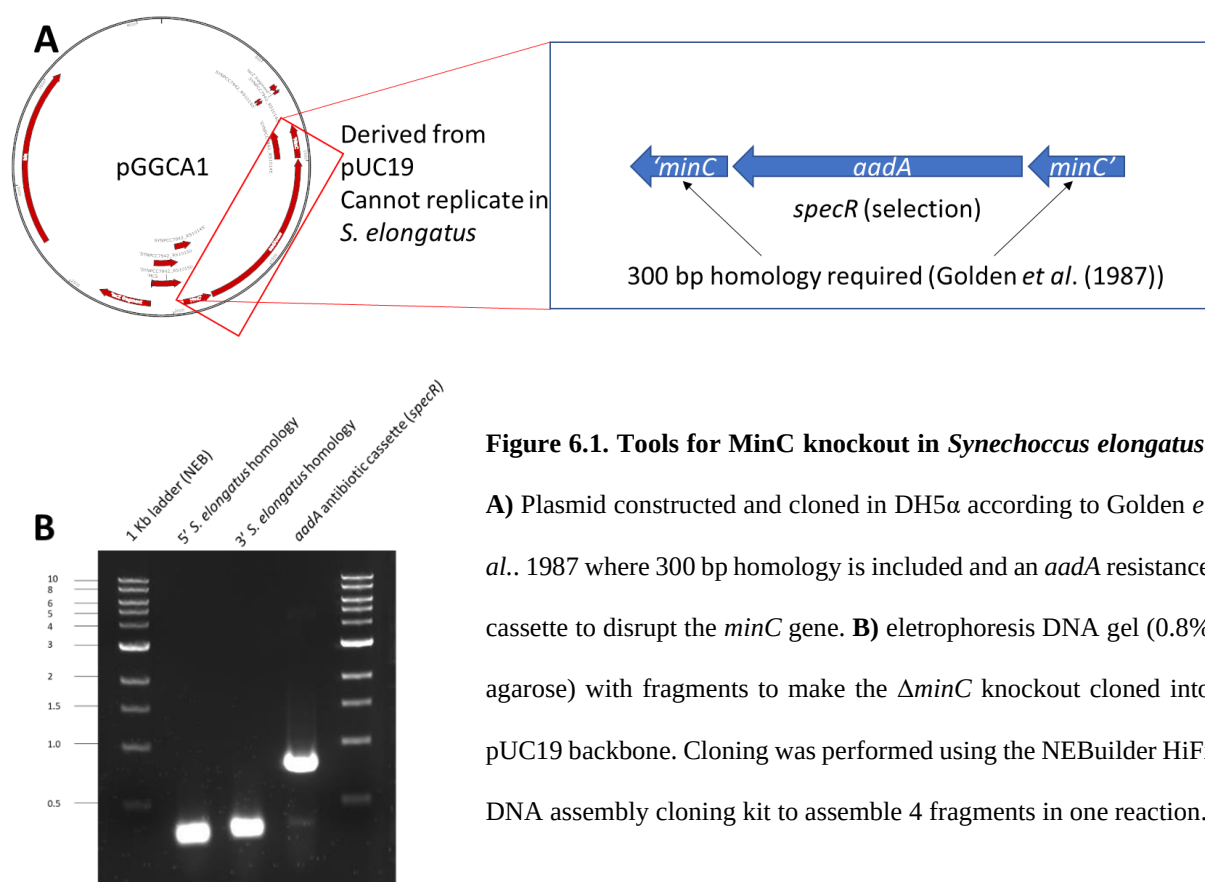


**Figure 6.1. Tools for MinC knockout in *Synechococcus elongatus*.**

**A)** Plasmid constructed and cloned in DH5 $\alpha$  according to Golden *et al.*, 1987 where 300 bp homology is included and an *aadA* resistance cassette to disrupt the *minC* gene. **B)** electrophoresis DNA gel (0.8% agarose) with fragments to make the  $\Delta minC$  knockout cloned into pUC19 backbone. Cloning was performed using the NEBuilder HiFi DNA assembly cloning kit to assemble 4 fragments in one reaction.

Transformants were gained after 6 weeks of growth, with constant illumination, on selective agarose plates. To verify transformants, phenotypic analyses were used via microscopy imaging, this allowed to see if *minC* had been disrupted efficiently by the appearance of minicells. Colonies yielded after *S. elongatus* transformation with pGGCA1 plasmid (fig.6.1) were grown with increasing doses of antibiotic to select for transformants with knockouts in all chromosomes. When spectinomycin concentration reached 80 µg/ml, cells were imaged to see if the minicell producing phenotype was present using thylakoid membrane autofluorescence (red fluorescence). In the wild type strain there were cells seen as chains with homogenously sized cells, the thylakoid membranes were noticable in all cells where some displayed brighter aggregates of fluorescence towards the poles (fig.6.2A). In contrast, strains transformed with  $\Delta minC$  plasmid showed the presence of small cells that possessed thylakoid membranes as they retained autofluorescence from the photosynthetic apparatus (fig.6.2A red arrows). All minicells seemed to remain attached to mother cells during exponential growth as no free floating minicells were observed. The rod shaped cells of the minicell producing strain also appeared to be heterogenous and larger in size, consistent with divisome misplacement with  $\Delta minC$  mutation.

To ensure all chromosomes had incorporated the spectinomycin resistance cassette and the phenotype was not linked to any other recombination events, colony PCR was performed. Wild type controls were used to see the expected size of the intact *minC* gene. If the gene was not incorporated into all chromosomes there would be 2 bands of different sizes to indicate this. Four transformants were screened using microscopy and colony PCR. Transformants 2-4 did exhibit the minicell producing phenotype shown in figure 6.2A, this was confirmed using colony PCR where the appropriate size difference was exhibited as the wild type was expected to show 1159 bp and the  $\Delta minC$  locus increased to 1462 bp. The one transformant which did not show a minicell producing phenotype (transformant 1) also did not show the correct size

of PCR product and the size of the band was clearly the same as wild type, indicating that the spectinomycin resistance cassette may have integrated elsewhere.

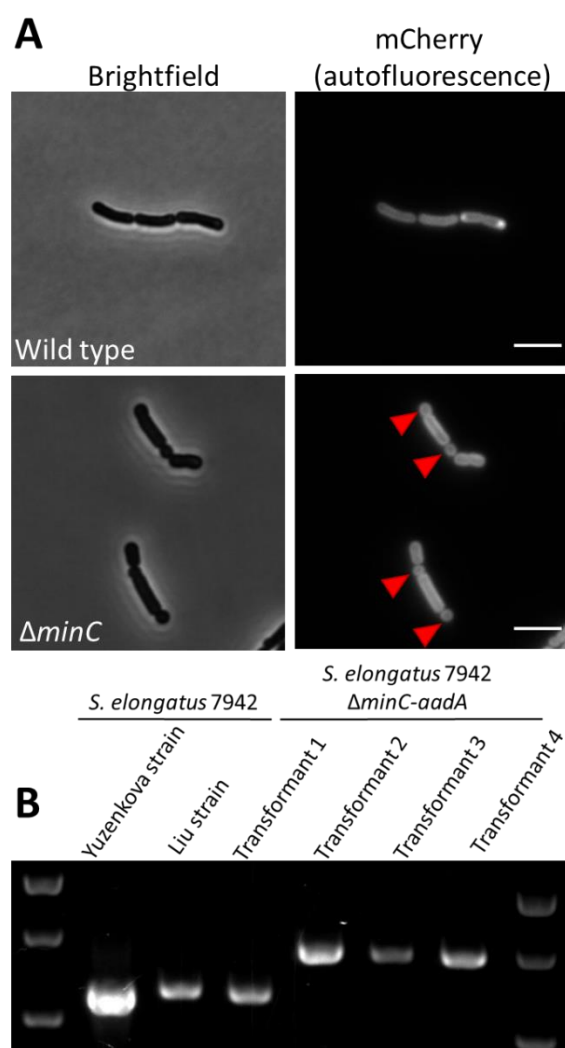


**Figure 6.2. Phenotypic and genotypic examination of transformants.** **A)** From single colonies on selective BG11 solid media, liquid culture was inoculated, and the antibiotic concentration gradually increased over each dilution. After reaching a concentration of 80  $\mu\text{g/ml}$ , the culture was used for microscopy analysis where cells were mounted onto 1.2% agarose pads. Red arrows indicate the presence of small round cells attached to the poles of rods which are not present in the wild type strain. The scale bar is representative of 4  $\mu\text{m}$  and is applicable to all images. **B)** From single colonies on selective BG11 solid media, liquid culture was inoculated, and the antibiotic concentration was gradually increased over culture dilution. After reaching a concentration of 80  $\mu\text{g/ml}$ , the culture was used for colony PCR using oligonucleotides outside the *minC* locus to see size changes if antibiotic resistance cassette had integrated at the correct locus. DNA was stained using SYBR gold and ladders are 1 Kb (NEB) also stained using SYBR gold.

## 6.3 Investigating ultrastructure of Cyanobacteria

### 6.3.1 Wild type structural analyses and division

To visualise the internal ultrastructures of *S. elongatus*, transmission electron microscopy (TEM) was used where electron beams passed through thin sections of bacterial culture stained using heavy metals to increase resolution. The wild type strain showed the internal structures expected to be seen such as carboxysomes and thylakoid membranes. Thylakoid membranes surrounded the inner cytoplasm of the cell close to the plasma membrane, also demonstrated by autofluorescence (fig.6.2A). In this example, the thylakoid membranes displayed 3 layers per side of the lateral body of the rod. However, in other examples it was seen that there were 3 layers on one side and only 2 on the other, demonstrating discontinuous lumen described by Nevo *et al.*, (2007). Cyanobacteria are a Gram-negative species which contain a single layer of peptidoglycan with lipid bilayers for the inner and outer membranes, that was obvious in electron microscopy images as the cell was surrounded by a double membrane structure (fig.6.3A). The icosahedral shaped carboxysome organelles were pronounced by grey staining within the cytoplasm, highlighted by red arrows (fig.6.3A & B). A rod cell cross section demonstrated how the thylakoid membranes surround the cytoplasm, leaving very little space for chromosomal DNA, and carboxysomes (fig.6.3B). White spaces within the cytoplasmic space were likely where chromosomal DNA would reside but was washed out within the stages of making/staining sections.

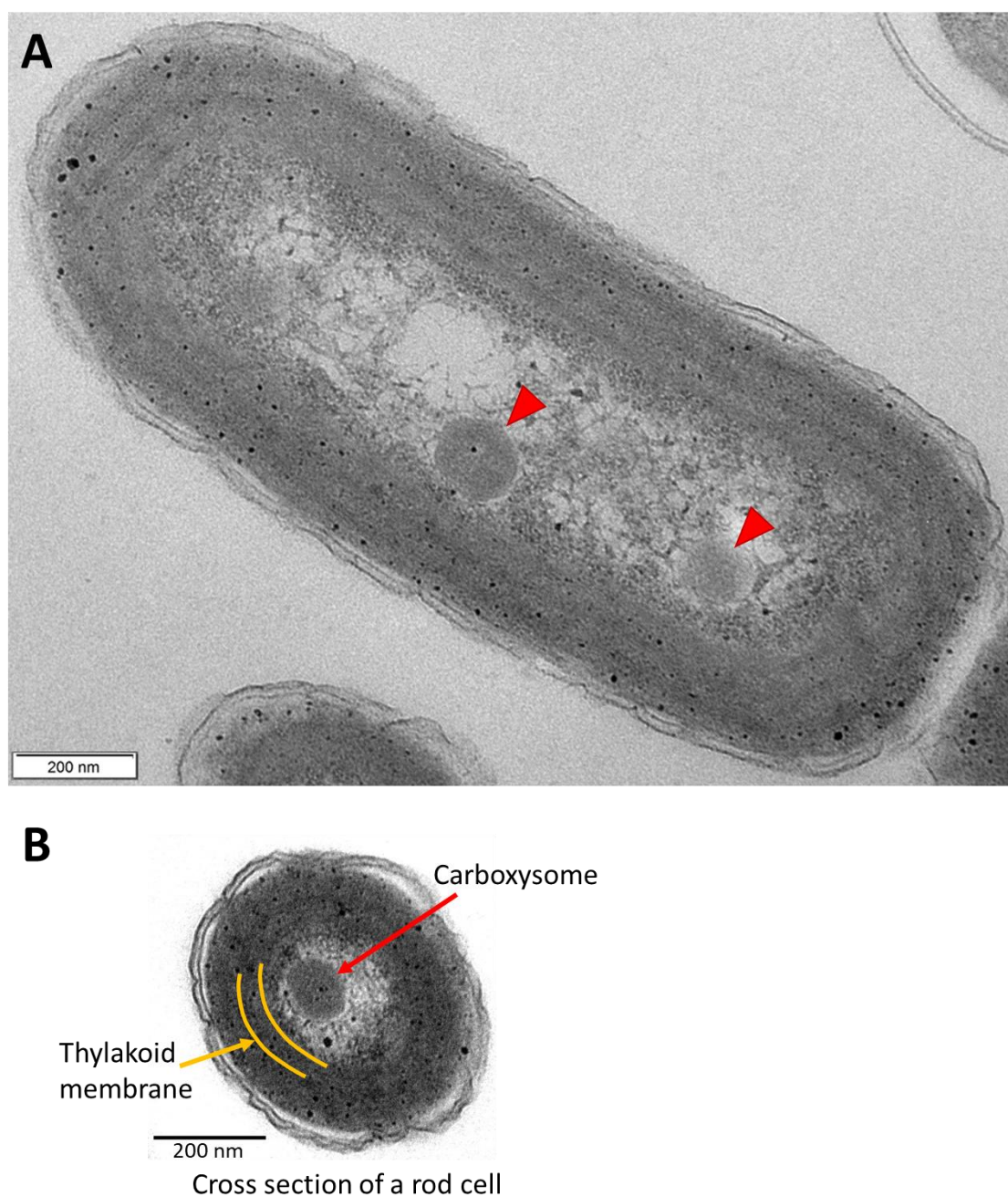


**Figure 6.3. *Synechococcus elongatus* wild type TEM internal structural analyses.** Wild type *S. elongatus* were grown in BG11 media with constant illumination until cultures started to turn green and were at around mid-exponential growth. Cells were harvested before resuspending in glutaraldehyde buffer, after epoxy resin embedding the sections were stained using heavy metals to increase sample density for better image contrasting (uranyl acetate and lead citrate). **A)** wild type rod cell, red arrows indicate carboxysomes **B)** cross section of a rod cell. Services were provided by the Electron Microscopy Research Services (EMRS) at Newcastle university. Images were acquired using Hitachi HT7800 120 kV transmission electron microscope. Scale bars are representative of 200 nm.

To investigate the minicell phenotype, the wild type division was firstly analysed to understand the native mechanism to understand polar divisions better. Dividing cells were found within sections which showed that thylakoid membranes curve inwards with the invagination cause by division, the outer thylakoid closest to the plasma membrane seemed to be disconnected (fig.6.4). The division septum was formed at the middle of one elongated cell which would have formed two equally sized daughter cells. Invaginations between rod cells resembled that of *E. coli* division where the membrane folded inwards (see chapter 5). In this example, there were only 2 thylakoid membranes on one side where there were 3 on the other showing these membranes are not one singular lumen. Between the two forming daughter cells there was a darker grey area where the thylakoid membranes were folding inwards, which shows a lack of DNA present. This lack of DNA shows that division did not occur over chromosomes and that the DNA segregation system is efficient at moving chromosomes into progeny cells. There was some punctate staining between the thylakoid membranes which were thought to be an artefact from the heavy metal staining, more wash steps would be required for future sections applying to all TEM images (fig.6.4).

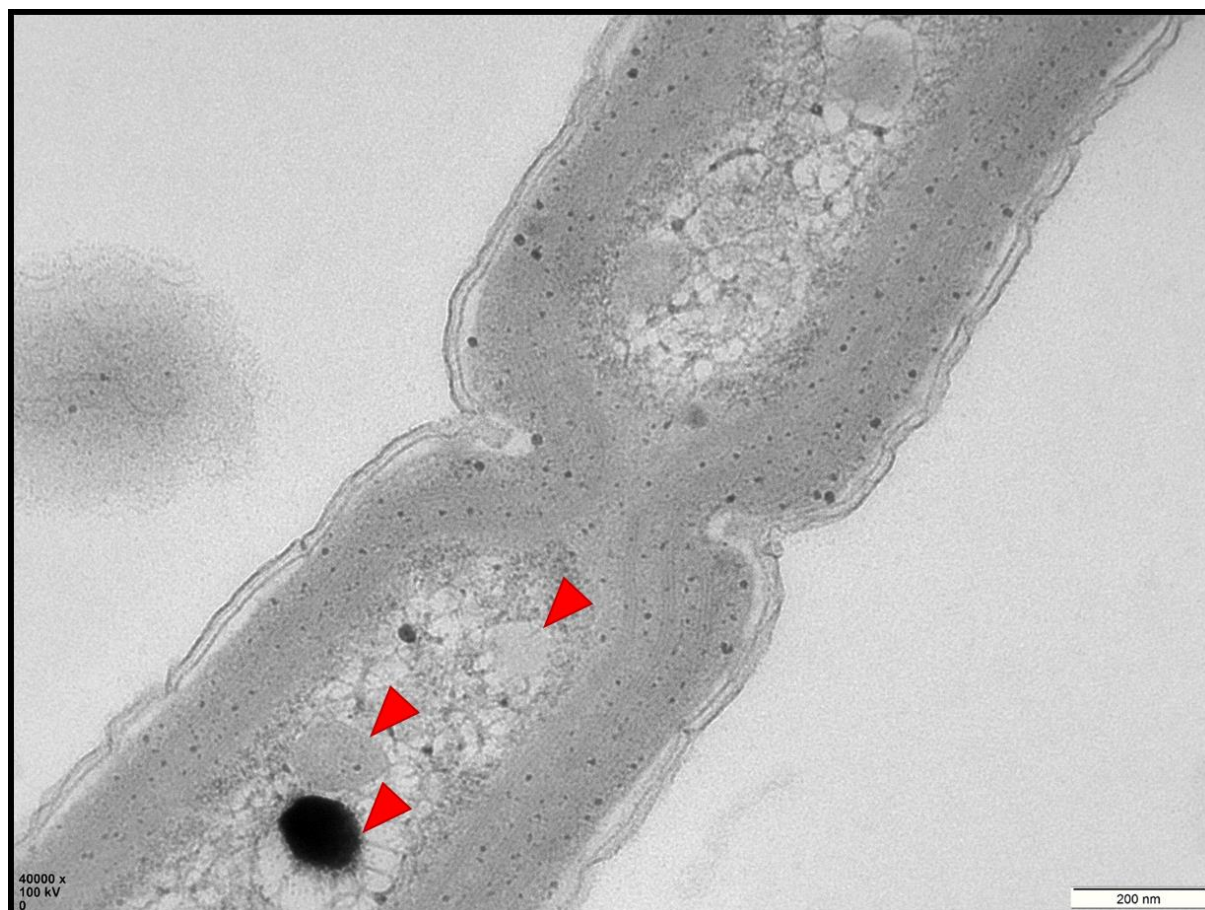


**Figure 6.4. TEM of wild type division showing septum constriction.** Wild type *S. elongatus* were grown in BG11 media with constant illumination until cultures started to turn green and were exponentially growing. Cell harvesting and processing was described in section 2.8.4 with aid of the Electron Microscopy Research Services (EMRS). Rod cell of *S. elongatus* wild type during division. Red arrows indicate internal carboxysomes used for carbon fixation and 3-PGA production.



During early stages of cytokinesis, it was seen that there was an asymmetric folding effect of the thylakoid membranes. This was not expected as it was thought that invaginations between cells were the driving force to move thylakoid membranes. Blue arrows indicated where invaginations were forming, simultaneous thylakoid folding indicated this event was linked to cell division and not random (fig.6.5; yellow lines). Thylakoid membranes on the cytoplasmic side always seemed to fold more prominently inwards, whether there was a signal for this folding to first occur is unknown, but it is possible a cytoplasmic protein/complex may play a role in causing thylakoid curvature. Between the two newly forming daughter cells there was a dark grey zone where it was assumed there was no chromosome, this could indicate that thylakoid membranes were invading chromosome free spaces as they seemed to protrude into the area (fig.6.5). The carboxysome internal structures were positioned away from the divisome which suggested there was a system to ensure successful segregation into daughter cells.

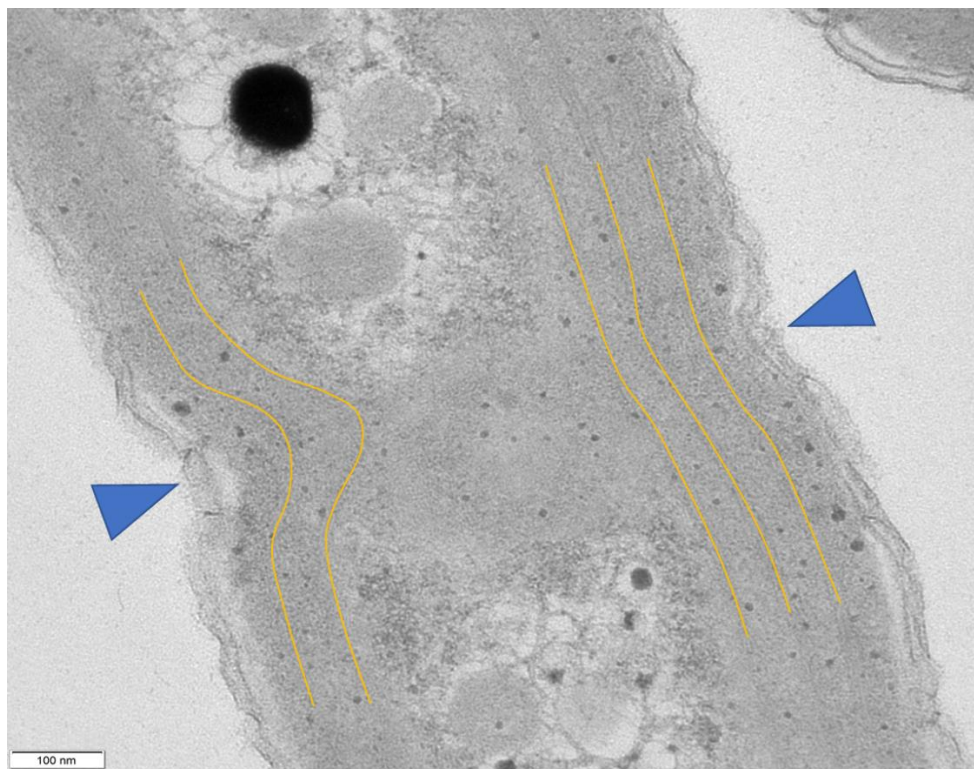


**Figure 6.5. Asymmetric thylakoid folding at the site of division.** Wild type *S. elongatus* were grown in BG11 media with constant illumination until cultures displayed green colouration and exponentially growing. Harvested

cells were fixed and processed in methods described in section 2.8.4 by EMRS. Blue arrows indicate the site of cytokinesis where there is a clear invagination forming at the mid-cell. Yellow lines trace thylakoid membranes to draw attention to the movement during constriction. Scale bars represent individual images.

Further images showed an exciting phenomenon with thylakoid folding (fig.6.6), where it was suggested that folding could be seen prior to physical division occurring as rod cells with no evident invaginations showed thylakoid membranes moving into the cytoplasm. Movement of the thylakoid membranes was prominent near the mid-cell, but not exactly where division would be expected (fig.6.6A). The white space, indicative of where the chromosome would have resided, showed that the chromosome had not yet been segregated so it is unlikely division had started. However, thylakoid movement was very obvious so it was hypothesised that some signalling may be required to initiate curvature and showed that thylakoid membranes were not simply invading chromosome free spaces. In previous figures it has been shown that the most inner cytoplasmic thylakoid membrane folded most prominently, however, in this example all membranes appear to be folding homogenously (fig.6.6B). Overall, thylakoid folding seems to occur before the site of division is obvious which led to more questions about how division in this organism was initiated.

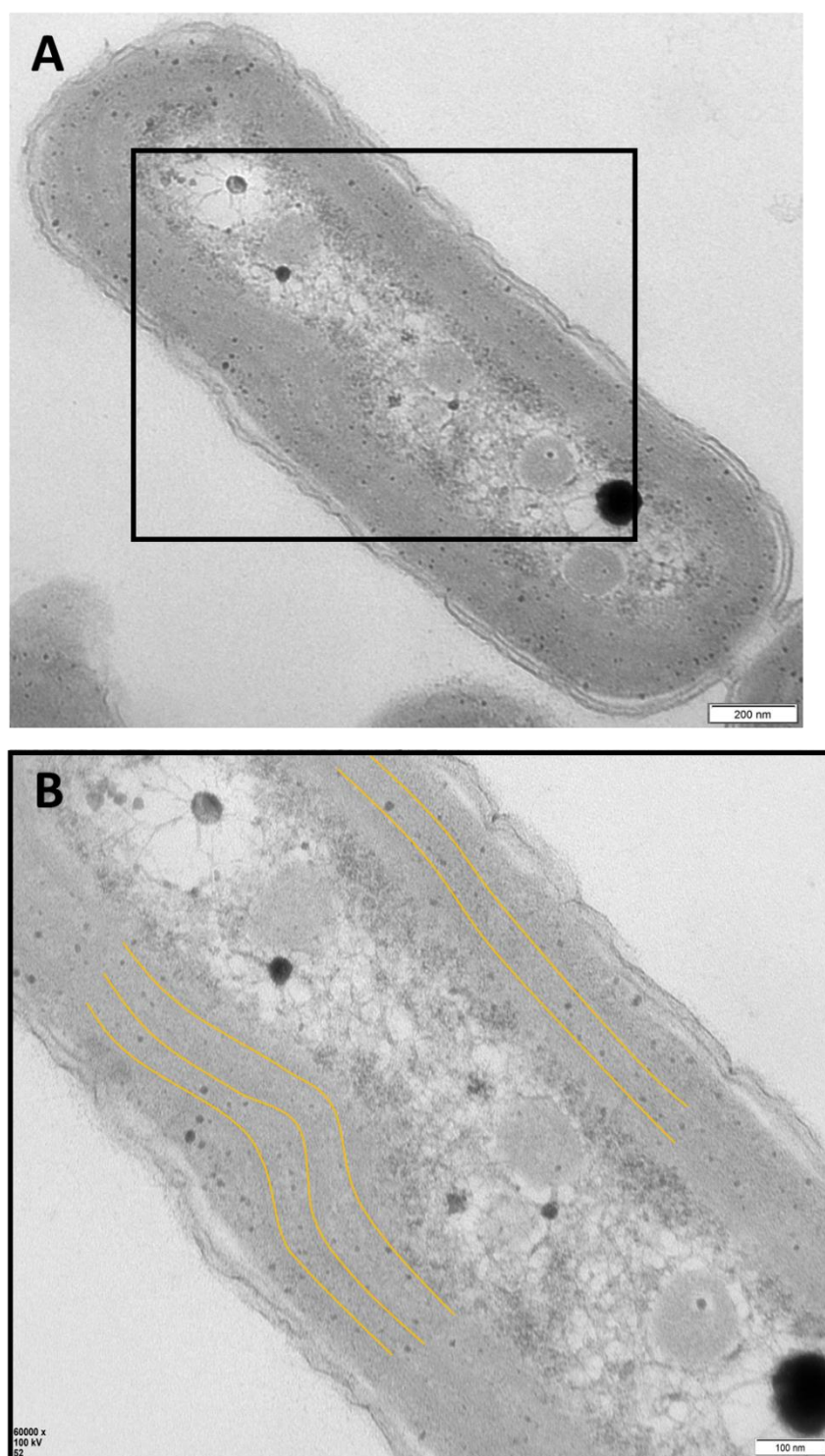


**Figure 6.6. Wild type asymmetric septum before invagination.** Wild type *S. elongatus* were grown in BG11 media with constant illumination until cultures started to turn green and samples were taken around mid-exponential growth. Cell fixation and processing was described in section 2.8.4 by Newcastle university EMRS. Yellow lines trace thylakoid membranes to draw attention to the movement during constriction. Scale bars are representative of nm in images individually.

### 6.3.2 Minicell ultrastructure

Structures of *S. elongatus*  $\Delta$ *minC* were analysed to understand the phenotype of asymmetric division, it was already known that minicells were produced but it was not known if they contained organelles. It was hypothesised that minicells did contain thylakoid membranes as epifluorescence microscopy revealed red-autofluorescence was retained. TEM analyses on  $\Delta$ *minC* strains of *S. elongatus* was a novel approach which confirmed that minicells do contain thylakoid membranes (fig.6.2A). Thylakoid membrane folding at polar division invaginations seems to be disorganised as outlined by the yellow lines (fig.6.7A). Although one minicell appeared to have continuous thylakoid membranes (fig.6.7C), there was evidence that these structures are discontinuous in most minicells. This is prominently demonstrated in fig.6.7B where there are 3 membranes on one side and 2 on the other. The formed minicell (fig.6.7C) still attached to the rod contained 2 layers of thylakoid membrane and demonstrates that the size of the minicells from this strain are a similar size to the cylindrical body of the rod, similar to *B. subtilis* and contradicts *E. coli*, which can make filtration for enrichment difficult. Thus, demonstrating that invagination at the cell poles did not always produce smaller sized minicells hypothesised in the *E. coli* model. This minicell producing strain showed that some minicells do gain carboxysomes, and in this case 2 outlined by red arrows (fig.6.7B). It is not clear how many minicells have carboxysomes, however, it could be beneficial to the lifespan of minicell expression as these structures produce sugars which may aid in longer bioproduction. The thylakoid folding was also more prominent on one side, which supported the data already shown prior where asymmetric folding occurred where division sites formed. Thylakoid membranes in the minicell demonstrated in fig.6.7C were wavy and did not appear to be as organised as they were within rod cells. There was also a larger space seen between the plasma membrane and thylakoid membrane (outlined with yellow lines), where the minicells thylakoid membrane did not sit close to the plasma membrane as it did within rod shaped cells.



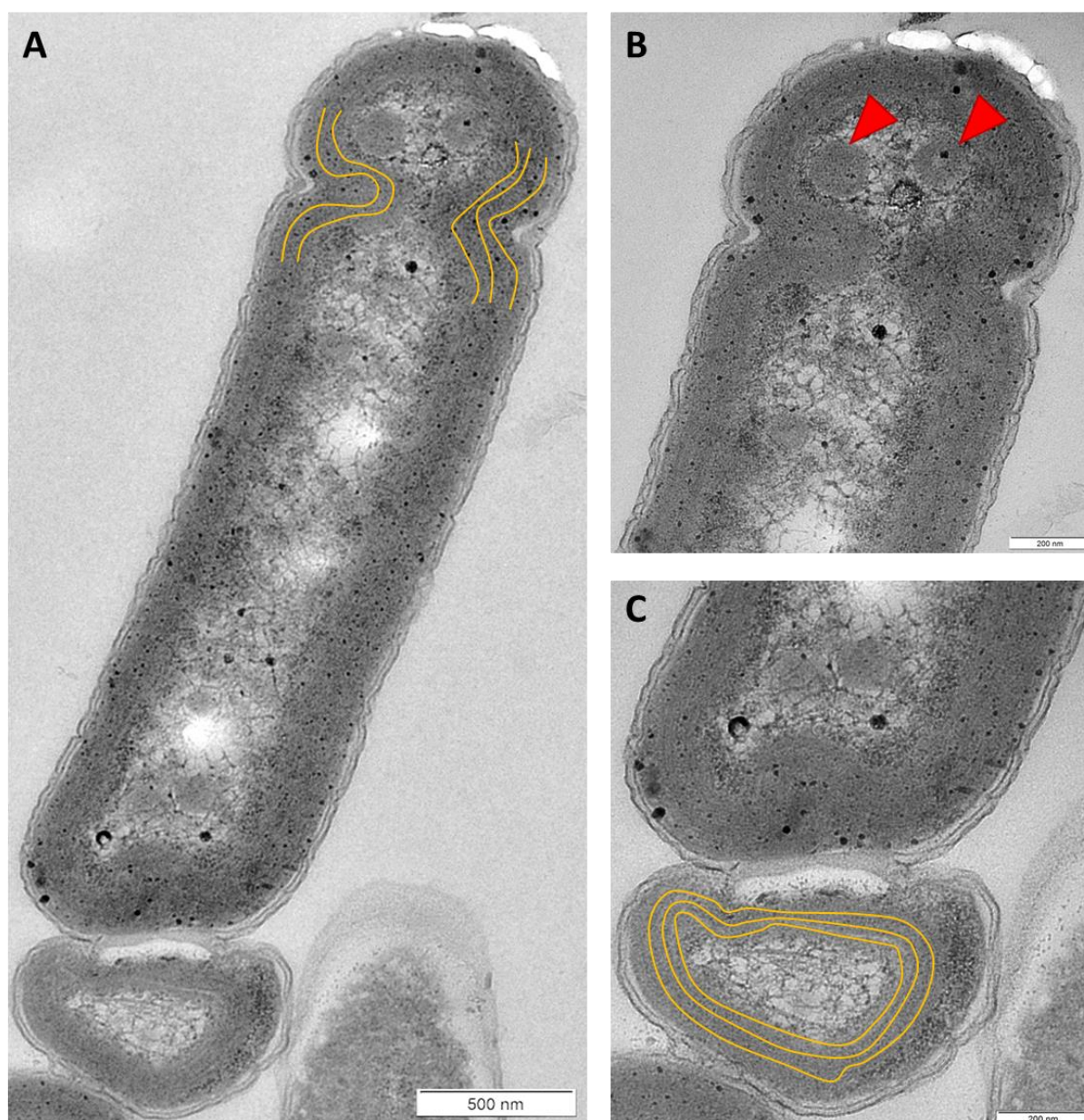


**Figure 6.7. Ultrastructures in a  $\Delta minC$  strain producing minicells.** The  $\Delta minC$  strain was grown in BG11 media at 30°C under constant illumination until cultures started to show green colouration and harvested during early exponential growth and minicells were seen using light microscopy. Fixed cells were processed for TEM in methods described in section 2.8.4. Yellow lines trace thylakoid membranes to draw attention to the movement during constriction. Red arrows draw attention to carboxysome structures. Scale bars are representative of nm in images individually.

Throughout minicell division analyses, it was found that the same asymmetric folding of the thylakoid membranes could also be observed at an early stage of division within the  $\Delta minC$  strain when the septum formed at the poles. This was interesting, as it showed that some examples of thylakoid folding were specific to division and not a random event as this never occurred at the poles of wild type cells. This provides further indications that the thylakoid membrane must receive a signal to fold at the specific site of cytokinesis whether it be at the mid-cell or the pole. Cell membrane invaginations were formed at both sides of the rod but were clearly asymmetric with respect to the thylakoid membrane. Interestingly the grey area surrounding the division site seen in wild type (fig.6.5) could not be seen which was previously hypothesised to be the chromosome free gap. This may suggest that minicell formation may occur over the chromosome as it is known that multiple copies of the genome are present within the cell.

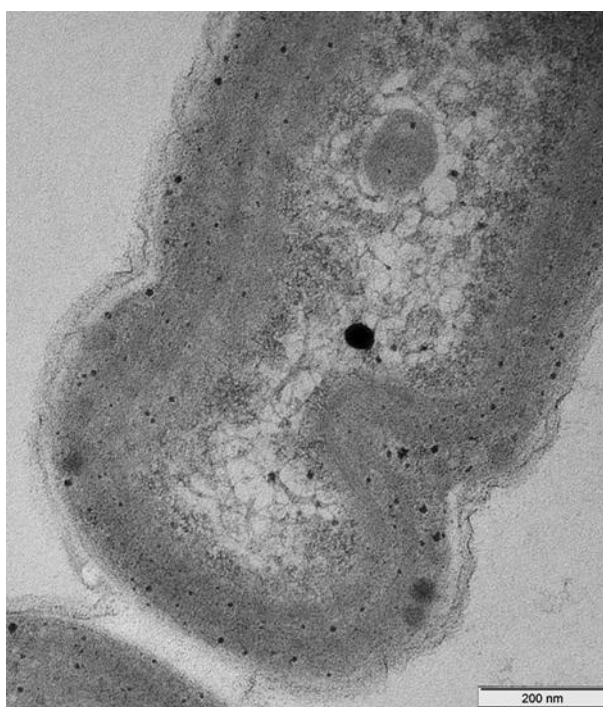


**Figure 6.8. Asymmetric thylakoid folding in a  $\Delta minC$  strain.** The  $\Delta minC$  strain was grown in BG11 media at 30°C under constant illumination until cultures were early in exponential growth and minicells were seen using light microscopy. TEM analyses were facilitated by Newcastle university EMRS, as described in section 2.8.4.

Yellow lines trace thylakoid membranes to draw attention to the movement during constriction. Scale bar is representative of 200 nm.

## 6.4 Thylakoid curvature in division

### 6.4.1 CurT localisation

CurT is a thylakoid associated protein known to have a role in arching membranes in *Synechocystis sp* PCC 6803, a coccoid cyanobacterial organism (Heinz *et al.*, 2016). As it is a known thylakoid curvature protein, it was thought that CurT may have a role in division associated thylakoid folding seen in TEM images. A strain was used from the laboratory of Prof. Luning Liu in collaboration to see how the protein localised in *S. elongatus* PCC 7942. The *curT-gfp* fusion was integrated at the native locus so the expression levels would be similar to wild type, it was confirmed that all chromosomes contained the correct fusion integration (T. Huokko, unpublished). Upon inspection using fluorescence microscopy, aggregates were seen similar to those in *Synechocystis sp* PCC 6803 (Heinz *et al.*, 2016). Aggregates were mainly associated with the poles of the cells where thylakoid membranes are negatively curved, however, some signals were found across the cylindrical body of the cell. Some aggregates were also associated with the mid-cell where division would be expected to occur, but it did not suggest that it was specific to division. Red autofluorescence from the photosynthetic apparatus showed there were some polar increases in fluorescence where CurT-GFP aggregates were associated within merged images. Overall, it seemed these aggregates were mostly found at the poles of the cells but did not seem to have any specific organisation as the structures present in cells differed broadly.

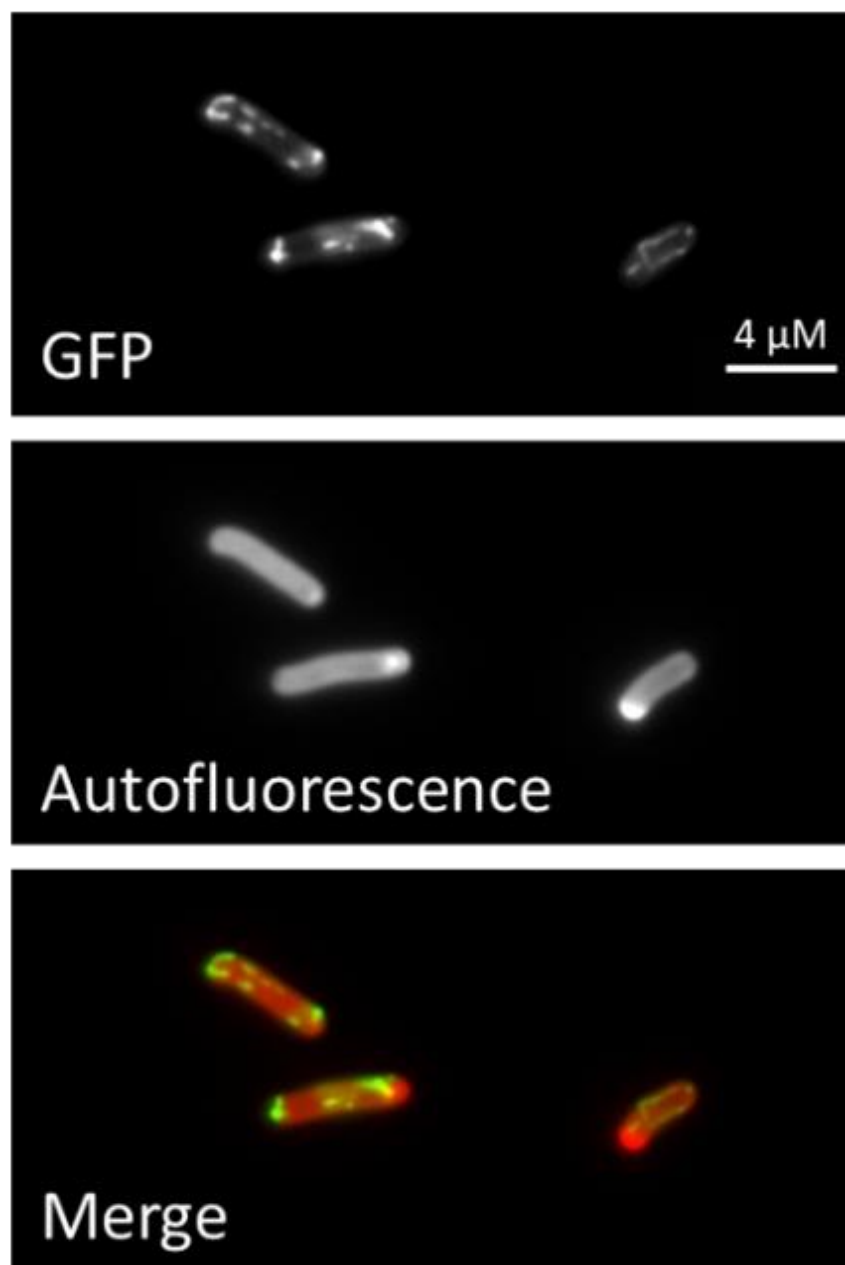


**Figure 6.9. Cyanobacterial thylakoid membrane localisation using CurT-GFP fusion.** *S. elongatus* CurT-GFP strains were incubated in BG11 media and grown at 30°C with constant illumination. Images were taken after mounting onto a 1.2% agarose pad, autofluorescence was excited using far-red filter. Images were processed and merged using ImageJ software. The scale bar is representative of 4  $\mu\text{m}$  and applicable to all images.

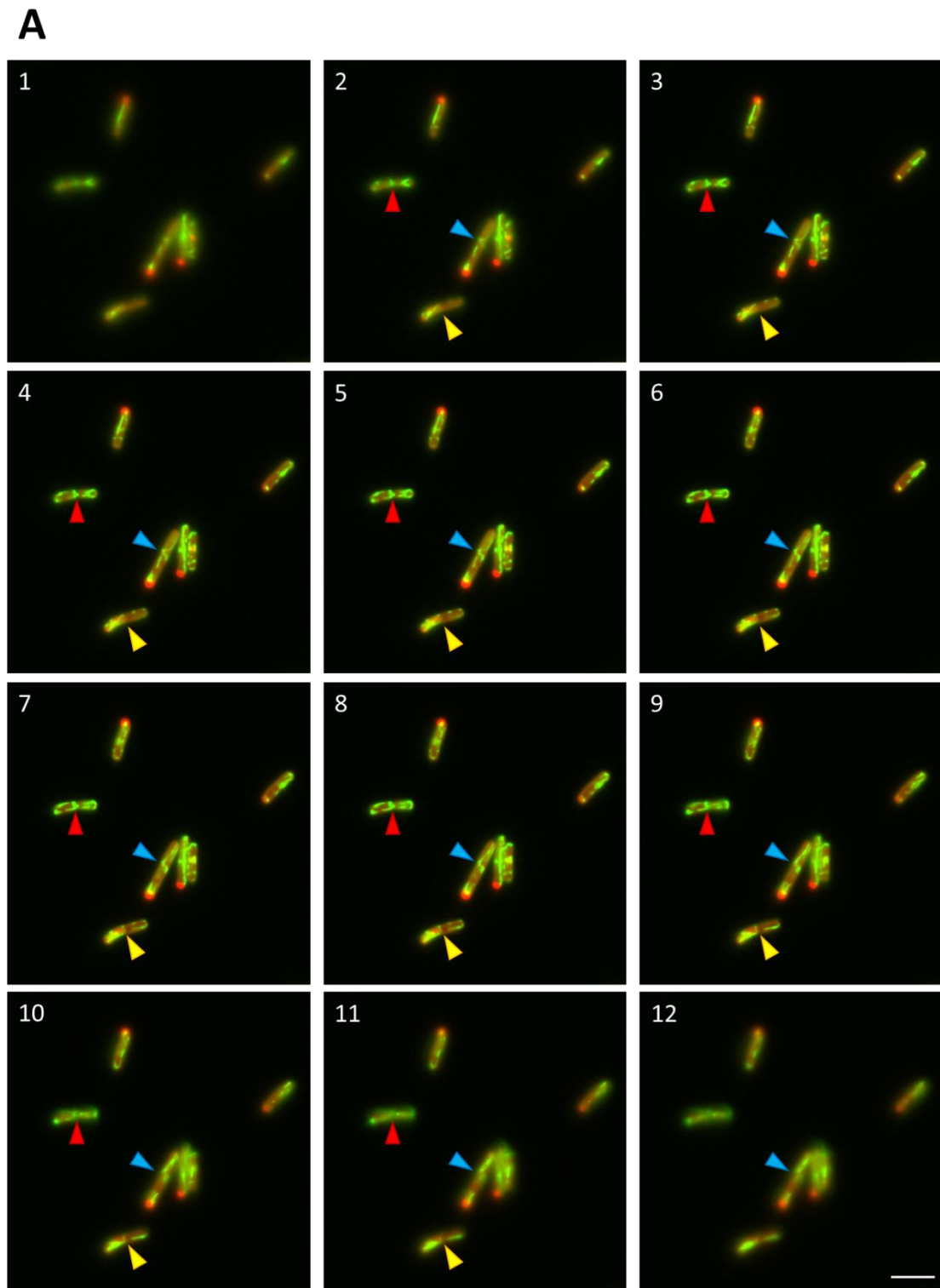


### 6.4.2 Z-stack imaging of CurT thylakoid associated protein

To see if CurT was associated with mid-cell thylakoid curvature, Z-stack imaging of CurT localisation was performed to see if there were any mid-cell associations through cells. Z-stack images were captured every 0.1  $\mu\text{m}$  to image fluorescence signals throughout all planes of the cell, images of fig.6.10 were representative of ~500 cells observed. Z-stack imaging revealed there were sections with some mid-cell localisation of CurT-GFP. There were 3 examples within the cluster of cells imaged indicated by coloured arrows where GFP signals appeared to be mid-cell and polar localised. It was clear that CurT-GFP structures remained localised in similar areas throughout the focal planes. The blue arrow draws attention to a cell where there are specific aggregated patterns across the divisome and cell poles, which may demonstrate thylakoid folding at the mid-cell. 3D modelling showed signals were more punctate across the cylindrical body, demonstrated by aggregates forming in different focal planes, such as those which appear in image 2 close to the mid-cell and no longer remain in image 9 (fig.6.10B). The cell indicated by the red arrow did show a stronger association with the mid-cell as CurT-GFP signals appeared to be strong across most image planes. This suggested there was an abundance of the fusion protein at that position. After image 10, the mid-cell fluorescence was not as intense which may indicate localisation to an asymmetrically folded thylakoid membrane (fig.6.10A). When signals were 3D modelled, the mid-cell localisation appeared to be present across all planes of the cell, which may be related to folding thylakoid membranes or invaginations pushing thylakoid membranes. Finally, the cell indicated with the yellow arrow showed a different pattern which was quite bright across the cylindrical body of the cell. There was not any specific mid-cell associated pattern to be seen within this particular cell.

When modelling the three cells, CurT-GFP aggregates were consistently associated with the cell poles and where negative curvature occurred. Although it was observed that there was mid-cell localisation, it did not seem that any aggregates were specifically associated with division

as CurT-GFP signals in the cylindrical body of the cells were heterogeneous. The different phenotypes may be related to cell cycle during growth if the population is asynchronous, as seen by Chen *et al.*, 2012 when chromosome segregation and cytokinesis seemed to occur heterogeneously.



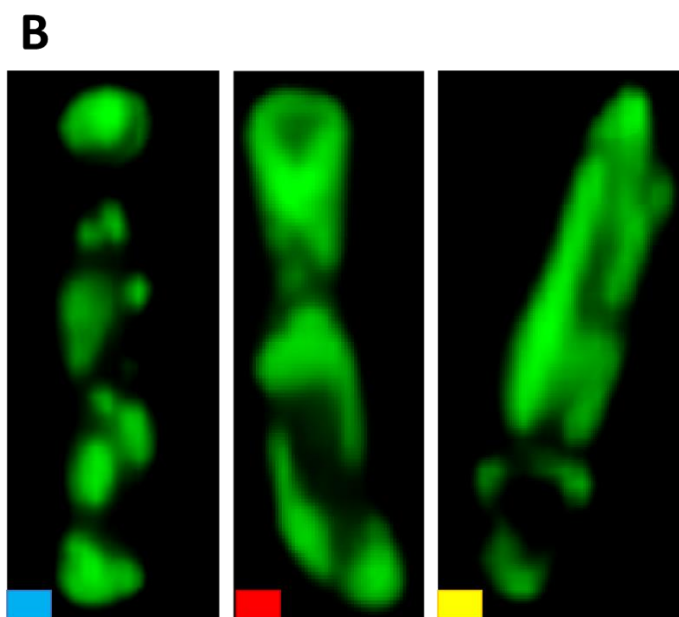


Figure 6.10. Cyanobacteria strains were incubated in BG11 media and grown at 30°C with constant illumination. Images were taken after mounting onto a 1.2% agarose pad, autofluorescence was excited using far-red filter. Z-stacks were achieved by focusing into the focal plane and the Nikon Ti with 22 0.1  $\mu\text{m}$  steps and therefore imaging 2.11  $\mu\text{m}$ . Images shows were of the central 12 steps showing 0.12  $\mu\text{m}$  through the cell. Images were processed and merged using ImageJ software. The scale bar is representative of 5  $\mu\text{m}$  and applicable to all images. B) Z-stacks were achieved by focusing into the focal plane and the Nikon Ti with 22 0.1  $\mu\text{m}$  steps and therefore imaging 2.11  $\mu\text{m}$ . 3D model determined by Z-stack imaging, model of the thylakoid membranes was assembled using ImageJ 3D viewer plugin.

### 6.4.3 CurT-GFP produce a minicell producing phenotype

While imaging strains with CurT-GFP, it was observed there was the occasional small cell comparable to the size of minicells (red arrows; fig.6.11). The minicells still harboured CurT-GFP signals, which confirmed they were from the rod cells of the strain provided by Dr T. Huokko. There were also “twin minicells” seen where 2 minicells have been formed and not separated before being released from the rod cell they were made from. These minicells were also seen attached to rod cells signifying that these are not contaminants. There was also a divisome-like aggregate seen outlined by blue arrows, this may be likely to be associated with

folding thylakoid membranes before division as hypothesised from TEM images. As GFP fusions were causing a division defect, it was hypothesised that the GFP tag was blocking movement of proteins through the thylakoid membranes. This might be related to CurT interactions with the thylakoid membrane which, when GFP tagged, blocks the Min system from working efficiently moving pole to pole and allowing FtsZ to polymerise at the poles. This may help to elucidate how proteins move across thylakoid membranes as the GFP tag clearly affected either pores or open gaps which would allow passage of proteins such as the Min system.

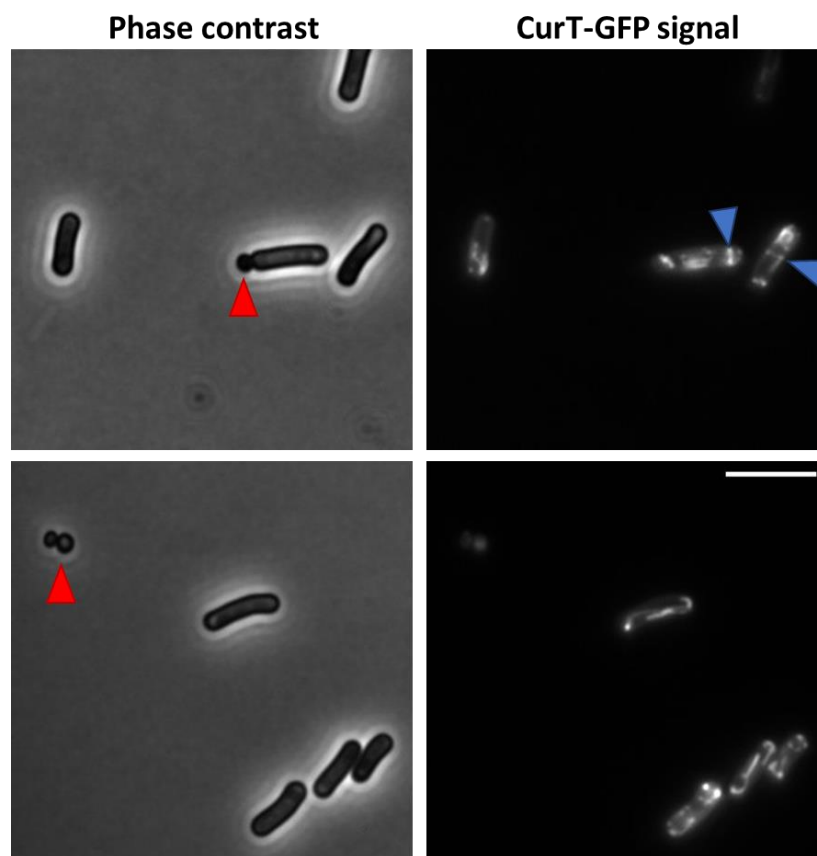


**Figure 6.11. CurT-GFP fusion causes a minicell producing phenotype.** Cyanobacteria strains were incubated in BG11 media and grown at 30°C with constant illumination. Images were taken after mounting onto a 1.2% agarose pad. Red arrows indicate minicells formed and 2 minicells attached together, the blue arrow draws attention to a mid-cell localisation. Images were processed and merged using ImageJ software. The scale bar is representative of 5  $\mu\text{m}$  and applicable to all images.

## 6.5 Chromosome localisation of a minicell producing strain

### 6.5.1 DNA distribution in Cyanobacteria

The aim of this study was to produce anucleated cells which could express from genetic circuits where expression in nucleated cells was controlled by a repressor expressed from the chromosome (see section 1.5). Therefore, as Cyanobacteria is a polyploid organism, it was questioned if the DNA partitioning system would move chromosomes away from asymmetric septa. To understand if minicells produced by polyploid organisms were anucleated, such as that seen in *B. subtilis* and *E. coli*, the nucleoid was stained with a fluorescent DAPI dye. The two strains outlined in figure 6.2 (transformant 1 and 2), where one had *minC* knockout and the other did not, were DAPI stained to see genome localisation. The wild type strain, showed that rod cells had an evenly distributed nucleoid staining within the cytoplasm of the cell. Mutant  $\Delta minC$  strains showed the presence of minicells, however, there was a heterogeneous population of minicells with (green arrows) and without (red arrows) nucleoid fluorescent staining. Quantification of DAPI fluorescence displayed that minicells fluorescence across 50 cells were mostly dimmer than that of rod cell equivalents. Therefore it was postulated that, if minicells did contain DNA, the frequency of minicells with a signal was low and concentration of DNA was not vast. Chromosome segregation in *S. elongatus* does have a spatially organised assemblance when the cell has committed to division (Chen *et al.*, 2012), however, asymmetric division may not facilitate this spatial alignment of chromosomes and may allow for constriction over the genome. As the minicell DAPI fluorescence was lower than that of rod cells, minicell staining could also be related to the two large native plasmids: 7.8 kb pANS and 46.2 kb pANL (Van der Plas *et al.*, 1992; Chen *et al.*, 2008; Chen *et al.*, 2016). These plasmids may be enough to exhibit a DNA stain if they were present at high enough concentrations within minicells.

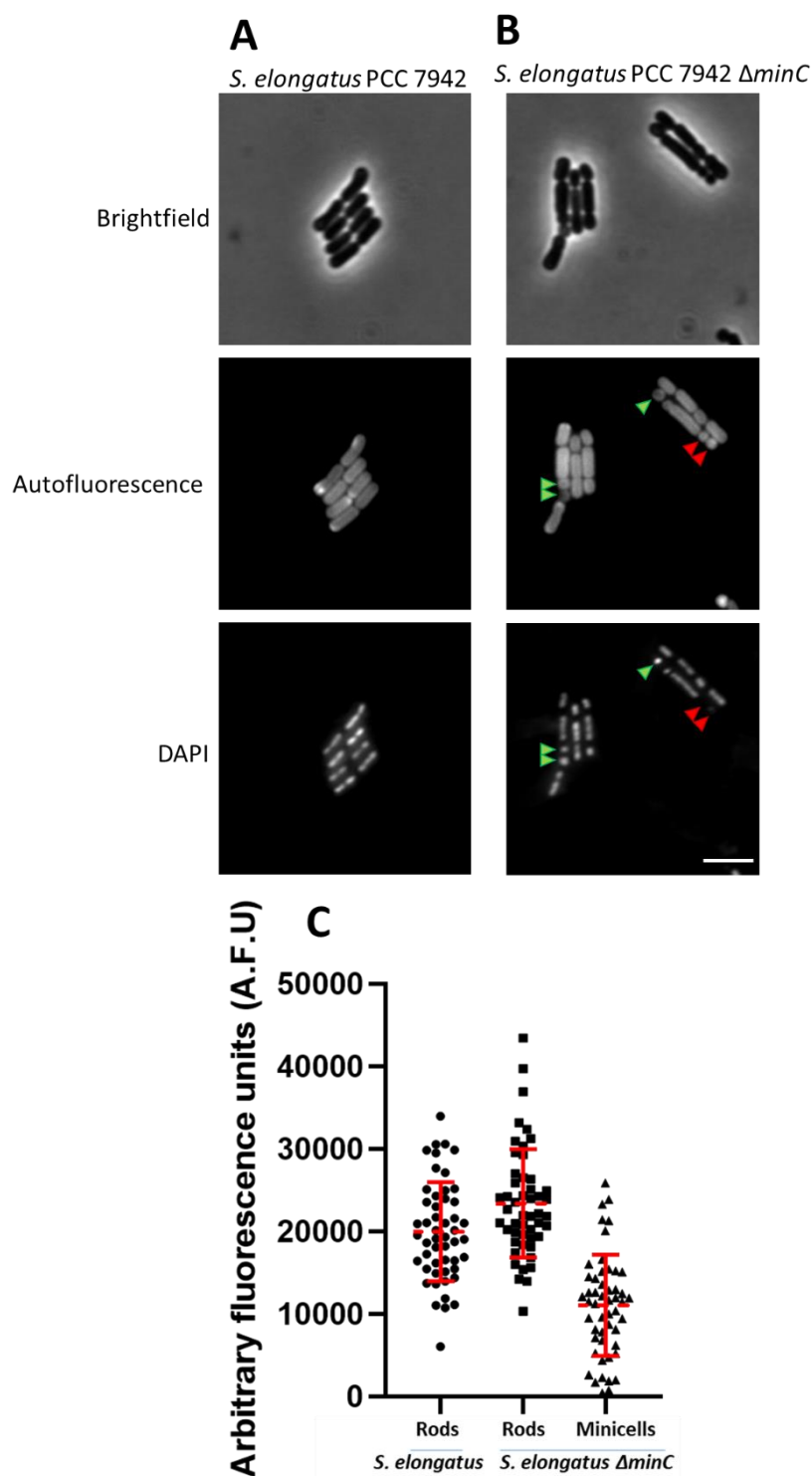


**Figure 6.12. Nucleoid localisation in wild type and minicell producing strains.** Cultures growing in BG11-media with constant illumination at 30°C were stained using 1  $\mu\text{g/ml}$  DAPI, using the far-red autofluorescence as a membrane marker. **A)** Strain without minicell phenotype. **B)** Minicells observed with nucleoid signal (green arrows) and some which had no DAPI signal (red arrows). **C)** fluorescence quantification of DAPI staining in rod cells and minicells.  $n=50$  cells per population (reproducible across 2 biological replicates). Red dashed line

indicated the mean of the data points, whereas vertical lines represented standard deviations. Images were made with Fiji, scale bar is representative of 4  $\mu\text{m}$  and is applicable to all images.

### 6.5.2 Chromosome origin localisation

Localisation of the chromosomes were carried out to determine if DNA signals from minicells were chromosomal. Fluorescence *in situ* hybridisation (FISH) was used in accordance with methods described by Watanabe *et al.*, 2018. Approximately 15 kb of either the origin or terminus were localised using fluorescein labelled probes. For each area, three PCR products of ~5 kb were amplified before denaturing and using a random primed DNA kit to integrate fluorescein labelled UTP. It was clear from microscopy analyses that FISH probes did not seem to enter fixed cells and therefore could not hybridise to their chromosomal target. Staining was mostly found around the cell wall/thylakoid membrane area of the cells and as there was a lack of inner cellular signals it was suggested that cells had not been permeabilised enough (fig.6.13A & B). There was similar staining on minicells where the probe was only able to show cell wall associated fluorescence. To ensure that the DNA probes were fluorescently labelled correctly, they were applied to a 0.8% agarose gel and separated via electrophoresis. The DNA products were detected for fluorescent nucleotide integration via 488 nm laser excitation, this revealed that there were lots of small products present which may also indicate that the probes were heavily degraded (fig.6.13C).

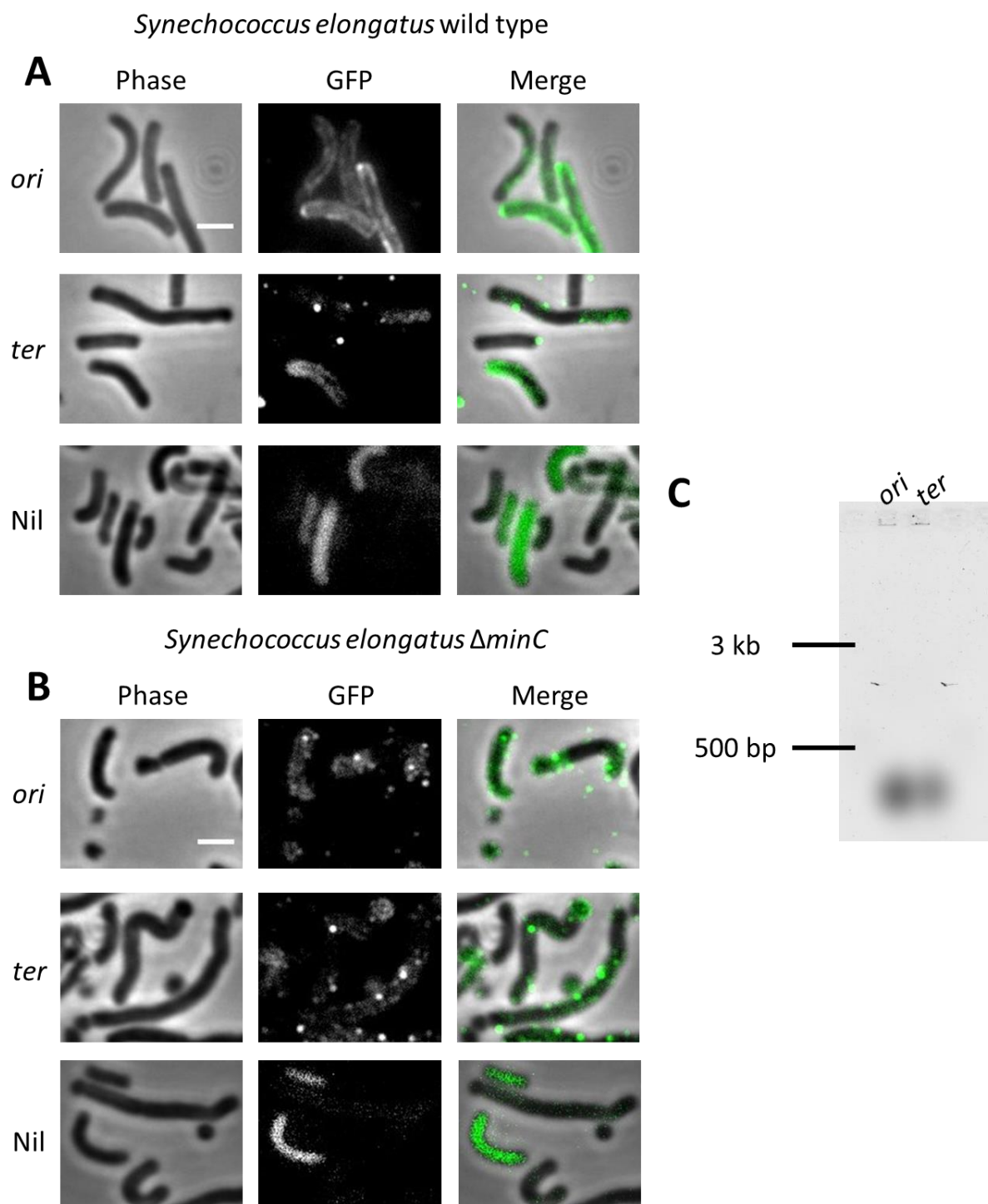


**Figure 6.13. Localisation of chromosomes in minicell producing *S. elongatus*.** *Synechococcus elongatus* was cultured for 1 week with constant illumination prior to back dilution to an OD<sub>750 nm</sub> of 0.1 24 hours prior to fixation. Cell pellets were fixed in paraformaldehyde solutions and applied to poly-L-lysine treated multi-well slides. Probes were labelled via random labelling kit with fluorescently labelled Fluorescein-12-UTP before cleaning via ethanol precipitation. **A)** wild type *S. elongatus* FISH **B)**  $\Delta minC$  variant FISH **C)** 0.8% agarose gel of fluorescently labelled probe excited at 488 nm emission.



## 6.6 Portabolomics chassis construction

### 6.6.1 Integration of a chromosome bound repressor

As a strain had been constructed which could make minicells, the next step was to integrate the LacI repressor to try and replicate the Portabolomics chassis demonstrated in *B. subtilis* (chapter 3) and *E. coli* (chapter 5). A plasmid was constructed using a published cloning vector provided by Prof. S. Golden on addgene (#40239), the LacI gene was placed under a promoter known to work constitutively in *S. elongatus*. A chloramphenicol antibiotic resistance cassette was integrated into the same site for selection of transformants and passage to select for those with a knockout in all multi-copy chromosomes. The genes were to be transformed into neutral site II which was close to the chromosomal origin of replication (*oriC*). Unfortunately, attempts made to transform this into *S. elongatus* were not successful and this aspect of the project had to be stopped due to time limits.

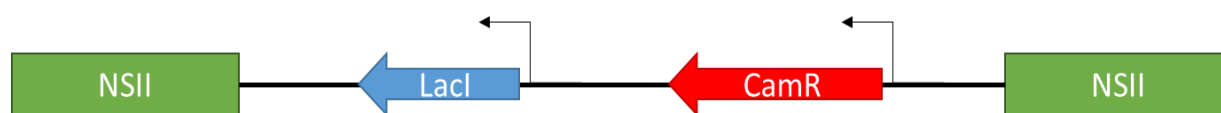


**Figure 6.14. Integrating the LacI repressor for the Portabolomics model.** Demonstration of the vector constructed to express the LacI repressor for the vector. The gene was cloned into a vector for neutral site II (NSII) integration in an integration vector originating from pAM1573 gifted by S. Golden on add gene (#40239). The chloramphenicol cassette was also integrated in order to select for transformants and passage for selection of knockout in all chromosomes.

### 6.6.2 Fluorescence analyses of the minicell producing strain with plasmid expression

Previous chapters demonstrated the use of flow cytometry to analyse cell populations and fluorescence intensity (Chapters 3 and 5). To try and replicate this, the CurT-GFP strain was utilised for its fluorescence to understand if GFP was a suitable reporter. Wild type strains with no fluorescent protein expression showed that there were two populations evident, one which showed high excitation to both GFP filters and one which was only reactive to the 488 nm 530/30 filter. This displayed that there was some native green fluorescence within *S. elongatus* (fig.6.15A). In the strain with CurT-GFP expression, the population of cells excited by 488 nm did increase (fig.6.15C). However, as there was some native GFP expression it was hard to distinguish which cells were GFP positive and which were natively fluorescent. Although GFP was expressed in all cells from *curT-gfp* fusions, there were still two populations of cells with varying fluorescence also described by the wild type control (fig.6.15A). As there were two populations it could be hypothesised to be a result of asynchronous growth. Regardless of this, if flow cytometry techniques were to be used for this strain, as seen with *E. coli*, a green fluorescent protein would not be a good reporter because of native green fluorescence. If GFP were to be used as a reporter, the number of minicells independently expressing from the plasmids would not be a true reflection because of native green fluorescence. After trying different filters, it was found that cyanobacteria are negative for blue fluorescent excitation, suggesting that a blue fluorophore would be best suited for analysis using flow cytometry (fig.6.15B & D). Both strains did show some outliers with blue fluorescent excitation, however, this was a much lower level than seen with green fluorescent excitation in the wild type control strain.

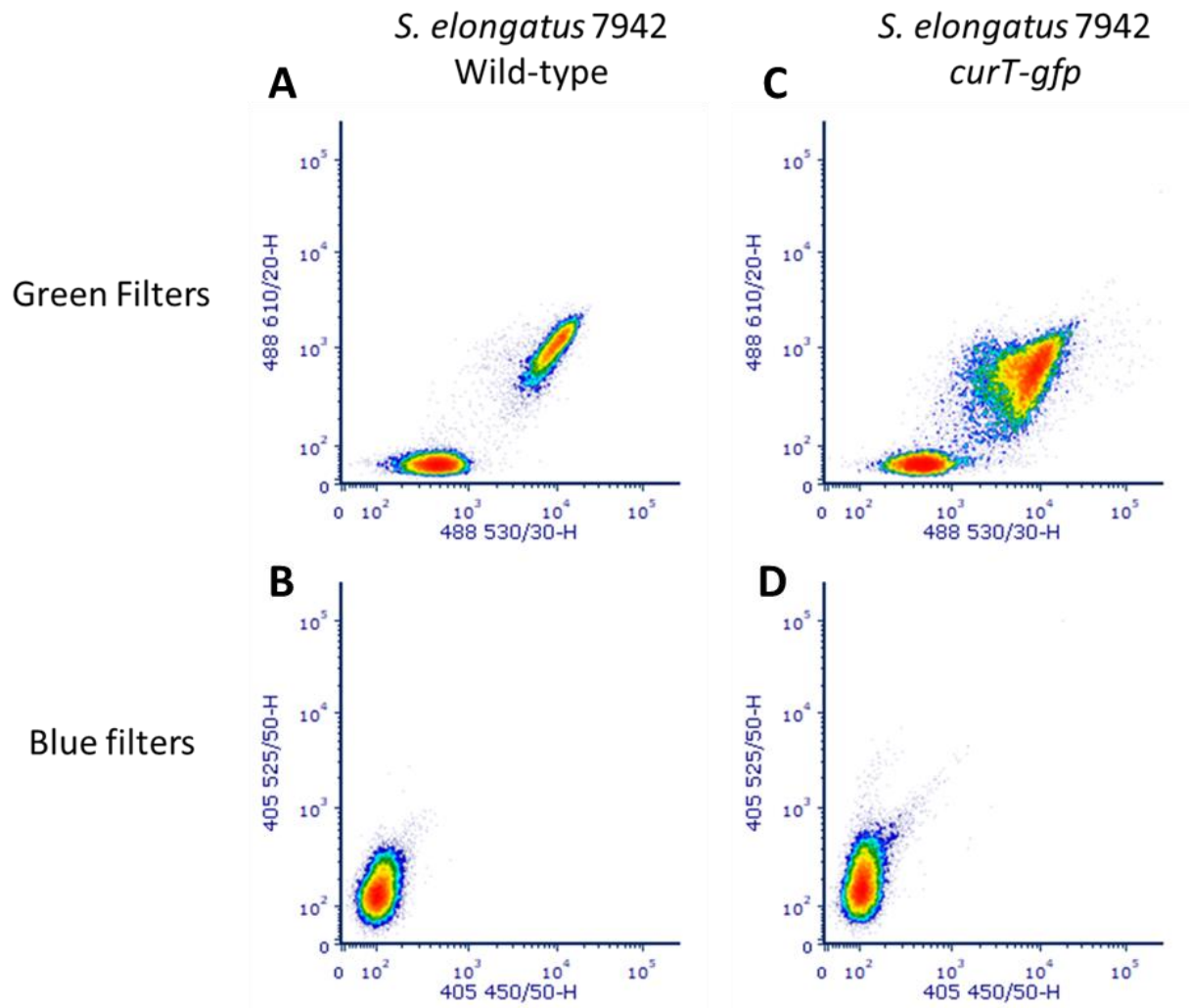


**Figure 6.15. Flow cytometry analyses of Cyanobacteria fluorescence.** Cyanobacteria strains were grown in BG11 media at 30°C under constant illumination. For flow cytometry analysis, 25 µl cells were mixed with filter sterile PBS sheath fluid before running through the chamber within different laser emissions for fluorophore excitation. 30,000 events were recorded per strain. **A)** Wild type *S. elongatus* exposed to green fluorescence excitation filters **B)** Wild type *S. elongatus* exposed to blue fluorescence excitation filters **C)** *S. elongatus curT-gfp* exposed to green fluorescence excitation filters **D)** *S. elongatus curT-gfp* exposed to blue fluorescence excitation filters. All populations were analysed using FCS express 6.

## 6.7 Discussion and conclusions

It was demonstrated in this chapter that *S. elongatus* minicell producing strains were able to be produced using a custom-made vector to disrupt the native *minC* gene, similar to works described by MacCready *et al.*, 2016. This minicell producing mutant was examined in further detail relating to ultrastructure, fluorescence and DNA localisation. As previously described, *S. elongatus* contains 2-8 chromosomes per cell, in this study the *minC* gene was knocked out of all chromosomes successfully by antibiotic passage and was confirmed via colony PCR.

### 6.7.1 Microscopy analyses of minicell producing strains

Microscopy studies exhibited the well-known red-autofluorescence produced by thylakoid membranes, commonly in chlorophyll, as carotenoids absorb light leading to emission of red fluorescence (Buschmann, 2007). This red autofluorescence was apparent within minicells and a clear indication that minicells of this species contain thylakoid membranes. The main role of thylakoid membranes are to house the photosynthetic apparatus which utilises light to aid in electron extraction from water, which is used within the electron transport chain to make energy (Hohmann-Marriott and Blankenship, 2011). As a result, cyanobacterial cells gain NADPH and ATP through the electron transport chain which are used to make carbohydrates (Rast *et al.*, 2015). As these membranes are within minicells it would be interesting to know if they are functional and how long for because this could be useful for industrial usage. It was suggested in the literature that there is a high turnover of photosystem II (PSII) as a result of D1 subunit degradation, however this was observed in *Synechocystis* PCC 6803 when fatty acids were restricted (Kanervo *et al.*, 1995). If minicells of *S. elongatus* are anucleated, then this could indicate that the photosynthetic apparatus may not function for a long period of time, potentially this could be evaluated if minicells could be isolated from the culture.

### 6.7.2 Transmission electron microscopy analyses

Structural analyses of Cyanobacterial strains showed there was a significant difference to *E. coli* minicell producing strains within this study (see section 5.2.3). TEM ultrastructural analyses confirmed the presence of thylakoid membranes in minicells and gave a better insight into division site placement. The images showed that the thylakoid membranes occupied a lot of space within cells as they encased the cytoplasm (see section 6.3). New thylakoid membranes are often inserted closest to the plasma membrane, pushing older membranes closer into the cell (Huokko *et al.*, 2021). Thylakoid membranes play the primary role in energy production using light as it houses the photosynthetic apparatus, therefore, it is understandable that the structures occupy most of the cellular space.

It was interesting in *S. elongatus* that the thylakoid membranes seemed to protrude into the cytoplasmic space at sites of wild type or polar  $\Delta minC$  asymmetric divisions (section 6.3.2). What causes this folding of thylakoid membranes is unknown, however, it could be hypothesised that there was an interaction with thylakoid membranes and the division complex in order to allow movement of membranes before invaginations start to form. It was known by cryoelectron tomography (cryo-EM) studies that the thylakoid membrane contacts the plasma membrane via convergence regions which connect discontinuous thylakoid structures, this could be a point of interest for thylakoid movement related to cytokinesis (Rast *et al.*, 2019; Huokko *et al.*, 2021). However, convergence regions do not seem to be found as obviously in *Synechococcus elongatus* as they are within *Synechocystis* PCC 6803 (Rast *et al.*, 2019). Future work to understand this mechanism would be interesting as it could be utilised to make more minicells or increase division times for faster growth of *S. elongatus*.

### 6.7.3 CurT interactions with the thylakoid membranes

The CurT-GFP strain was visualised for a collaboration effort with Dr T. Huokko and Prof. Lu-Ning Liu. CurT-GFP patterns were like those seen in previous studies of *Synechocystis* PCC 6803 with mostly inner membrane-like associations, where some CurT signals crossed into the cytoplasm (Heinz *et al.*, 2016). As CurT is a known thylakoid curvature protein, GFP localisation was analysed to see if there was any association with division which could explain how asymmetric thylakoid folding was observed in TEM experiments. However, the localisation of the protein did not seem to be very specific, although there was some division localisation outlined most signals appeared to be polar. 3D reconstruction imaging did help to define localisation across all planes of the cell but also did not show division specific localisations (see section 6.4). CurT localisation was not consistent between cells, with the patterns observed differing significantly. Thus, the idea that it may be located at the mid-cell at any specific stage in the cell cycle is questionable or potentially CurT distribution is random. However, from this work (see section 7.4.3) it was suggested that there was an accumulation of CurT-GFP signal at the poles of wild type cells, therefore, it was postulated that minicell formation was a result of blocked thylakoid permeabilisation. This was supported by work presented by MacCready *et al.*, 2016 where modelling showed that the thylakoid membrane must be >12% permeability to allow MinCD oscillations to occur efficiently. Therefore, the functionality of CurT may be improved by the fusion of GFP, leading to reduced permeability of the thylakoids. This may then result in division occurring at polar positions, consequently making minicells.

### 6.7.4 DNA & carboxysomes positioning within minicells

Characterisation of the DNA distribution in the  $\Delta minC$  strain showed some minicells appeared to have a nucleoid signal, this indicated that minicells could hold chromosomal or plasmid DNA as well as potentially being artefacts. Fluorescence intensity analyses of DAPI staining

did show that most minicells were dimmer than rod cells, this either indicated that staining was artefactual or that minicells gaining a chromosome was infrequent (see section 6.5.1). As mentioned within the introduction, there are chromosome segregation pathways identified in *S. elongatus* such as ParA-like proteins (Watanabe *et al.*, 2018). Though, it is not known how this system functions with multiple genomes and when asymmetric division occurs. It was also hypothesised by this study that the minicell asymmetric septa could divide over chromosomes. Experiments to confirm if DNA staining was chromosomal were not successful as FISH experiments did not seem to penetrate the cell wall efficiently. Therefore, this study did not determine if chromosomal signals were present in minicells. However, using knowledge gained from TEM images it has been hypothesised that DNA found in minicells was chromosomal based on carboxysome positioning.

Previous studies into carboxysome placement in *S. elongatus* PCC 7942 showed that positioning was not a random event and that partitioning proteins were involved in ensuring daughter cells received equal quantities (Savage *et al.*, 2010). The ParA-like protein was found to be directly involved in carboxysome placement and segregation, however, the filament formation hypotheses was later disproved and a different mechanism was established comprising of self-organising proteins utilising nucleoid positioning (Savage *et al.*, 2010; MacCready *et al.*, 2018). The McdAB complexes were established to be carboxysome specific positioning complexes, McdB interacts directly with carboxysome outer shell proteins in order to aid in positioning (MacCready *et al.*, 2018). McdB drives McdA oscillations by disrupting its attachment to the nucleoid via ATPase stimulation, McdA moves from pole-pole rapidly and carboxysomes were found to follow high protein gradients of oscillating McdA aiding in equal placement in daughter cells (MacCready *et al.*, 2018). Therefore, as McdA is associated with the chromosome, it was hypothesised that the carboxysomes found within minicells were nucleoid associated. These McdAB systems are known to be found widespread within the  $\beta$ -

cyanobacteria family, further studies of the protein complex also found that it was able to work upon various carboxysomes constructed in different ways (MacCready *et al.*, 2020; MacCready *et al.*, 2021).

Future work to successfully identify if minicell compartments contained chromosomal DNA would be desirable, with *in vivo* origin labelling shown by Chen *et al.*, 2012 a promising method. The authors showed that chromosomes aligned spatially across the rod cell by using GFP tagged LacI which bound to the *lacO* operator sequence at neutral site I (Chen *et al.*, 2012). This method could also be beneficial as multiple chromosome locations could be tracked in living cells, this would aid in visualising if minicells have chromosomal DNA and how frequently the event occurs, as current data suggests not all minicells display DAPI staining.

#### **6.7.5 Portabolomics chassis work**

To make *S. elongatus* into a Portabolomics minicell producing chassis equal to that of *B. subtilis* and *E. coli*, the LacI repression system was required to be integrated into the chromosome. Strains from other studies were requested as it was published that the LacI system was operational within *S. elongatus*, however, strains were not obtained. When the system was attempted to be constructed within this study, the LacI repressor was not able to be transformed into *S. elongatus* (indicated section 6.6.1). It was demonstrated by Camsund *et al.*, 2014 that the LacI repressor, although functional, was leaky when coupled with the  $P_{trc}$  promoter on plasmids. To combat this the authors utilised a DNA looping mechanism in order to increase repression as the authors believed *Synechocystis* RNAP was sensitive to looping (Camsund *et al.*, 2014). DNA was looped by increasing the gaps between the *lacO* operator sequences to 11 bp apart which were found to repress best ( $P_{trc}$  2O) (Bond *et al.*, 2010; Camsund *et al.*, 2014). The use of the LacI repressor has also been demonstrated in synthetic biology approaches such as Niederholtmeyer *et al.*, which used the LacI repressor and IPTG induction for expression of hydrophilic products (Niederholtmeyer *et al.*, 2010). As a result of common leaky expression,



there are other promoters available for *S. elongatus* optimised specifically to tightly regulate expression based upon the araBAD system with  $P_{ara}$  promoter and *araC* repressor. Cao *et al.*, 2017, who determined the toolkit in Cyanobacteria, describe that the araBAD mechanism in plasmids creates a DNA loop and, when induced, it was found that there was greater biomass expressed. Whereas, encouragingly, homogenous expression and metabolism was not hindered (Cao *et al.*, 2017).

It was clear by flow cytometry that alterations would be required to analyse a Cyanobacteria chassis as there was endogenous green fluorescence at an excitation of 488 nm. With CurT-GFP expression there was a difference in the population's fluorescence, however, this was not greatly distinguishable from the wild type control (section 6.6.2). Therefore, the reporter would be required to be a blue fluorescent protein for fluorescence analyses as it was shown in Figure 6.15 that there was no significant shifts in fluorescence wild type and CurT-GFP expressing strains. Cyan fluorescent protein (CFP) and mTurquoise are often used for tagging and localisation of proteins within cyanobacteria such as Heinz *et al.*, 2016 with CurT labelling. Examples of expression to remain in the cytosol are shown in the literature such as work by Cao *et al.*, 2017 with GFP expression, however, similar experiments did not seem to be found using blue fluorescent proteins.

### **6.7.6 Conclusions**

Conclusions drawn from this chapter are that the minicell producing strain of *S. elongatus* can be made but more work was required before it could be used as a Portabolomics chassis. To achieve this, further work would be required to introduce appropriate modules for plasmid repression in nucleated cells. Work within this part of the study did not progress rapidly because of the slow doubling times of *S. elongatus*. Overall, this study did answer one question postulated to distinguish if organelles were present in minicells. TEM revealed the ultrastructures of minicells, showing some contained carboxysomes and thylakoid membrane

structures that were postulated to be beneficial to expression longevity. However, there was also evidence to suggest some minicells were not anucleated, requiring further study to understand this. Although it was discussed that, because of carboxysome positioning relying on a nucleoid-associated protein, it was likely that some minicells did possess a chromosome.

## Chapter 7 – Concluding remarks and future directions

The work presented within this thesis has explored a number of intricacies within synthetic biology such as, characterising anucleate producing chassis' in different host organisms and genetic circuit optimisation. The purpose of this study was to produce strains that could compartmentalise genetic circuits into anucleate cells. Minicell producing strains of *B. subtilis*, *E. coli* and *S. elongatus* were all characterised. It was found that *E. coli* minicells were smaller than the cylindrical body of rod cells, whereas, *B. subtilis* and *S. elongatus* showed minicells diameter was a similar size to the rod cells. Minicells of *S. elongatus* also had a specialised morphology as there were organelle-like structures present such as thylakoid membranes and carboxysomes. It was hypothesised that these structures could be beneficial as both processes could provide continual energy for minicell expression. However, without the chromosome, it was unclear how long these systems would work without replacement of complex components (as discussed in chapter 6).

The minicell model depicted in the introduction (see section 1.5) was implemented within both *B. subtilis* and *E. coli*, where it was confirmed that minicells were able to independently express the GFP reporter from the plasmid. In *B. subtilis*, it was shown that up to 50% of the minicell population were expressing GFP from the plasmid and the nucleated cells were repressed very well with a small proportion (3%) counted as GFP positive (section 3.2.1). However, the equivalent model in *E. coli* demonstrated that, with an RSF1010 driven plasmid, there was only 35% of minicells which were GFP positive. The true number of minicells expressing from the plasmid was thought to be masked by high expression in nucleated cells. To make the models in *B. subtilis* and *E. coli* comparable, efforts were made to reduce nucleated cell repression using small RNA's but unfortunately did not have any affect.

Going forward, as discussed in section 5.7, the repression in *E. coli* needs to be improved to eliminate expression in nucleated cells, this could be done by exploring different promoters and repression systems. Advancing from the original Portabolomics aims, regulation in each strain may be better if it were customised for each species as opposed to using a one-size fits all approach. Potentially, the *lac* system may have been more efficient in *B. subtilis* as it is heterologous, whereas the native *lac* system in *E. coli* was more likely to fail. In *E. coli*, the *lac* promoter was known to have leaky expression, mainly with high copy number plasmids, which clearly effected how the model system worked. Alternative hybrid/synthetic promoters could be used to increase repression, similar to what was attempted with the universal replicon collaboration in appendix 10 (Rosano and Ceccarelli, 2014).

Although the maxicell model (section 1.5) was only constructed within a *B. subtilis* background, it was found to be a very efficient chassis and was able to express 7.4-fold greater than minicells in  $\beta$ -galactosidase activity experiments (see section 3.3.5). The amount of maxicells expressing GFP remained similar to that of minicells (~50%), therefore, it was hypothesised that these larger compartments were better equipped to express greater yields. Maxicells responded better to the changes in plasmid copy number (chapter 4) compared to minicells as there was good nucleated mother cell repression and an increase in maxicells expressing GFP. In fact, when the copy number increased 3-fold compared to the native pLS20 replicon, while 9% of nucleated cells were GFP positive, the maxicell population increased to 70% expressing GFP (see section 4.4.2). As this chassis demonstrated great potential for expression and repression, future directions would be to transfer this system to *E. coli*. Maxicells have been described in *E. coli* via various mutations that make anucleate cells, such as chromosome condensing mutations or *oriC* mutations (Mäkelä *et al.*, 2021; Niki *et al.*, 2000, respectively).

In *B. subtilis* minicells, maxicells and *E. coli* minicells it was demonstrated that all three could express heterologous protein A and showed evidence of secreting the protein into the cell wall/periplasmic space. More work is required for this section of the study, but it was a promising result as it could indicate that minicells and maxicells have an application for expression of industrially relevant proteins. The ability to decorate the outside of the cell is important because, as mentioned in Chapter 1, the use of minicells for vaccination purposes has become particularly encouraging, especially since it has been demonstrated that minicells can express, secrete and insert type III secretion needles (Carleton *et al.*, 2013).

One of the main aims of the Portabolomics project was to have genetic circuit portability throughout microbial bioreactors. Two attempts were made within this study which did not show success, one being a collaborative effort with Dr Alex Johns using the universal replicon between *B. subtilis* and *E. coli*. It was demonstrated by Dr Alex Johns that the plasmid copy number was ~10 copies per genome, similar to RSF1010 used in this work, but it was clear that the universal replicon was not regulated as tightly as GFP expression in *E. coli* rod cells was much greater. Although, it was confirmed by time-lapse microscopy that some minicells did gain a plasmid and independently expressed GFP, this was a very infrequent event. Overall, it was shown that plasmid compartmentalisation into *E. coli* minicells was inefficient (see appendix 10). It was hypothesised that regulation issues were because the plasmid was diluting out the LacI repressor through single stranded intermediates, produced because of rolling circle replication, though it was already known that expression was leaky in *E. coli* (see section 1.4.1). As it was known pLS20 and RSF1010 replicons worked in *B. subtilis* and *E. coli* respectively, pGGR01 first designed for *E. coli* chassis was tested within *B. subtilis*. It was demonstrated that this system did not repress well, when RSF1010 was present on the plasmid there was a significant increase in fluorescence intensity for both nucleated cells and minicells (see section 5.6). This indicated that the RSF1010 replicon was having an effect on plasmid replication in

*B. subtilis*, furthermore qPCR data revealed a significant increase in copy number. It was hypothesised that the mobility proteins could be functional in *B. subtilis* and causing an accumulation of single stranded DNA (see section 5.7.4). This requires more work in order to achieve portability goals, firstly by understanding why RSF1010 had an effect on plasmid copy number, GFP expression and to develop a system that works in all Portabolomics model organisms.

Development of a Cyanobacterial chassis is of importance as these CO<sub>2</sub> dependent organisms were shown to be effective at producing biofuels or chemicals without negatively impacting the environment (Atsumi *et al.*, 2009). The model system, described in section 1.5, was not able to be entirely constructed within *S. elongatus* during the time of this study. However, further characterisation of minicells from *S. elongatus* showed that some minicells potentially possess chromosomes (see section 6.5). Consequently, it is important to understand this before constructing the model system as, if minicells of *S. elongatus* are nucleated, then the model would not be effective since the transcriptional repressor would be expressed from the chromosome. Overall, this component of the project has provided better characterisation of minicells from *S. elongatus* and an insight into ways to exploit *S. elongatus* genetically and established the method in the laboratory for the future. With all considered, this project has met most of the aims set out with any future work being highlighted on how to improve or achieve certain aims in all species studied.

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## Appendix 1 – media recipes

### Complex defined media (CDM) for *Bacillus subtilis*

1x Spizizen's minimal salt (1 litre):

| Chemical compound                                                                   | Weight for 1 L volume (g) | Final concentration in 1 L |
|-------------------------------------------------------------------------------------|---------------------------|----------------------------|
| Ammonium sulphate<br>( $(\text{NH}_4)_2\text{SO}_4$ )                               | 2                         | 15 mM                      |
| di-Potassium hydrogen<br>phosphate ( $\text{K}_2\text{HPO}_4$ )                     | 14                        | 80 mM                      |
| Potassium phosphate<br>monobasic ( $\text{KH}_2\text{PO}_4$ )                       | 6                         | 44 mM                      |
| Sodium citrate dihydrate<br>( $\text{Na}_3$ citrate. $2\text{H}_2\text{O}$ )        | 1                         | 3.4 mM                     |
| Magnesium sulphate<br>heptahydrate<br>( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) | 0.2                       | 811 $\mu\text{M}$          |

\*sterilised in 100 ml batches before adding further supplements to make minimal media

Minimal media recipe for *B. subtilis*:

| Supplement                    | Amount added for 100 ml | Final concentration in 100 ml |
|-------------------------------|-------------------------|-------------------------------|
| SMM salts                     | 100 ml                  | 1x                            |
| 40 % glucose                  | 1.25 ml                 | 0.5%                          |
| Tryptophan                    | 1 ml                    |                               |
| 1 M $\text{MgSO}_4$           | 600 $\mu\text{l}$       | 10 mM                         |
| 20% Casamino acids            | 100 $\mu\text{l}$       | 0.02%                         |
| 0.22% $\text{FeNH}_4$ citrate | 50 $\mu\text{l}$        | 0.00011%                      |

To make agarose pads for *B. subtilis* time lapse microscopy imaging, this recipe was made with addition of 1.2% agarose.

Starvation media used for transformations of *B. subtilis*:

| Supplement          | Amount added for 100 ml | Final concentration in 100 ml |
|---------------------|-------------------------|-------------------------------|
| SMM salts           | 100 ml                  | 1x                            |
| 40 % glucose        | 1.25 ml                 | 0.5%                          |
| 1 M $\text{MgSO}_4$ | 600 $\mu\text{l}$       | 10 mM                         |

**M9 minimal media for *Escherichia coli***

5x M9 salts stock (1 litre):

| Chemical compound                                                | Weight for 1 L volume (g) | Final concentration in 1 L |
|------------------------------------------------------------------|---------------------------|----------------------------|
| Disodium phosphate (Na <sub>2</sub> HPO <sub>4</sub> anhydrous)  | 30                        | 211 mM                     |
| Potassium phosphate monobasic (KH <sub>2</sub> PO <sub>4</sub> ) | 15                        | 110 mM                     |
| Sodium chloride (NaCl)                                           | 2.5                       | 42 mM                      |
| Ammonium chloride (NH <sub>4</sub> Cl)                           | 5                         | 93 mM                      |

\*Autoclaved on a sensitive cycle before used to make media.

| Ingredient                      | Amounts added for 50 ml | Final concentration |
|---------------------------------|-------------------------|---------------------|
| M9 5x salts                     | 10 ml                   | 1x                  |
| 40% Glucose                     | 500 µl                  | 0.4%                |
| 1M MgSO <sub>4</sub>            | 100 µl                  | 2 mM                |
| 20% CAA casamino acids          | 250 µl                  | 0.1%                |
| 0.1M CaCl <sub>2</sub>          | 50 µl                   | 0.1 mM              |
| 0.22% FeNH <sub>4</sub> citrate | 5 µl                    | 0.00002%            |
| H <sub>2</sub> O (autoclaved)   | 39.1 ml                 |                     |

\*all components were autoclaved separately before mixing together into sterile water.

To make agarose pads for *E. coli* time lapse microscopy imaging, this recipe was made with addition of 1.2% agarose.

**BG11 media for *Synechococcus elongatus***

Stock solutions required to make batches of BG-11:

| Chemical compound                                                                      | Weight for 500 ml volume (g) | Final concentration in 500 ml |
|----------------------------------------------------------------------------------------|------------------------------|-------------------------------|
| Sodium carbonate (Na <sub>2</sub> CO <sub>3</sub> )                                    | 10.016                       | 189 mM                        |
| di-Potassium hydrogen phosphate (K <sub>2</sub> HPO <sub>4</sub> )                     | 15.24                        | 175 mM                        |
| Ferric Ammonium Citrate (Ammonium iron(III) citrate 18 % Fe)                           | 3                            | 6 mg/ml                       |
| Sodium Thiosulphate (Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> ·5H <sub>2</sub> O) | 150                          | 30%                           |
| Hepes                                                                                  | 119.15                       | 1M                            |

100x BG11 salts:

| Chemical compound                                                      | Weight for 1 L volume (g) | Final concentration in 1 L |
|------------------------------------------------------------------------|---------------------------|----------------------------|
| Sodium nitrate (NaNO <sub>3</sub> )                                    | 149.6 g                   | 1.76 M                     |
| Magnesium sulphate heptahydrate (MgSO <sub>4</sub> ·7H <sub>2</sub> O) | 7.5 g                     | 30 mM                      |
| NaEDTA 0.5 M pH 8.0                                                    | 554 µl                    | 0.27 mM                    |

## Appendix 1 – Media recipes

|                                                                      |        |         |
|----------------------------------------------------------------------|--------|---------|
| Calcium chloride dihydrate<br>(CaCl <sub>2</sub> ·2H <sub>2</sub> O) | 3.6 g  | 24.5 mM |
| Citric acid<br>(HOC(COOH)(CH <sub>2</sub> COOH) <sub>2</sub> )       | 0.65 g | 3.4 mM  |

1000x Trace metal mix:

| Metal compound                                                                        | Weight for 1 L volume (g) | Final concentration in 1 L |
|---------------------------------------------------------------------------------------|---------------------------|----------------------------|
| Dihydrogen borate (H <sub>2</sub> BO <sub>3</sub> )                                   | 2.86 g                    | 47 mM                      |
| Manganese(II) chloride tetrahydrate (MnCl <sub>2</sub> ·4H <sub>2</sub> O)            | 1.81 g                    | 9.1 mM                     |
| Zinc sulphate heptahydrate (ZnSO <sub>4</sub> ·7H <sub>2</sub> O)                     | 0.221 g                   | 768 µM                     |
| Sodium molybdate dihydrate (Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O)      | 0.39 g                    | 1.61 mM                    |
| Copper sulphate pentahydrate (CuSO <sub>4</sub> ·5H <sub>2</sub> O)                   | 0.08 g                    | 320.4 µM                   |
| Cobalt(II) nitrate hexahydrate (Co(NO <sub>3</sub> ) <sub>2</sub> ·6H <sub>2</sub> O) | 0.049 g                   | 168.3 µM                   |

\*all autoclaved with sensitive cycle prior to adding to liquid or solid media

Liquid BG11 growth medium

| Ingredient                                                                | Amount added for 1 L |
|---------------------------------------------------------------------------|----------------------|
| Deionised H <sub>2</sub> O                                                | 966 ml               |
| 100x BG-11                                                                | 10 ml                |
| 189 mM Sodium carbonate (NaNO <sub>3</sub> )                              | 1 ml                 |
| 175 mM di-Potassium hydrogen phosphate (K <sub>2</sub> HPO <sub>4</sub> ) | 1 ml                 |
| 6 mg/ml Ferric Ammonium Citrate (Ammonium iron(III) citrate 18 % Fe)      | 1 ml                 |
| Trace metal solution                                                      | 1 ml                 |
| 1 M Hepes                                                                 | 20 ml                |

BG11 solid media:

| Ingredient                                                                                 | Amount added for 100 ml |
|--------------------------------------------------------------------------------------------|-------------------------|
| Deionised H <sub>2</sub> O                                                                 | 92 ml                   |
| BactoAgar                                                                                  | 1 g                     |
| 100x BG-11                                                                                 | 1 ml                    |
| 189 mM Sodium carbonate (NaNO <sub>3</sub> )                                               | 0.5 ml                  |
| 175 mM di-Potassium hydrogen phosphate (K <sub>2</sub> HPO <sub>4</sub> )                  | 0.5 ml                  |
| 6 mg/ml Ferric Ammonium Citrate (Ammonium iron(III) citrate 18 % Fe)                       | 0.5 ml                  |
| Trace metal solution                                                                       | 0.5 ml                  |
| 1 M Hepes                                                                                  | 2 ml                    |
| 30% Sodium Thiosulphate (Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> ·5H <sub>2</sub> O) | 2 ml                    |

## Appendix 1 – Media recipes

### **Luria-Bertani broth (LB)**

| <b>Supplement</b> | <b>Amount added for 1 litre volume</b> |
|-------------------|----------------------------------------|
| Tryptone          | 10 g                                   |
| Yeast extract     | 5 g                                    |
| NaCl              | 10 g                                   |
| H <sub>2</sub> O  | 1 L                                    |

\*mixtures autoclaved before use.

### **Penassay broth (PAB)**

Dehydrated culture medium Antibiotic medium No.3 assay broth (Oxoid) diluted into water before autoclaving (17.5 g per 1 L).

## Appendix 2 – Buffers

### Laemmli SDS loading dye

| Ingredients                           | Amount added to 100 ml | Final concentration |
|---------------------------------------|------------------------|---------------------|
| Sodium dodecyl sulfate (SDS)          | 10 g                   | 10%                 |
| 0.125 M Tris-HCL (pH 6.8)             | 25 ml                  | 1.25 mM             |
| Bromophenol blue                      | 0.01 g                 | 0.01%               |
| Glycerol                              | 40 ml                  | 40% glycerol        |
| Dithiothreitol (DTT)                  | 6.17 g                 | 400 mM              |
| Deionised H <sub>2</sub> O            | 25 ml                  |                     |
| cOmplete EDTA free protease inhibitor | 1 tablet per 50 ml     |                     |

### Western blot/protein gel buffers

#### Biorad transblot transfer buffer:

| Ingredients                  | Amount added to 1 L | Final concentration |
|------------------------------|---------------------|---------------------|
| Tris                         | 72.68 g             | 300 mM              |
| Glycine                      | 45.04 g             | 300 mM              |
| Tricine                      | 50.16 g             | 140 mM              |
| 10% SDS (dissolved in water) | 5 ml                | 0.05%               |
| 0.5 M EDTA                   | 5 ml                | 2.5 mM              |

#### Wash solutions:

PBS: 1 tablet of phosphate buffered saline pH7.2 (Oxoid) in 100mL water

PBST: PBS + 0.1% Tween20 (Sigma)

#### Blocking buffer:

PBST + 5% powdered skimmed milk

#### Tris acetate-EDTA (TAE) electrophoresis buffer

10x concentrate - 0.4 M Tris acetate, pH approx. 8.3, containing 0.01 M EDTA.

1x used, diluted 10-fold in deionised water prior to use.

**Appendix 3 – oligonucleotide table**

| Name            | Sequence                                                    | Restriction site | Purpose                                                            |
|-----------------|-------------------------------------------------------------|------------------|--------------------------------------------------------------------|
| oGGN01          | CAGTGAATACAGGCTATTTGAG                                      | NA               | Sequence pLS20 origin                                              |
| oGGN02          | GGGTACCCATAGTAGTTCAC                                        | NA               | Sequence pLS20 origin                                              |
| oGGZ01          | TTCTGCTAGTTTCAAGTTCTCAAAG                                   | NA               | Sequence reporter gene in pAG-P08/pGG08Z/pGGR01/pGGS01/pGGR04      |
| oGGZ02          | GAAGCATTTATCAGGGTTATTGTC                                    | NA               | Sequence reporter gene in pAG-P08/pGG08Z/pGGR01/pGGS01/pGGR04      |
| GG-RepN-IM F    | GATCGGATCTTCATGAAAAAC                                       | NA               | For cloning RepN-iteron mutant into pSHP1                          |
| GG-RepN-IM R    | CTTATCGATACCGTCGACCTCGAGGGATCCTT<br>AATTATCTAACCAATTATAAAAC | NA               | For cloning RepN-iteron mutant into pSHP1                          |
| GG-N32-IT-seq-F | TAGGGATCTAGACATTTCTCCGATTAGAGGA<br>TCCG                     | Xba I            | Remove 683 bases from pGGN32 of RepN protein and promoter sequence |
| GG-N32-IT-seq-R | GATGCATCTAGAGGGATCGGTAAACAATGA<br>AGTTG                     | Xba I            | Remove 683 bases from pGGN32 of RepN protein and promoter sequence |
| oGG011          | TAGGTGACGGCCGGGGCAAACCTTTTTTCAT<br>ATATTC                   | Eag I            | Remove 147 bases downstream of RepN iteron sequences               |
| oGG012          | GATGCACGGCCGCAAGTGCCGTTTTATAATT<br>GG                       | Eag I            | Remove 147 bases downstream of                                     |

### Appendix 3 – Oligonucleotide table

|             |                                                |       | RepN iteron sequences                                                     |
|-------------|------------------------------------------------|-------|---------------------------------------------------------------------------|
| oGG005<br>F | CTCATGACCAAAATCCCTTAACG                        | NA    | Sequence <i>E. coli</i> origin on pAG-P08, pGG08Z, pGGS01, pGGR01, pGGR04 |
| oGG006<br>R | CACTCAAAGGCGGTAATACG                           | NA    | Sequence <i>E. coli</i> origin on pAG-P08, pGG08Z, pGGS01, pGGR01, pGGR04 |
| oGG013      | TCTGACGCTCAGTGGAAACCGCGGTACCTCTGGCGGTGATAATG   | SacI  | Amplify SC101 origin to replace pBR322 origin                             |
| oGG014      | GGCCTTTTGCTCACATGTTCTCTAGAAGACAGCTGGACAGTAAGAC | XbaI  | Amplify SC101 origin to replace pBR322 origin                             |
| oGG020      | TATGCTCTAGAGCAACGCGGCCTTTTTACGG                | XbaI  | Remove pBR322 <i>E. coli</i> origin for replacement with SC101 or RSF1010 |
| oGG021      | TTGACCGCGGTCTGACGCTCAGTGGAAAC                  | SacI  | Remove pBR322 <i>E. coli</i> origin for replacement with SC101 or RSF1010 |
| oGG022      | TTCCCTTCTGGGCCAAAC                             | NA    | Used to amplify from outside <i>minC</i> locus to check for deletion      |
| oGG023      | CAGCGCCATTTATCACAG                             | NA    | Used to amplify from outside <i>minC</i> locus to check for deletion      |
| oGG1a       | GTACTCTAGACTCCGCTTCGAGCTGGATTGG                | EcoRI | Amplify around <i>minC</i> locus for gene disruption (pGGCA1)             |
| oGG1b       | TAGCGAATTCGGGCAAGGGCGATCACTGTATTCTGG           | XbaI  | Amplify around <i>minC</i> locus for gene disruption (pGGCA1)             |
| oGG2a       | TGCAGGTACCGACGCGACAATTTTTGCCCTGTGCTGG          | KpnI  | Amplify around <i>minC</i> locus for                                      |

### Appendix 3 – Oligonucleotide table

|        |                                           |           |                                                               |
|--------|-------------------------------------------|-----------|---------------------------------------------------------------|
|        |                                           |           | gene disruption (pGGCA1)                                      |
| oGG2b  | TTGAGCATGCCGGGCGATCGCTCATCTGCCA<br>ACCTAG | Sph<br>I  | Amplify around <i>minC</i> locus for gene disruption (pGGCA1) |
| oGG3a  | TTAGTCTAGATTAAACATCATGAGGGAAGCG<br>GTG    | Xba<br>I  | Amplify <i>aadA</i> spectinomycin cassette for pGGCA1         |
| oGG3b  | GACTGGTACCTTAGGCGGGCTGGCCGGCCTT<br>GCC    | Kpn<br>I  | Amplify <i>aadA</i> spectinomycin cassette for pGGCA1         |
| oGG036 | TCATTAGGCACCCCAGGC                        | NA        | Sequence pGGCA1                                               |
| oGG037 | CGCCAGCTGGCGAAAGGG                        | NA        | Sequence pGGCA1                                               |
| oGG038 | GATCCGATATCGTTGTTG                        | NA        | Sequence after Pspac sequence of pAG-P08, pGG08Z, pGGZ32      |
| oGG039 | TAGTTCACCACCTTTTCC                        | NA        | Sequence towards origin of pAG-P08, pGGZ32, pGG08Z            |
| oGG040 | GATCCGCGGCCGTTGCTAGACATTGCCAGC            | SacI<br>I | Amplify RSF1010 replicon from pRL1383a                        |
| oGG041 | AGGTCTAGATTAGCAGGAAAATAAGC                | Xba<br>I  | Amplify RSF1010 replicon from pRL1383a                        |
| oGG042 | GGCATTGAATTCTGGTTCTAG                     | NA        | Amplify pLS20 origin 'cluster 1' mutation for cloning         |
| oGG043 | TTGATACCCTCCAAACAGTGG                     | NA        | Amplify pLS20 origin 'cluster 1' mutation for cloning         |
| oGG044 | CTATGGCCAAGCTTCTATGCA                     | NA        | Amplify pLS20 origin 'cluster 2' mutation for cloning         |
| oGG045 | ACTGTTCTAGGAAACAGGGCG                     | NA        | Amplify pLS20 origin 'cluster 2'                              |



### Appendix 3 – Oligonucleotide table

|        |                                                    |          | mutation for cloning                                                           |
|--------|----------------------------------------------------|----------|--------------------------------------------------------------------------------|
| oGG046 | GGACGACAGAAAACCACT                                 | NA       | Amplify vector with overhangs for cluster 1 integration                        |
| oGG047 | TTGTCTGCAAACAACAGG                                 | NA       | Amplify vector with overhangs for cluster 1 integration                        |
| oGG058 | AGGCGATTGTCCGCAACC                                 | NA       | Sequencing <i>arsB</i> locus of <i>E. coli</i>                                 |
| oGG059 | CGGAGTTTCCAGATAATGGATAATAG                         | NA       | Sequencing <i>arsB</i> locus of <i>E. coli</i>                                 |
| oGG068 | GCCCCTCCTAAGGGCTAATTG                              | NA       | Amplify <i>intS</i> site from HL713 for integration of <i>lacI<sup>q</sup></i> |
| oGG069 | ACCACCCAGTGTATAAGGGTG                              | NA       | Amplify <i>intS</i> site from HL713 for integration of <i>lacI<sup>q</sup></i> |
| oGG074 | ACTTTGCGGGGCTTCCCAACC                              | NA       | Sequencing of <i>intS neo lacI<sup>q</sup></i> integration                     |
| oGG075 | ACCCAGTTGATCGGCGCGAGA                              | NA       | Sequencing of <i>intS neo lacI<sup>q</sup></i> integration                     |
| oGG076 | TGGTACCACTGGCCGTCGTTTTACAAC                        | NA       | Amplify pUC19 for integration of pTet asRNA (micF7.4)                          |
| oGG077 | AAGGATCCGGCGTAATCATGGTCATAGCTGT<br>T               | NA       | Amplify pUC19 for integration of pTet asRNA (micF7.4)                          |
| oGG078 | ATTACGCCGGATCCTTTTCAGCAGGACGCACT<br>GACCTCCCTAT    | Xba<br>I | Primer dimerising to make start of pTet (has HiFi sites for cloning)           |
| oGG079 | TATCACTGATAGGGATGTCAATCTCTATCACT<br>GATAGGGAGGTCAG | Xba<br>I | Primer dimerising to make start of pTet (has HiFi sites for cloning)           |
| oGG080 | TTGACATCCCTATCAGTGATAGAGATACTGA<br>GCACATCTAAAGTT  | NA       | Primer dimerising to make end of pTet and TBR (has                             |

### Appendix 3 – Oligonucleotide table

|                  |                                                                                   |       | HiFi sites for cloning)                                                    |
|------------------|-----------------------------------------------------------------------------------|-------|----------------------------------------------------------------------------|
| oGG081           | CATCCTTGCGGGACGTGAGCAAAGGAGAAGA<br>ACTTTAGATGTG                                   | NA    | Primer dimerising to make end of pTet and TBR (has HiFi sites for cloning) |
| oGG082           | CGTCCCGCAAGGATGCGGGTCTGTTTACCCCT<br>ATTTCAACCG                                    | Kpn I | Primer dimerising to make end of MicF 7.4 (has HiFi sites for cloning)     |
| oGG083           | CGGCCAGTGGTACCAAAAAAAAAACCGGCCGC<br>GAGGCGGCCGGTTGAAATAGG                         | Kpn I | Primer dimerising to make end of MicF 7.4 (has HiFi sites for cloning)     |
| oGG092           | CAGCCCGCCTAACTGTCAGACCAAGTTTACTC<br>ATATATACTTTAGATTGATTTAAAAC                    | NA    | Amplify to remove <i>bla</i> for integration of <i>aadA</i>                |
| oGG093           | TCATGATGTTTAAACTCTTCCTTTTCAATATT<br>ATTGAAGCATTATCAGG                             | NA    | Amplify to remove <i>bla</i> for integration of <i>aadA</i>                |
| oGG094           | CTTGGTCTGACAGTTAGGCGGGCTGGCCGG                                                    | NA    | Amplify <i>aadA</i> spectinomycin cassette to make pGGAS2                  |
| oGG095           | AAAAGGAAGAGTTTAAACATCATGAGGGAA<br>GCGGTGATCG                                      | NA    | Amplify <i>aadA</i> spectinomycin cassette to make pGGAS2                  |
| oGG140           | CCAAGCTTGCATGCCTGCAGGTCGACTCTAG<br>AGGATCCCCCCCATCCAATTGTTCAAAATTCT<br>TGCTGATG   | NA    | Integrate <i>arsB</i> locus to make pGG007                                 |
| oGG141           | GTAAAACGACGGCCAGTGAATTCGAGCTCGG<br>TATCCCAGCGGCGTCACCACAATCGGGCGAT<br>TAATCAGAATC | NA    | Integrate <i>arsB</i> locus to make pGG007                                 |
| oGG148           | CCCATCCAATTGTTCAAAATTCTTGCTGATGA<br>AAC                                           | NA    | Sequence <i>arsB</i> locus (annealing in <i>arsR</i> )                     |
| oGG149           | TCCAGCGGCGTCACCACAATCGGGCGATTA<br>ATC                                             | NA    | Sequence <i>arsB</i> locus (annealing in <i>arsC</i> )                     |
| oGG150<br>- lacZ | GCGTTTATCGCCGTCATTATCATCAGCCTGCT<br>GACCATGATTACGCCGGATCCTTTTC                    | NA    | For amplification and integration                                          |

### Appendix 3 – Oligonucleotide table

|        |                                                                                        |    |                                                                                                                                                                                                      |
|--------|----------------------------------------------------------------------------------------|----|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|        |                                                                                        |    | of asRNA<br>(amplified from<br>pGGAS2)                                                                                                                                                               |
| oGG151 | GCGCCAACCAGTACCGTCGGCATATTGTTCC<br>GCCAGCTGGCGAAAGGG                                   | NA | For amplification<br>and integration<br>of asRNA<br>(amplified from<br>pGGAS2)                                                                                                                       |
| oGG152 | TCTCTATCACTGATAGGGATGTCAATCTCTAT<br>CACTGATAGGGAGGATCCGATCTTTTGTTTAT<br>AAGTGGGTAAACCG | NA | Amplify <i>aadA</i><br>cassette for<br>integration to<br>make pGG011                                                                                                                                 |
| oGG153 | GCGTTTATCGCCGTCATTATCATCAGCCTGCT<br>G                                                  | NA | Amplify <i>aadA</i><br>cassette for<br>integration to<br>make pGG011                                                                                                                                 |
| oGG155 | GCGCCAACCAGTACCGTCGGCATATTGTTCC                                                        | NA | pGG011<br>sequencing                                                                                                                                                                                 |
| oGG156 | TTCTATAGAACTTCTCTCAATTAGGCTAATT<br>TTATTGC                                             | NA | pGG011<br>sequencing                                                                                                                                                                                 |
| oGG157 | AGTATCCACAGAGGTTATCGACAACATTTTC<br>ACATTAC                                             | NA | Amplify <i>dnaA</i><br>locus for<br>sequencing                                                                                                                                                       |
| oGG158 | GAATCGTGAATTTTCATTTTTATCCTCCTAACG<br>GATAATG                                           | NA | Amplify <i>dnaA</i><br>locus for<br>sequencing                                                                                                                                                       |
| oGG159 | TGTGTACGAATGGTAAGCGCC                                                                  | NA | Sequence <i>dnaA</i><br>PCR product                                                                                                                                                                  |
| oGG160 | GTAGGGCCTGTGGATTTGTGGATAAGTTG                                                          | NA | Sequence <i>dnaA</i><br>PCR product                                                                                                                                                                  |
| oGG161 | CGACGCTGTATTTGAGGAAAAGGAATAAGTG<br>ATTAGAAATGAATACAAATATGGTAGCAAGT<br>G                | NA | Amplify the<br>RacA<br>MEGAPRIMER<br>for integration<br>into pBR322<br>diviva from<br>Henrik to make a<br>divIVA/racA<br>operon under the<br>Para promoter<br>(inducible<br>arabinose<br>expression) |
| oGG162 | CCAAAACAGCCAAGCTTGCATGCTTAGGTTT<br>GAAATTTGAACAGTGAC                                   | NA | Amplify the <i>racA</i><br>MEGAPRIMER<br>for integration<br>into pBR322<br><i>divIVA</i> from<br>Henrik to make a<br><i>divIVA/racA</i>                                                              |

### Appendix 3 – Oligonucleotide table

|                |                                                                          |    |                                                                                          |
|----------------|--------------------------------------------------------------------------|----|------------------------------------------------------------------------------------------|
|                |                                                                          |    | operon under the Para promoter (inducible arabinose expression)                          |
| oGG163         | TGCTGATTGAAGCTCAGCTTGATC                                                 | NA | Sequence pGG017 (internal primer in <i>divIVA</i> seq)                                   |
| oGG164         | CTACTGCCGCCAGGCAAATTC                                                    | NA | Sequence pGG017 (internal primer in pBAD322 backbone)                                    |
| oGG165         | TATAGGGAAAAGGTGGTGAAGTACTTTGAAA<br>AAGAAAAACATTTATTCAATTCGTAACTAG<br>GTG | NA | Protein A amplification for RSF1010 vector                                               |
| oGG166         | CGCCATTCAGGCTGCGCAACTTATAGTTCGCG<br>ACGACGTCCAG                          | NA | Protein A amplification for RSF1010 vector                                               |
| oGG167         | AGTAGTTCACCACCTTTTCCCTATATAATTG                                          | NA | pGGR01 vector amplification for HiFi cloning (makes pGGR04)                              |
| oGG168         | GTTGCGCAGCCTGAATGGCG                                                     | NA | pGGR01 vector amplification for HiFi cloning (makes pGGR04)                              |
| s.el Ori1<br>F | TTTTACACCAGCGCCCTAAA                                                     | NA | Watanabe <i>et al</i> , 2018 - make <i>S. elongatus</i> FISH probes for origin detection |
| s.el Ori2<br>F | CCGATCTAGTAATCGCACCGT                                                    | NA | Watanabe <i>et al</i> , 2018 - make <i>S. elongatus</i> FISH probes for origin detection |
| s.el Ori3<br>F | GCTGACGATCGCCTCAGTTT                                                     | NA | Watanabe <i>et al</i> , 2018 - make <i>S. elongatus</i> FISH probes for origin detection |
| s.el Ori1<br>R | TGGTCATGATAAAGCGCGA                                                      | NA | Watanabe <i>et al</i> , 2018 - make <i>S. elongatus</i> FISH probes for origin detection |

### Appendix 3 – Oligonucleotide table

|                |                                      |          |                                                                                                        |
|----------------|--------------------------------------|----------|--------------------------------------------------------------------------------------------------------|
| s.el Ori2<br>R | AGCTCTTGGCGCAACTCAA                  | NA       | Watanabe <i>et al</i> ,<br>2018 - make <i>S. elongatus</i> FISH<br>probes for origin<br>detection      |
| s.el Ori3<br>R | GGCTGGAAAAGCATAGTCACG                | NA       | Watanabe <i>et al</i> ,<br>2018 - make <i>S. elongatus</i> FISH<br>probes for origin<br>detection      |
| s.el Ter1<br>F | CTTGATCGATGATCTGCAAGC                | NA       | Watanabe <i>et al</i> ,<br>2018 - make <i>S. elongatus</i> FISH<br>probes for<br>terminus<br>detection |
| s.el Ter2<br>F | TATTCCGCCAGTAGCGGATTA                | NA       | Watanabe <i>et al</i> ,<br>2018 - make <i>S. elongatus</i> FISH<br>probes for<br>terminus<br>detection |
| s.el Ter3<br>F | CGTGCCAATCCTGCTTGTT                  | NA       | Watanabe <i>et al</i> ,<br>2018 - make <i>S. elongatus</i> FISH<br>probes for<br>terminus<br>detection |
| s.el Ter1<br>R | AACTACCCTGTCTGGCGATCT                | NA       | Watanabe <i>et al</i> ,<br>2018 - make <i>S. elongatus</i> FISH<br>probes for<br>terminus<br>detection |
| s.el Ter2<br>R | TCGCAACCAAGCAAAATCAG                 | NA       | Watanabe <i>et al</i> ,<br>2018 - make <i>S. elongatus</i> FISH<br>probes for<br>terminus<br>detection |
| s.el Ter3<br>R | TGATAACATTCGCCTCGTCG                 | NA       | Watanabe <i>et al</i> ,<br>2018 - make <i>S. elongatus</i> FISH<br>probes for<br>terminus<br>detection |
| oGG181         | GACTCTAGAATAACCACTAGGTTGCTTGGCG<br>C | Xba<br>I | Remove DnaA<br>box identified in<br>pAG-P08 and<br>replacing with                                      |

### Appendix 3 – Oligonucleotide table

|        |                                 |          |                                                                                                         |
|--------|---------------------------------|----------|---------------------------------------------------------------------------------------------------------|
|        |                                 |          | XbaI (+3 bases )<br>to make pGG018                                                                      |
| oGG182 | GACTCTAGATTCTCCGGACCGTTAATGGCCG | Xba<br>I | Remove DnaA<br>box identified in<br>pAG-P08 and<br>replacing with<br>XbaI (+3 bases )<br>to make pGG018 |
| oGG183 | CGTCAGGAGTTGCCCCGTCC            | NA       | Sequencing<br>primer for<br>removal of DnaA<br>box                                                      |
| oGG184 | TGTCTCGTGTTCTCTCACACGG          | NA       | Sequencing<br>primer for<br>removal of DnaA<br>box                                                      |

## Appendix 4 – $\Delta$ dnaA for pLS20 replication and immunostaining controls

### $\Delta$ dnaA plasmid copy number

In the DnaA depletion strain the stability was grown in rich liquid media (LB) with different inducers and antibiotic selection conditions. After 8 hours of growth all cultures were diluted back to an OD600 of 0.3 before further dilution series 4-fold in a 96-well plate followed by a spot titre as mentioned in section 2.12. Plates were incubated at 37°C overnight.

Supplementary table 4.1. *Bacillus subtilis* strains used for the DnaA depletion studies.

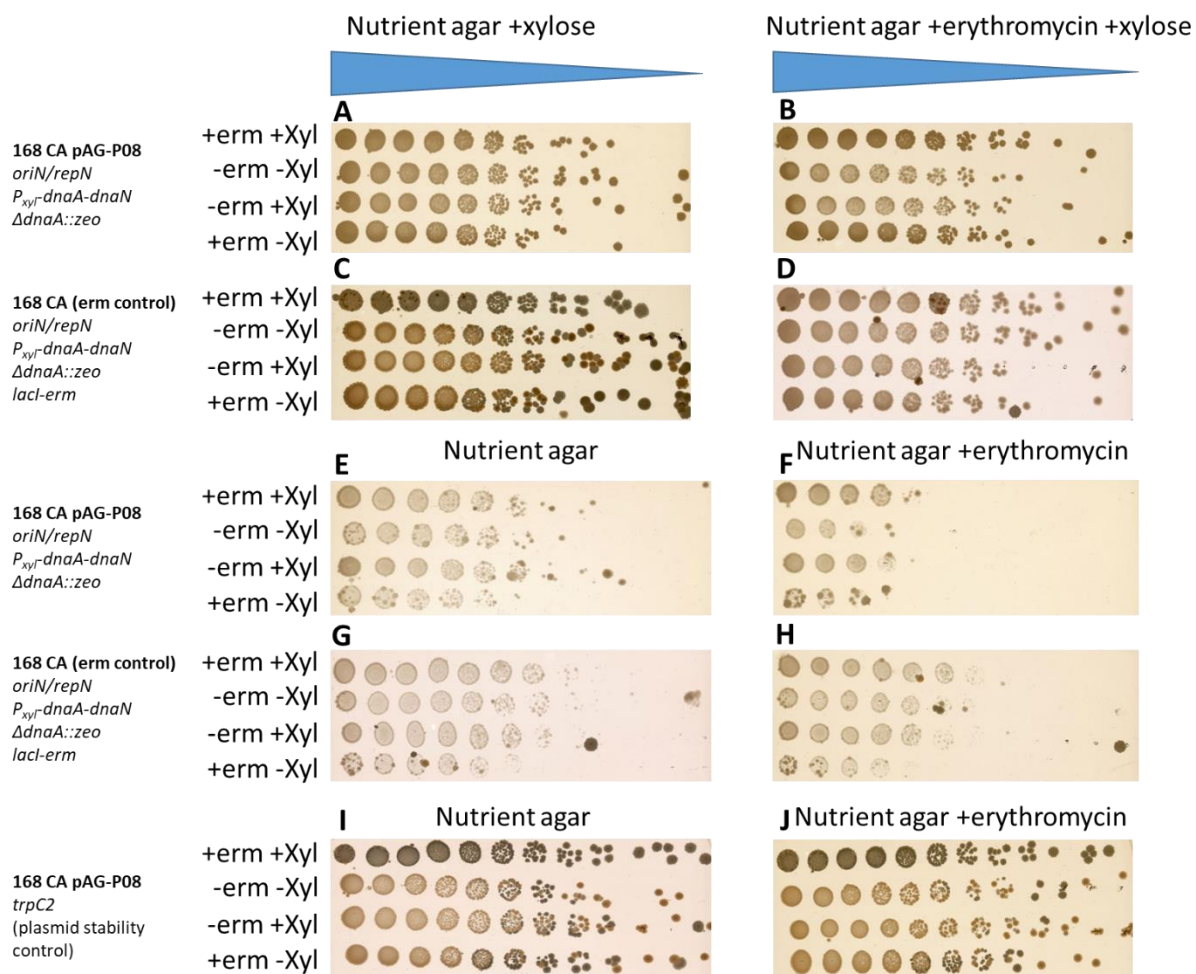
|             |                                                                                                                                          |                               |               |
|-------------|------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------|---------------|
| HM949       | <i>spoIIIJ(359°)::(oriN kan tet)</i>                                                                                                     | -                             | H. Murray     |
| HM1423      | <i>spoIIIJ(359°)::(oriN kan tet)</i><br><i><math>\Delta</math>dnaA::zeo</i>                                                              | -                             | H. Murray     |
| TR273       | <i>amyE::spc(Pxyl-dnaA-dnaN)</i>                                                                                                         | -                             | T. Richardson |
| MR01        | <i>spoIIIJ(359°)::(oriN kan tet)</i><br><i>amyE::spc(Pxyl-dnaA-dnaN)</i>                                                                 | TR273 gDNA                    | GGoldsmith    |
| HM949(lacI) | <i>spoIIIJ(359°)::(oriN kan tet)</i><br><i>aprE::lacI-erm (erythromycin control for experiment)</i>                                      | pAPNC-erm                     | GGoldsmith    |
| MR03        | <i>spoIIIJ(359°)::(oriN kan tet)</i><br><i>amyE::spc(Pxyl-dnaA-dnaN)</i><br><i><math>\Delta</math>dnaA::zeo</i>                          | TR273, HM1423 gDNA            | GGoldsmith    |
| MR04        | <i>spoIIIJ(359°)::(oriN kan tet)</i><br><i>aprE::lacI-erm</i><br><i>amyE::spc(Pxyl-dnaA-dnaN)</i>                                        | pAPNC-erm, TR273 gDNA         | GGoldsmith    |
| MR05        | <i>spoIIIJ(359°)::(oriN kan tet)</i><br><i>aprE::lacI-erm</i><br><i>amyE::spc(Pxyl-dnaA-dnaN)</i><br><i><math>\Delta</math>dnaA::zeo</i> | pAPNC-erm, TR273, HM1423 gDNA | GGoldsmith    |
| MR03 pAGP08 | <i>spoIIIJ(359°)::(oriN kan tet)</i><br><i>amyE::spc(Pxyl-dnaA-dnaN)</i><br><i><math>\Delta</math>dnaA::zeo pAG-P08</i>                  | TR273, HM1423 gDNA            | GGoldsmith    |

#### Appendix 4 – $\Delta$ dnaA for pLS20 replication and immunostaining controls

To see if DnaA was essential for replication of the pLS20 plasmid, a DnaA depletion *B. subtilis* strain was constructed using strains gifted from the Murray lab. These strains, when depleted of DnaA, were dependent the minimal replicon on RepN/*oriN* derived from pLS32 (such as those published by Hassan *et al.*, 1997). This secondary replicon system described by Richardson *et al.*, 2016 was constitutive within the constructed strain while DnaA expression was placed under a  $P_{xyl}$  inducible promoter. This would allow the strain to survive but if DnaA from the host is required for upkeep of the plasmid then depletion would reduce stability of the plasmid and the strain would not confer the erythromycin resistance from the vector. A positive control strain with erythromycin resistance expressed from the chromosome was also used to show no loss of stability by comparison. The spot titre assay showed a lot of suppressor mutants from all strains including the positive control. When spotted onto plates supplemented with xylose, to permit DnaA expression, all strains grew well even when strains had been grown in liquid without erythromycin or xylose. This indicates that the plasmid had not been lost as the dilution series did not demonstrate any erythromycin sensitivity. On plates supplemented with erythromycin, the growth was slightly affected across the more dilute cultures. This indicates that, on plates, a reduction of DnaA may affect erythromycin resistance but not profoundly, the positive control did manage to display resistance in the diluted cultures but growth was not dense. As the dilution series contains a lot of suppressor mutations, it makes this data hard to interpret and cannot conclude anything about the plasmid's stability.



## Appendix 4 – $\Delta$ dnaA for pLS20 replication and immunostaining controls

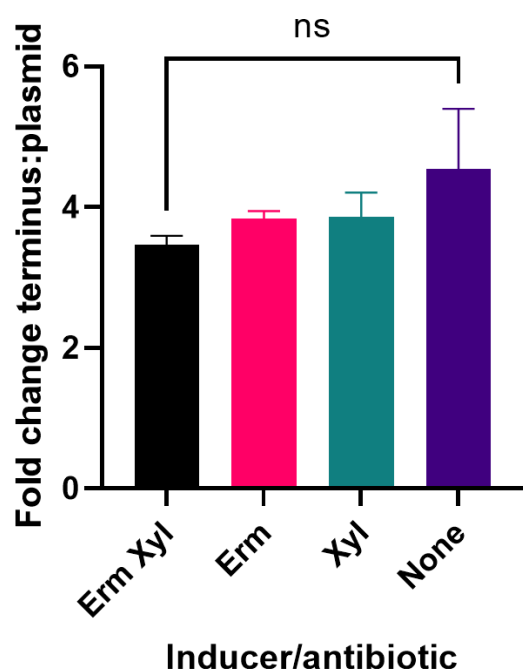


Supplementary figure 4.1. pLS20 stability in a DnaA depletion strain. The stability of the plasmid in the absence of host DnaA was tested where there was 16 hours growth in liquid culture containing different induction and antibiotic selection conditions. After 16 hours, the cultures were diluted back to an OD600 of 0.3 and further diluted across a series 4-fold. 5 µl of the dilution series were dropped onto nutrient agar with different antibiotic and induction. Plates were incubated at 30°C for 48 hours before imaging.

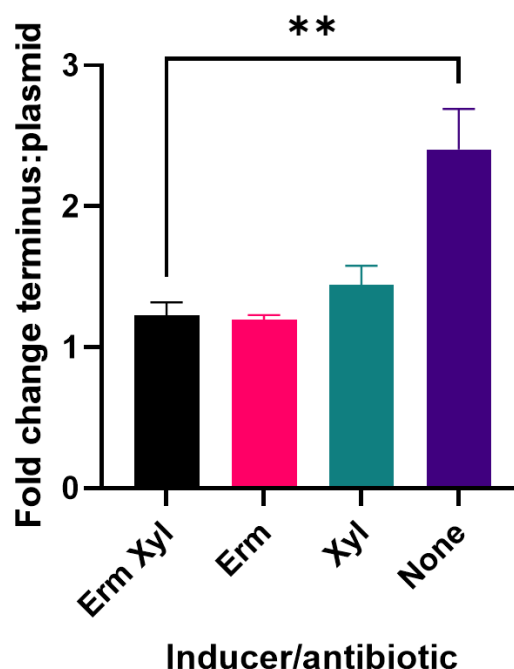
As spot titre assays were not successful, further tests using qPCR were carried out to see if the DnaA depletion was able to detect any fluctuations in plasmid replication. Cultures were grown in liquid medium for 4 (mid exponential) and 16 hours (stationary phase). After 4 hours (fig.4.2A) there was no significant increase in the plasmid copy number across all induction conditions and supplements into the media. However, after 16 hours of growth, there was a significant increase when an unpaired T-test showed significance between the strain with both

inducers and with none. Interestingly, with just the addition of erythromycin there was still no increase in plasmid copy number which would be expected as the xylose inducer was removed and depleted DnaA. This makes the data hard to interpret as there should be a similar copy number increase with no inducer. The increase from no inducer or antibiotic may indicate that DnaA is a negative regulator but also could just be a result of more anucleated cells being produced as a result of the RepN/oriN mini-replicon not working as successfully as DnaA and skewing the chromosome:plasmid ratio.

### A – 4 hours



### B – 16 hours

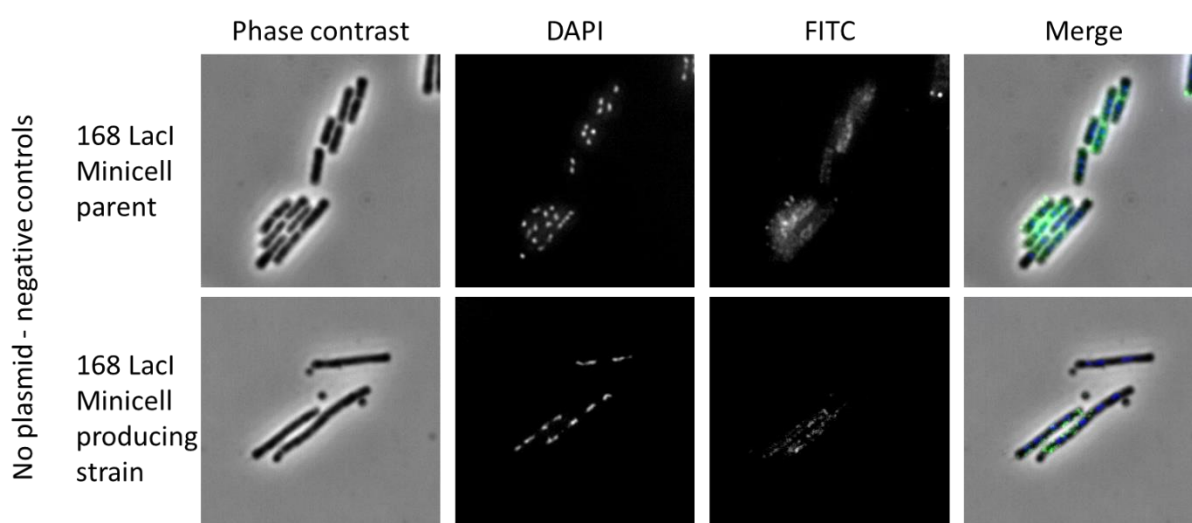


Supplementary figure 4.2. DnaA depletion strains with constitutive RepN/*oriN* system for survival were grown in minimal media at 30°C with xylose inducer, next day pellets were washed and inoculated into fresh liquid minimal media with different induction conditions. Cultures were placed at 30°C with shaking and aliquots were taken after **A)** 4 hours and **B)** 16 hours of growth. Pellets were processed in lysis buffer before dilution and running qPCR. Terminus:plasmid ratios were calculated using Microsoft Excel. Error bars represent triplicate technical repeats.

## Protein A no plasmid controls

### *Bacillus subtilis* no lysozyme:

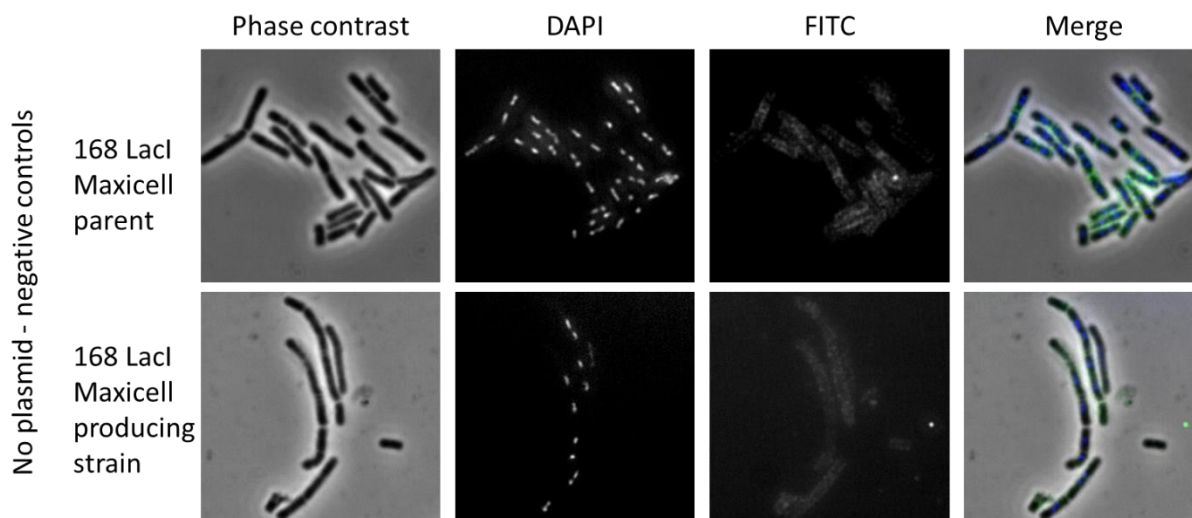
Negative controls without any expression of protein A show that there is some immunostaining observed. This is especially obvious within clusters of cells where there seems to be non-specific signals. For the minicell producing strain, there was no minicells with signals which was encouraging as it indicates signals seen in the test strain are real. There was some rod cell signals which are obvious around the two cells close to one another.



Supplementary figure 4.3. Parental and minicell producing strains negative control immunostaining. Strains were fixed in paraformaldehyde solution before mounting to glass slides with poly-L-lysine, blocking with 2% BSA. Primary antibodies ( $\alpha$ -Protein A, 1:250) were incubated overnight at 4°C and for detection FITC labelled secondary antibodies were used. Cells were imaged using Metamorph 6 software with a Nikon Eclipse Ti with cool LED light source.

#### Appendix 4 – $\Delta$ dnaA for pLS20 replication and immunostaining controls

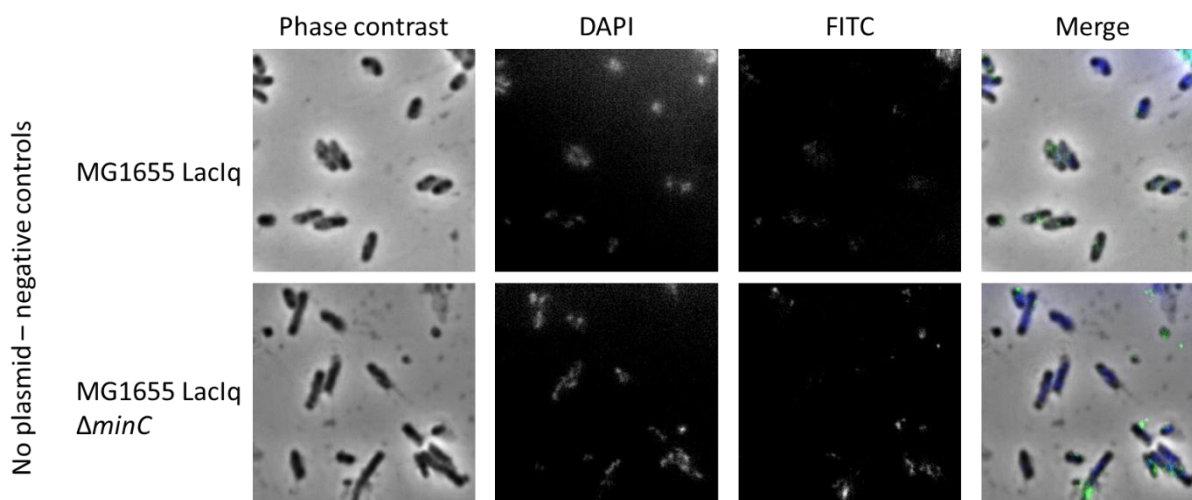
A similar effect was seen in the parental and maxicell producing strains, there was obvious staining around groups of cells which was not especially bright. The staining in these strains looked more regular over the cell surface and stronger at some of the septal peptidoglycan.



Supplementary figure 4.4. Parental and maxicell producing strains negative control immunostaining. Strains were fixed in paraformaldehyde solution before mounting to glass slides with poly-L-lysine, blocking with 2% BSA. Primary antibodies ( $\alpha$ -Protein A, 1:250) were incubated overnight at 4°C and for detection FITC labelled secondary antibodies were used. Cells were imaged using Metamorph 6 software with a Nikon Eclipse Ti with cool LED light source.

***Escherichia coli* – lysozyme treated:**

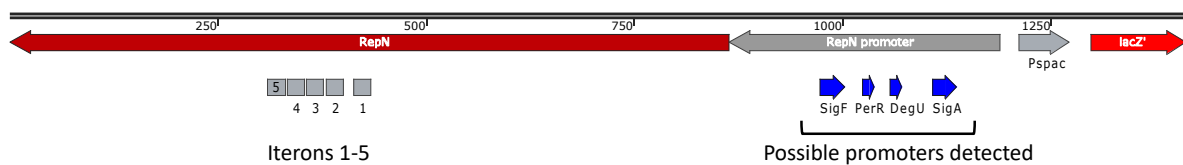
*Escherichia coli* strains negative controls (no plasmids or protein A expression) were also probes to visualise non-specific staining. There was some non-specific staining see around groups of cells, especially in the minicell producing strain, but overall, not many FITC staining. This is reassuring as it shows there is no cross-contamination from *E. coli* proteins which may have been recovered from the lysates when overexpressed to make rabbit antibodies.



Supplementary figure 4.5. *Eschechia coli* negative controls for protein A immunostaining experiments. Strains were fixed in paraformaldehyde solution before mounting to glass slides with poly-L-lysine, treating with 10 mg/ml lysozyme (Sigma) to lyse cells, and blocking with 2% BSA. Primary antibodies ( $\alpha$ -Protein A, 1:250) were incubated overnight at 4°C and for detection FITC labelled secondary antibodies were used. Cells were imaged using Metamorph 6 software with a Nikon Eclipse Ti with cool LED light source.

## Appendix 5 - pGGZ32 RepN/*oriN* region promoter scan

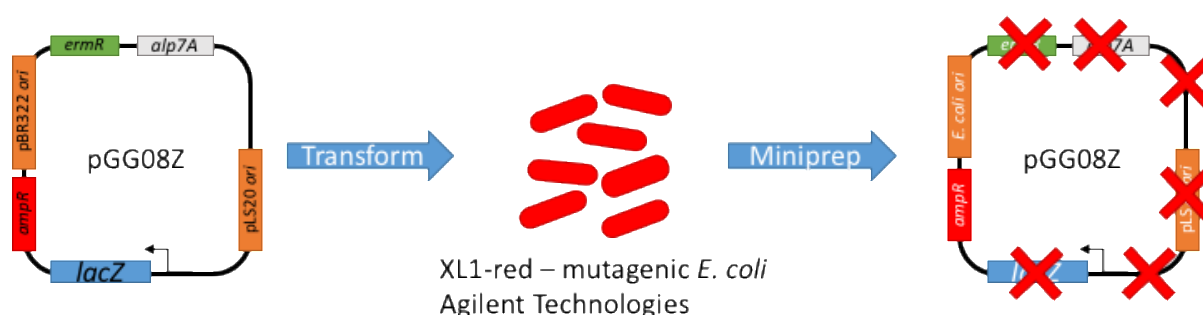
Since pGGZ32 was seen to have much higher expression of  $\beta$ -galactosidase a search using DBTBS (release 5) using the RepN sequence and promoter showed there were several possible promoters which may be strong enough to permit expression of  $\beta$ -galactosidase. This may explain why the transformants with pGGZ32 had dark blue colouration on plates or it may also be from increased plasmid copy number from 2 RepN proteins being expressed. The minimal replicon plasmid (pGGP02) had this sequence deleted so it would not be an issue for those plasmids, but it may be important to note if using the full RepN sequence plasmid in future. The promoters predicted were SigF, PerR, DegU and SigA. SigA is present during vegetative growth of *Bacillus subtilis* and therefore may result in a stronger expression of *lacZ*.



Supplementary figure 5.1. *in silico* analyses of the RepN/*oriN* locus in relation to the  $P_{spac}$  promoter. The full length RepN/*oriN* and promoter sequence were analysed using DBTBS (release 5) to see if there were any potential strong promoters affecting expression of  $\beta$ -galactosidase. 4 possible promoters were detected which may be able to force expression of the *lacZ* gene and cause an increase in expression.

## Appendix 6 – XL1-red mutagenesis

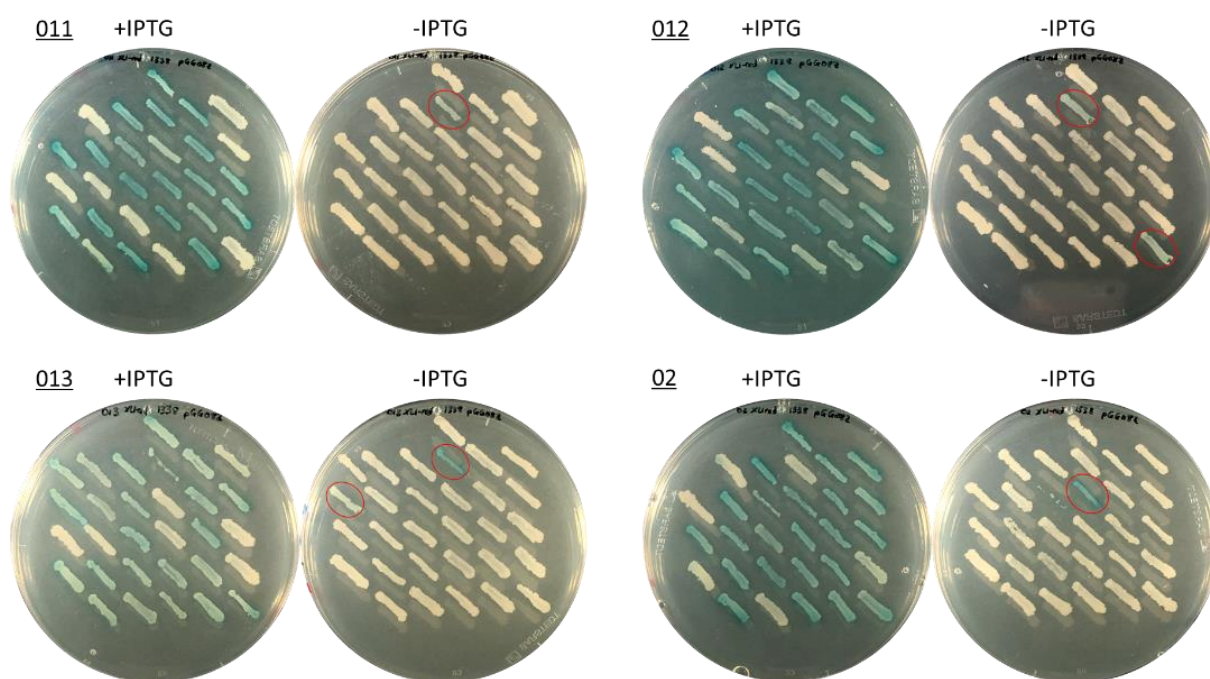
**pLS20 replicon mutagenesis.** As the functionality of the pLS20 replicon is not fully understood, the origin was subjected to random mutagenesis. Using the pGG08Z, mutagenesis was performed with the XL1-red mutagenesis kit (Agilent technologies) following manufacturer's instructions. Transformants were grown on selective media and colonies were pooled for miniprep to give a mixed population when transformed into sAG1338. Colonies, chosen at random, were patched in duplicate onto erythromycin, X-gal plates with or without IPTG in order to see if there is any increase in *lacZ* expression via colorimetric change as copy number increase may out compete LacI and be produced in the absence of IPTG. Patched plates were incubated overnight at 30°C and left at room temperature overnight to increase blue development before imaging using FujiFilm FinePix S9500 digital camera. Any *Bacillus* colonies which exhibited blue colouration in the absence of IPTG were miniprepped and subsequent plasmids used to re-transform JM109. The subsequent preps will be used to ensure that the phenotype remains in *B. subtilis*.



**Supplementary figure 6.1. Characterising the origin of pLS20.** The current plasmid used was clone to have a  $\beta$ -galactosidase reporter which will produce a colourimetric change in the presence of increased copy number. These plasmids were mutated via XL1-red mutagenic *E. coli* transformation. The plasmids were pooled and transformed into the minicell producing *B. subtilis* strain (sAG1338). 'X' depicts where mutations may occur as the whole plasmid may be mutagenized via XL1-red transformation.



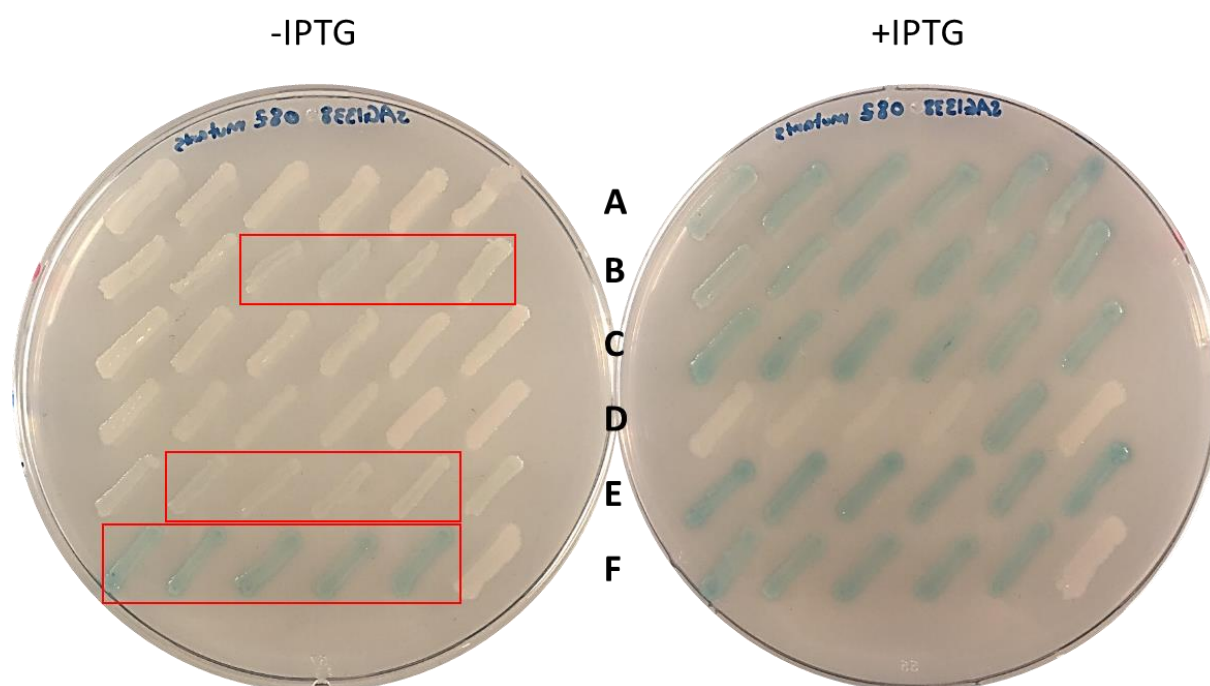
**Selection of potential pLS20 replicon mutants.** The mutant pool of plasmids was transformed into 168 LacI  $\Delta minC$  and single colonies patched onto selective agar with X-gal to observe colourimetric changes which may indicate increased copy number. Six mutants were chosen (circled in red) for their increased  $\beta$ -galactosidase activity without the presence of IPTG. This may be indicative of increased copy number in comparison to the control transformed with unmutated pGG08Z. There is a range of colour variations when IPTG is absent, these mutants were treated with caution in case inducible promoter damage reduced LacI repressor affinity.



**Supplementary figure 6.2 Selection of pLS20 mutations.** Numbers 011-02 indicate different pooled plasmids from XL1-red transformations. After extracting plasmids from the mutagenic *E. coli* strain, they were transformed to a *B. subtilis* strain with LacI repression and minicell producing mutation, single colonies from transformations were patched onto plates with X-gal and +/- IPTG to see if the expression of  $\beta$ -galactosidase was increased as a result of increased copy number. Plates were incubated at 30°C overnight before scanning with the Epson scanner.



**Phenotype observation and sequencing data.** The 6 patches highlighted in red circles in fig.6.3 were cultured to extract the plasmids which were transformed into *E. coli* DH5 $\alpha$ . When reintroduced to sAG1338 the phenotype was checked from 6 random colonies for each transformation. Mostly, the phenotype had not be retained as most colonies were white in the absence of IPTG. However, patches gated by the red boxes in fig.6.3B, E and F did retain some blue colouration in the absence of IPTG. Sequencing revealed there were no mutations within the origin sequence or within the *P<sub>spac</sub>* inducible promoter. Some plasmids have lost all blue colouration which may be a result of rearrangement in the plasmid. The cause of the blue colouration is not fully understood but it may be a result of some mutagenesis within the *lacZ* gene allowing better expression.

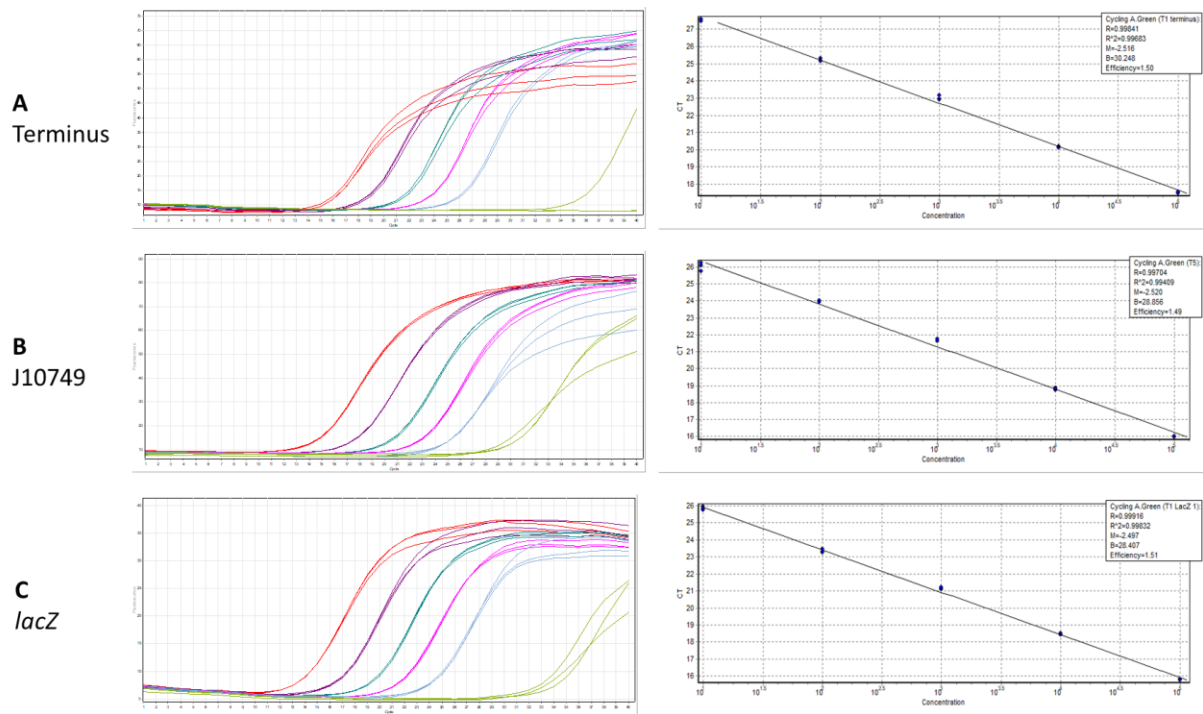


**Supplementary figure 6.3. pLS20 mutant phenotype check.** After choosing mutants with the expected phenotype, plasmids were extracted and transformed to *E. coli* and transformed back into 168 LacI  $\Delta$ *minC*. 6 random transformants were patched onto selective media with X-gal in duplicate to show results with and without IPTG induction. Plates were grown overnight at 37°C. Each row (A-F) is a different plasmid transformation which were from colonies outlined in red in figure 4.2.4. Images were taken using a Fuji FinePix camera after 24 hours of growth.

## Appendix 7 – qPCR standards traces

The trace from the qPCR showed an even distribution across the gDNA dilutions in triplicate. Standard curves represent the triplicate repeats for each dilution and shows a consistent linear reduction in the Ct values, these standard curves were used for quantification of test Ct values. The water control in all 3 primer pairs (light green) showed some activity between 28-32 cycles but overall was conclusive that there was no immediate primer dimerisation to affect the final data given. The terminus primers (Supp.fig.7.1, Fenyk, unpublished) displayed a good dilution series and this standard curve was used for all subsequent terminus quantifications. To probe for the plasmid, 2 sets of primers were chosen with different targets. The first pair, J10749 (Supp.fig.7.1B), originated from a publication by Lee *et al*, 2006 and is used to amplify from the ampicillin cassette of the plasmid (*bla*). The qPCR trace showed a consistent reduction in the amplification as the gDNA was diluted. The standard curve, produced by RotorGene-Q software, echoes the linear reduction in plasmid target amount over the 10-fold dilutions. A secondary probe for plasmid detection was also used which would amplify from the *lacZ* gene (for pGG08Z detection specifically) (Sup.fig.7.1C). The same 10-fold dilutions were used from the control gDNA prep, over the dilution series it is obvious that there is a linear reduction in quantity. The light green control (no gDNA) shows minimal signals indicating there is no primer dimerization to account for within the experiment. The standard curve produced from the RotorGene-Q software reduces consistently across the serial dilutions and was used for quantification of the plasmid in further assays.

## Appendix 7 – qPCR standards traces



**Supplementary figure 7.1 qPCR standard curves for terminus and plasmid quantification.** The gDNA of the control strain (sAG1392 with pGG08Z) was diluted 10-fold 5 times to provide a linear reduction in qPCR amplification. qPCR amplification was carried out using Luna qPCR master mix (NEB), gDNA was quantified by nanodrop before 10-fold serial dilution 5 times. Data and standard curves are from RotorGene-Q (Qiagen) software. **A)** standard plots of the terminus amplification primers with control gDNA **B)** standard plots and curve for the J10749 primer pair detecting the plasmid **C)** standard plot and curve for the *lacZ* specific primer pair. Standard curves were used for all quantification of the chromosome terminus and plasmids in later experiments.

## **Appendix 8 - RSF1010 replication in *B. subtilis***

There is no literature relating to the use of “broad host-range” RSF1010 plasmid replicon in *B. subtilis*. However, it has been recorded that this replicon is able to work within some Gram-positive bacteria such as *Streptomyces lividans* and *Mycobacterium smegmatis* (Gormley & Davies, 1991). Firstly, the pBR322 origin was replaced with that of RSF1010 (pGGR01) and the pLS20 origin was subsequently removed using restriction cutting and gel extraction of the correct sized plasmid without pLS20 replicon (pGGR02), these extracts were religated. Controls were used/constructed where both pLS20 origin and pBR322 origin remained (pAG-P08) and one with pLS20 origin removed and pBR322 origin remaining (pAG-P08 $\Delta$ oriLS20). Transformation into *B. subtilis* showed the following number of colonies were gained for each plasmid. Interestingly, there was only 1 transformant this time for the RSF1010 only plasmid but none in either of the negative controls so it is unlikely a persisting cell. What was consistent was the preference to grow on lower concentration of antibiotic compared to the positive controls. This 1 pGGR02 transformant could grow on own made 1  $\mu$ g/ml plates but not lab stock erythromycin/lincomycin still. However, plasmid extraction of these cultures used for further *E. coli* transformation yielded no colonies leading to hypotheses that the plasmid is either not there or too low in concentration to retrieve using column binding plasmid extraction or RSF1010 may be modified by *Bacillus* in order to replicate and survive antibiotic pressure.

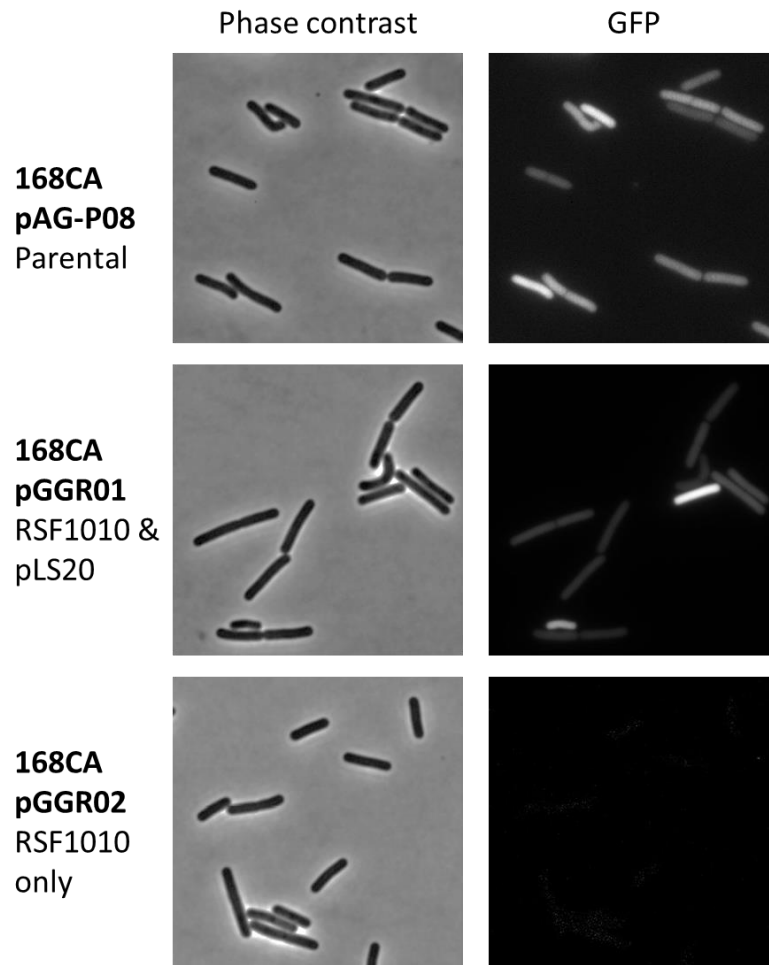
## Appendix 8 – RSF1010 replication in *B. subtilis*

Supplementary table 8.1. Transformation efficiency in sAG1333 with different plasmids replicons. sAG1333 was made competent by methods described in Chapter 2 using 1 µg of plasmid DNA per transformation, plates were incubated overnight at 37°C before counting colonies.

|                                                       | 1 µg/ml<br>erythromycin | 1 µg/ml<br>erythromycin | 0.5 µg/ml<br>erythromycin |
|-------------------------------------------------------|-------------------------|-------------------------|---------------------------|
| pGGR01<br>( <i>oriRSF1010</i> and<br><i>oriLS20</i> ) | 4                       | 5                       | 3                         |
| pGGR02<br>( <i>oriRSF1010</i> only)                   | 0                       | 0                       | 1                         |
| pAG-P08 ( <i>oriBR322</i><br>and <i>oriLS20</i> )     | 12                      | 11                      | 33                        |
| pAG-P08Δ <i>oriLS20</i><br>( <i>oriBR322</i> only)    | 0                       | 0                       | 0                         |
| 168 CA only                                           | 0                       | 0                       | 0                         |

To see if the plasmid was present, the strains were grown with IPTG for the induction of *P<sub>spac</sub>-gfp* which should show if the plasmid specific fluorescence was present. After growth overnight and subsequent dilution with IPTG induction, during mid-exponential phase the cultures were imaged. However, with microscopy analyses of GFP fluorescence there was no signal from the cells which was indicative that there was no plasmid. When the strain was used in plasmid extraction there was no colonies after transformation into *E. coli* whereas the positive controls showed >300 colonies per plate. Spontaneous resistance to low levels of erythromycin were not further studied but there is potential a recombination event may have occurred in order to withstand antibiotic selective pressure.

## Appendix 8 – RSF1010 replication in *B. subtilis*

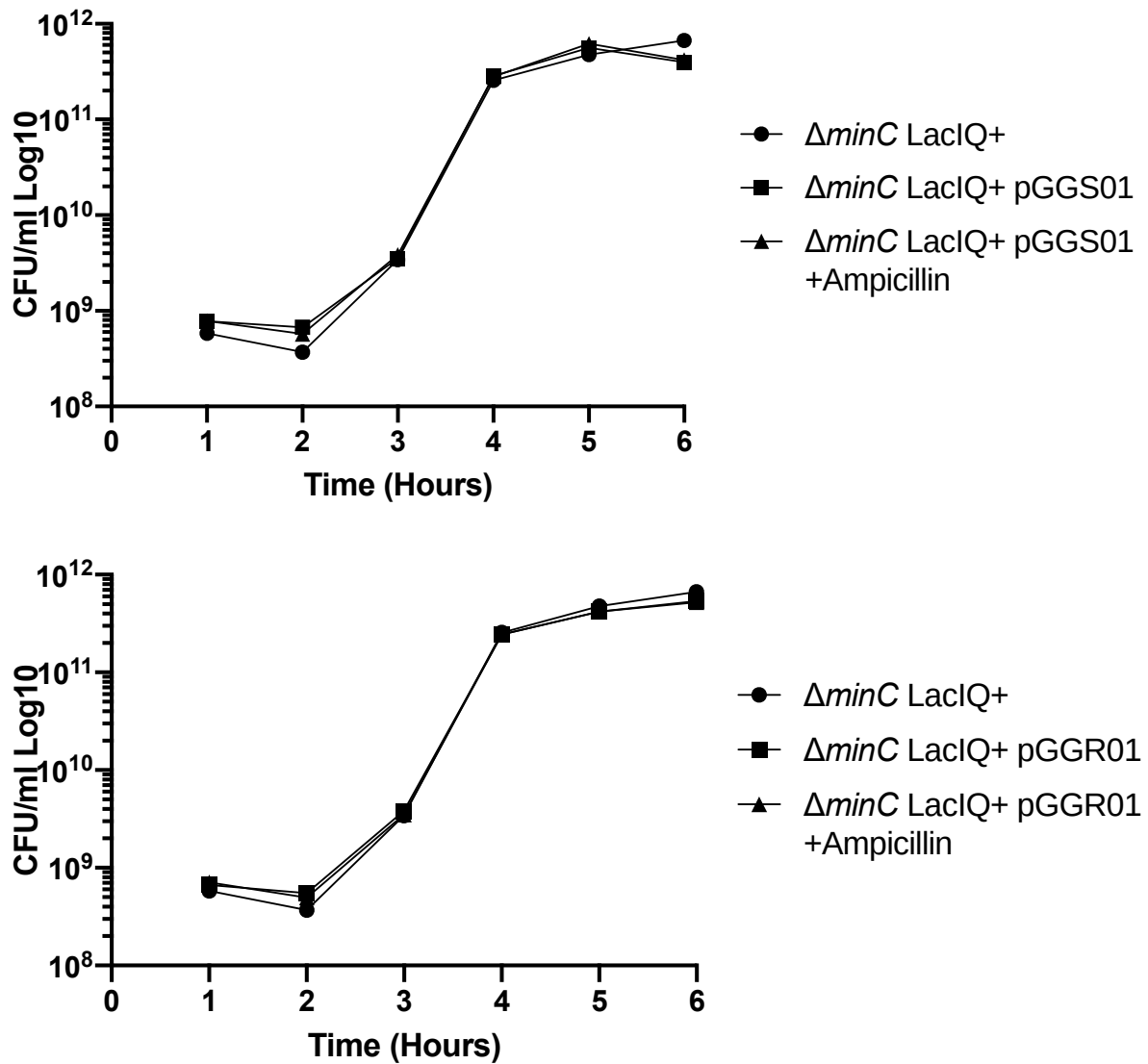


Supplementary figure 8.2. Plasmid specific GFP induction. Strains were grown in minimal media at 30°C with 0.5 µg/ml erythromycin selection. Images were taken during mid exponential growth with GFP excitation. pAG-P08 +*oriBR322* +*oriLS20*; pGGR01 +*oriRSF1010* +*oriLS20*; pGGR02 +*oriRSF1010*  $\Delta$ *oriLS20*.

## **Appendix 9 – 6-hour stability of plasmids in *E. coli***

**Short-term stability of plasmids with different replicons.** To ensure the new plasmids would be stable at low copy number, an assay was performed over a 6-hour period to distinguish if the absence of antibiotic selection shows a stability impairment. Liquid cultures were grown without the presence of antibiotic and plated onto nutrient agar and agar supplemented with ampicillin to calculate the colony forming units/ml, if the units decrease on antibiotic plates it indicates the plasmid is being lost. Negative control (*E. coli* without plasmid, data not shown on Logarithmic scale) did not yield any colonies on Ampicillin plates over the whole-time course. Both RSF1010 and SC101 driven plasmids increased the CFU/ml over the time course as expected, CFU/ml on both media followed one another very closely which indicated that there were no significant stability issues over 6 hours of growth in liquid without antibiotic selection in liquid.

## Appendix 9 – 6-hour stability of plasmids in *E. coli*



**Supplementary figure 9.1. Stability of plasmids over a 6-hour growth cycle.** Strains were grown in liquid culture with the absence of antibiotic selective pressure and culture were diluted each hour before spreading over nutrient agar plates with and without selection. Plates were grown overnight at 37°C and colonies counted next day, the colony forming units per ml (CFU/ml) were calculated and plotted using Graphpad prism 8 on a Logarithmic scale. (Technical repeats = 2 as plates were inoculated in duplicate, averages were obtained between plates and error bars represent standard deviation).

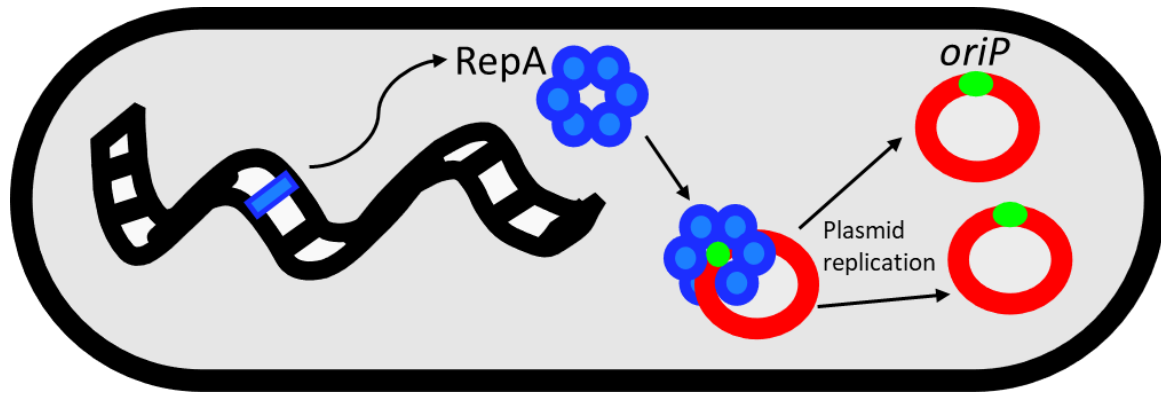


## **Appendix 10 - Universal replicon integration *E. coli***

### **Supplementary 10.1 Introduction**

As a collaboration within the Portabolomics project, the universal replicon strand of the project was integrated into the minicell producing system with LacI repressor (see section 1.5). The plasmid system was based on a plasmid known as pSH71, native to *Lactococcus lactis*, which is known to have broad host range and replicates in both *E. coli* and *B. subtilis* (Gasson, 1983; De vos, 1987). The minimal requirements for replication were determined via transposon mutagenesis and identified that RepA, which contains the replication origin, and RepB are essential (Hayes *et al*, 1990). The *L. lactis* plasmid pSH71 is known to use rolling circle replication, where replication first requires a replication protein (Rep) to nick the DNA at a site-specific location (Biswas *et al*, 2008; Kahn, 1997). The end result is an accumulation of linear concatenated plasmid which then requires recombination and DNA synthesis to resolve into circular double stranded replicons (see section 1.4.1).

As a response to Responsible Innovation (RI), the universal replicon system was designed with replication restrictions to specific hosts by splitting replication mechanisms into two parts. The replication initiation protein (RepA) was located on the chromosome, whereas the iteron sequences resided on the plasmid (*oriP*). This will ensure the broad host range plasmid cannot replicate in any other organisms than those engineered to possess the cognate replication protein, expressed from the chromosome. This was demonstrated in figure10.1 where the RepA protein is expressed, this could then interact with iteron sequences on *oriP* region of the plasmid. The plasmids are then able to replicate using host machinery such as DNA polymerase in methods shown by supplementary figure 10.1.



**Supplementary figure 10.1. Universal replicon split system.** Image adapted from Alex Johns (unpublished).

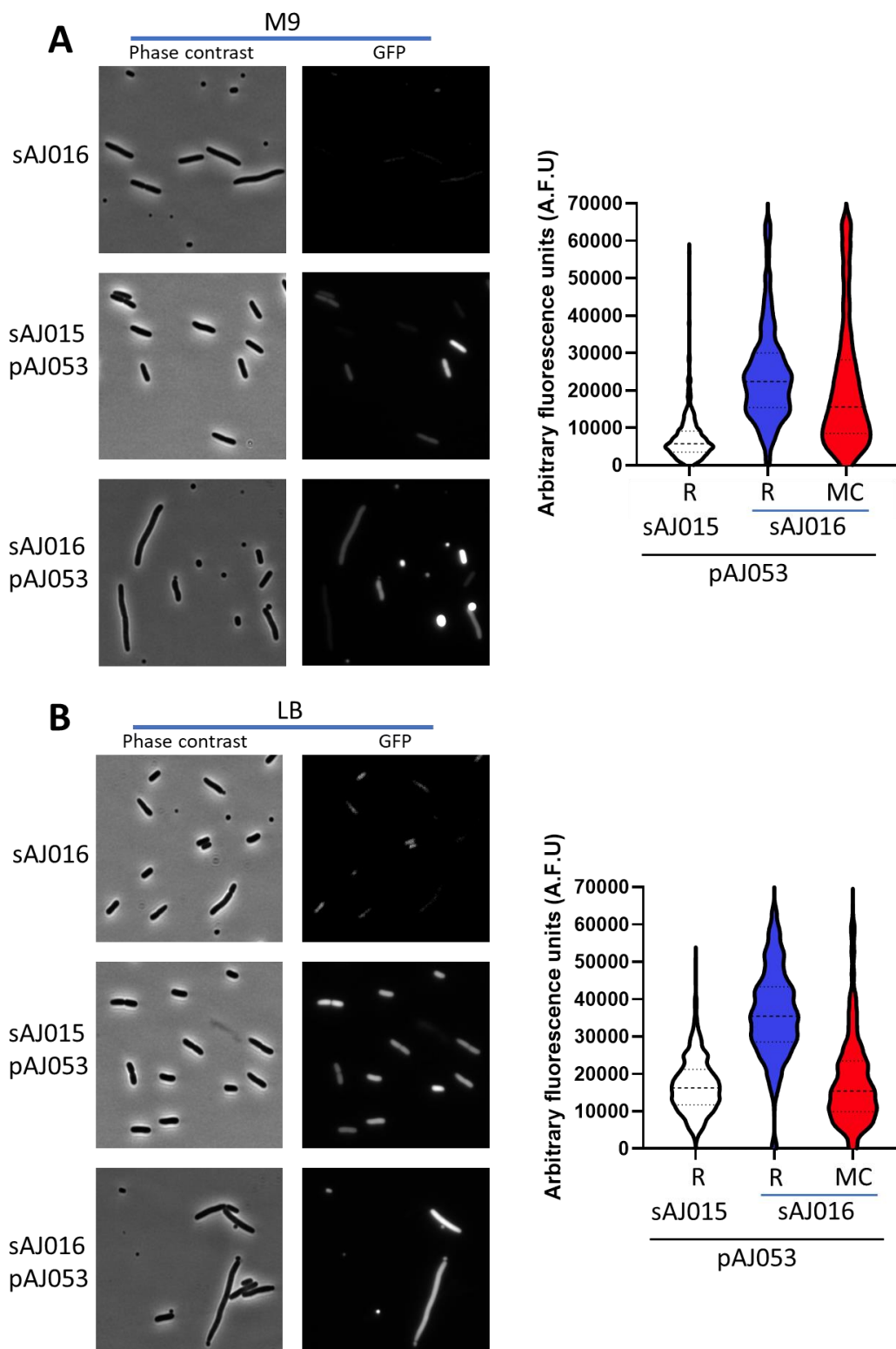
The replication initiation protein was expressed from the chromosome and nicks the plasmid DNA within the *oriP* iteron sequence. The plasmid then utilises host replication components to replicate the plasmid.

This system was integrated into the *B. subtilis* chassis with some issues overcome and low frequency of minicells gaining a plasmid but repression in rods was satisfactory. The aim of this study was to transfer and analyse this system in the minicell producing strain of *E. coli* and see if the minicells will independently express GFP from this minimal replicon successfully, similar to that seen in Chapter 5.

Unpublished work by Alex Johns showed that the native plasmid, in *E. coli*, was found to be present at ~25 copies per chromosome origin. However, when *repA* was relocated onto the host strain chromosome (fig.10.1) the plasmid copy number decreased to ~20 copies per cell. Later work indicated that inserting the coding sequence for *P<sub>spac</sub>-gfp* (pAJ053) reduced the copy number further, resulting in 10 copies/chromosome (A. Johns, unpublished).

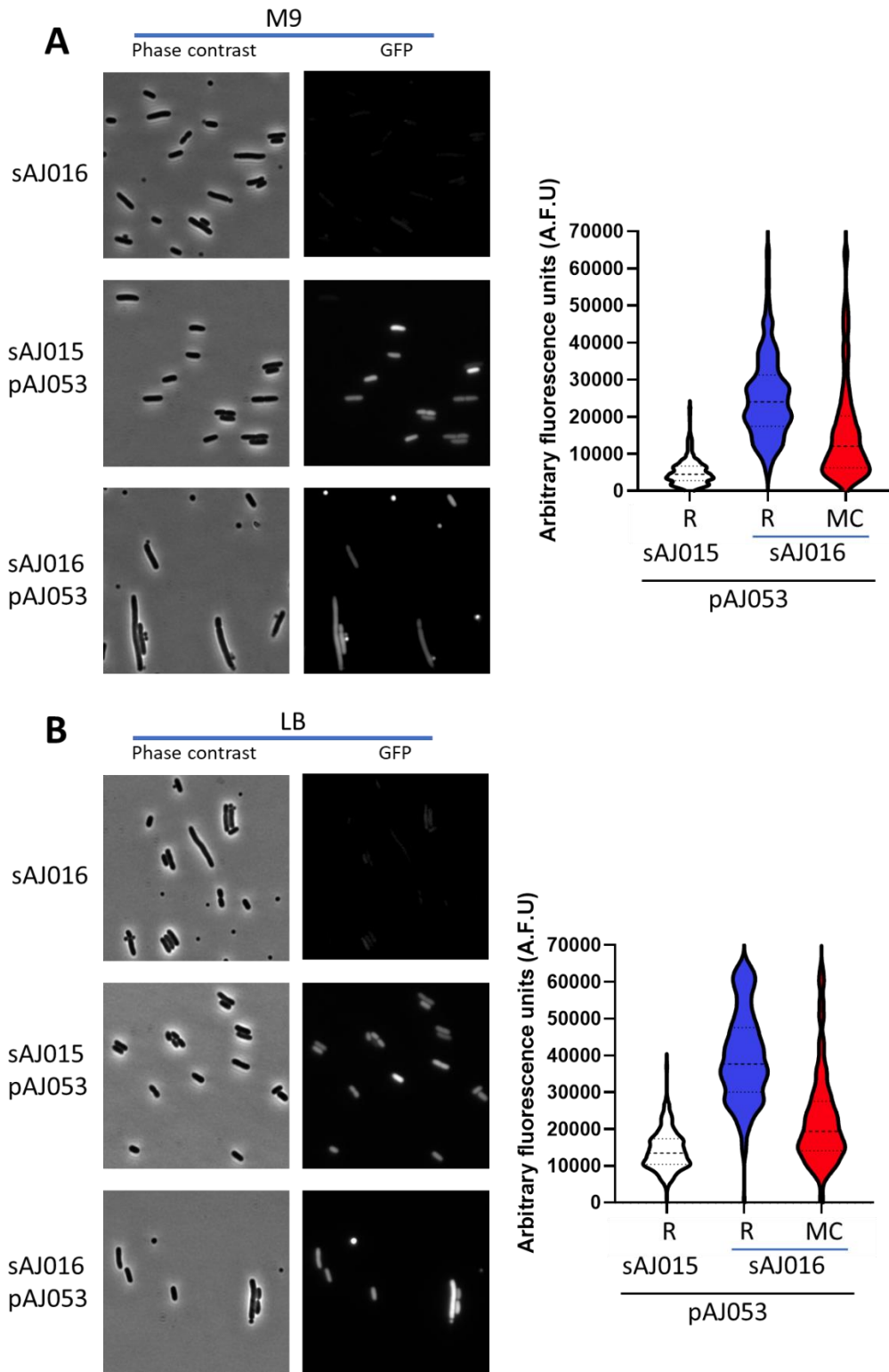
## 10.2 Time course microscopy in different media

Microscopy images were taken over 4 and 6 hours of growth to see how the fluorescence may change as growth progressed in the *E. coli* chassis. Different media were also tested to see how fluorescence intensity changed in populations as growth rates were different. After 4 hours incubation in LB media, sAJ015 pAJ053 was fluorescent but this was notably heterogeneous (middle panel fig.10.2B). This was even more evident in M9 media as there was noticeable variation between bright and dim rods. In the minicell producing strain (sAJ016 pAJ053 – bottom panel fig.10.2A), this variation was similar again in both media. As the culture was grown with antibiotic selective pressure fluorescence variation was not likely owing to plasmid instability. In M9 media, there were some minicells which were seen to be saturated (giving highest GFP intensity units). Cell population fluorescence measurements were analysed to see the effects of expression on these strains, it was immediately obvious that the M9 media (fig.10.2A) did show better repression as GFP wasn't as bright as LB grown cultures (fig.10.2B). However, minicells in either media did not seem to surpass the GFP signals determined for the rod cell population. This indicated that minicells are not gaining the plasmid and that fluorescence seen is likely compartmentalised cytosolic GFP from mother cells.



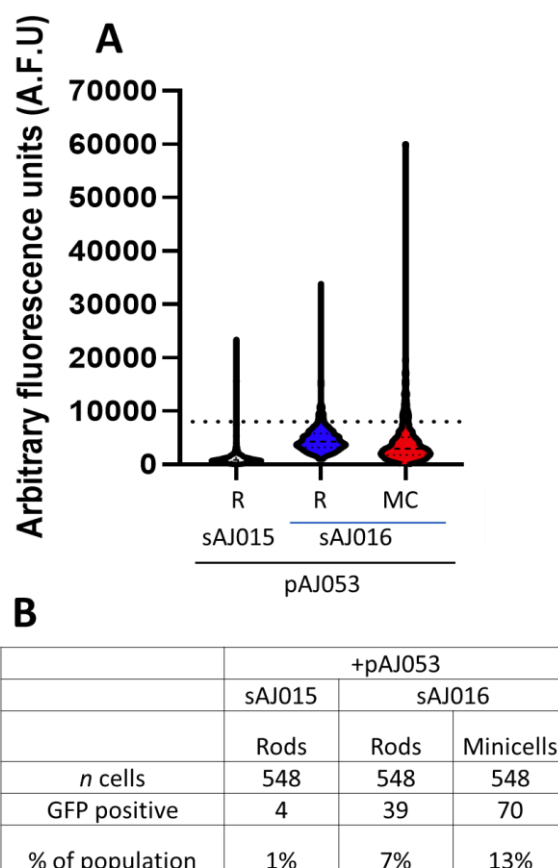
**Supplementary figure 10.2. Universal replicon fluorescence counting.** Cultures were grown in M9 minimal or LB rich media at 30°C. Mid-exponentially growing cells were imaged using a Nikon Ti eclipse with cool LED light source and the fluorescence was analysed using imageJ software. Graphs were plotted using Graphpad prism 9. **A)** images of cultures and fluorescence intensity from cultures grown in M9 media **B)** images of cultures and fluorescence intensity from cultures grown in LB media. N=500 cells per population.

When the cultures had grown longer and were at late exponential, almost at stationary phase, the strains were imaged again to see if there may have been any regulatory responses throughout growth. After 6 hours, sAJ015 pAJ053 was still showing heterogeneous expression in both rich and minimal media (fig.10.3A & B). Similarly, rod fluorescence of sAJ016 pAJ053 remained similar after 6 hours and remained heterogeneous. The minicell population also looked to be dimmer with less minicells reaching saturation that was likely a result of degradation of cytosolic GFP from rod cells. However, to determine this accurately, fluorescence measurements were analysed by measuring the mean fluorescence for each rod cell. This determined that growth in M9 media exhibited better repression compared to LB. In both media the populations looked similar to that observed after 4 hours of growth, and disappointingly, there was still an absence of minicells showing independent expression. The heterogeneous population seen by microscopy was hypothesised to be potentially from a burst of expression in some cells which may be mediated by increased replication of the plasmid. The RepA protein responsible for replication of the plasmid is controlled under a  $P_{spac}$  inducible promoter so it may also be possible there are too many binding sites for LacI which affects the regulation of GFP from the plasmid.



**Supplementary figure 10.3. Universal replicon fluorescence counting – late exponential phase.** Cultures were grown in M9 minimal or LB rich media at 30°C. Cells at late-exponential phase were imaged using a Nikon Ti eclipse with cool LED light source and the fluorescence was analysed using imageJ software. Graphs were plotted using Graphpad prism 9. **A)** Cultures grown in M9 media **B)** Cultures grown in LB media. N=500 cells per population.

The microscopy time course showed that the best conditions were to grow the strain in M9 minimal media into mid-exponential growth. As many cells were reaching saturation, the fluorescence excitation exposure was reduced 10-fold for a reproduction. Reducing the exposure helped to distinguish the populations better, there were no cells reaching saturation. The WT RepA<sup>+</sup> strain had a lower fluorescence intensity compared to the rods from the minicell producing strain. This could indicate that the  $\Delta minC$  mutation is affecting the strains expression that may be related to stress or other factors. Rod cell mean fluorescence increased greater than that of minicells mean, however, it was evident that the minicell fluorescence was distributed over a wider range with a significant number of outliers. This indicated that the plasmid segregation into minicells was not frequent.



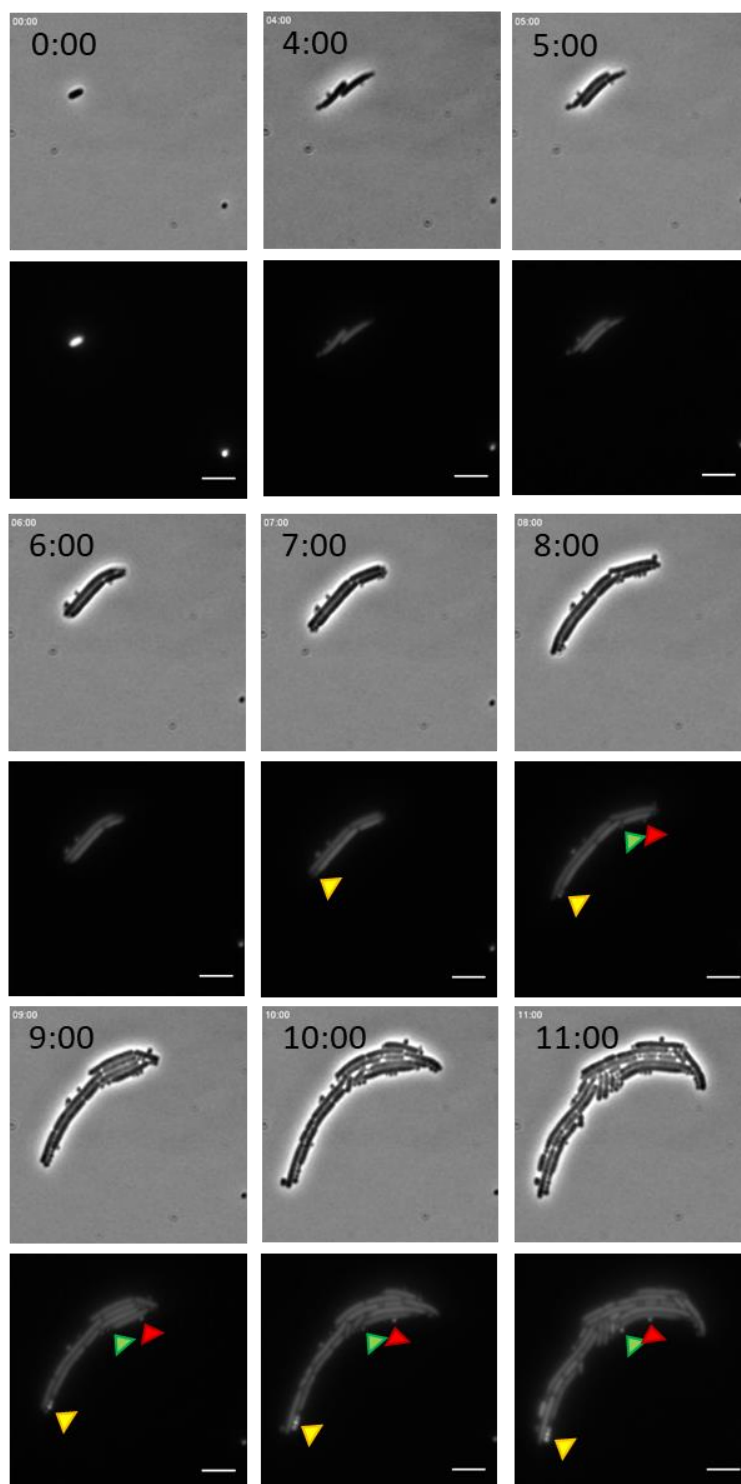
**Supplementary figure 10.4. Universal replicon fluorescence counting.** Cultures were grown in M9 minimal at 30°C. Mid-exponentially growing cells were imaged using a Nikon Ti eclipse with cool LED light source (10-fold reduced exposure) and the fluorescence was analysed using ImageJ software. Graphs were plotted using

GraphPad prism. **A)** images of cultures for phenotypic analyses **B)** plots of cell counting **C)** table of cells counted and calculation of those measured as ‘GFP positive’.

### 10.3 Time lapse microscopy

Analyses at the single cell level was done to see if minicells did express GFP independently and to understand the cause of rod cell fluorescence heterogeneity seen in fig.10.2. This time-lapse would determine if GFP was expressed independently from minicells or if all signals seen were derived from rod cell cytoplasmic GFP compartmentalised at division. The time-lapse clearly demonstrated minicells independently expressing GFP; yellow, green and red arrows indicate minicells with increasing fluorescence intensity over time. It was also clear when a minicell was made, it reflected the same GFP fluorescence properties as the rod showing that minicells in these images do independently express GFP. This would also explain why average minicell fluorescence was much lower than rod cells as a minicell was produced with the same GFP intensity. Subsequently, the signal was rapidly reduced as GFP seemed to be degraded when no plasmid was present to express more. At the beginning it was interesting to see that the rod cell was very bright, but fluorescence reduced rapidly. This may be due to bleaching or potentially, since there was a varying increases and decreases in random cells, there may have been a surge of plasmid replication which then diluted out as the cell elongated before dividing. Then the process occurred again which gave the appearance of cells “turning off and on”. This may explain the heterogeneity of the population fluorescence, exhibited in microscopy at single time points as some images captured cells before GFP was titrated. After 11 hours, it was evident that this plasmid was extremely inefficient at segregating into minicells for independent expression. From this time-lapse population it was shown, out of 29 minicells, only 17% of this population were positive for GFP expression.

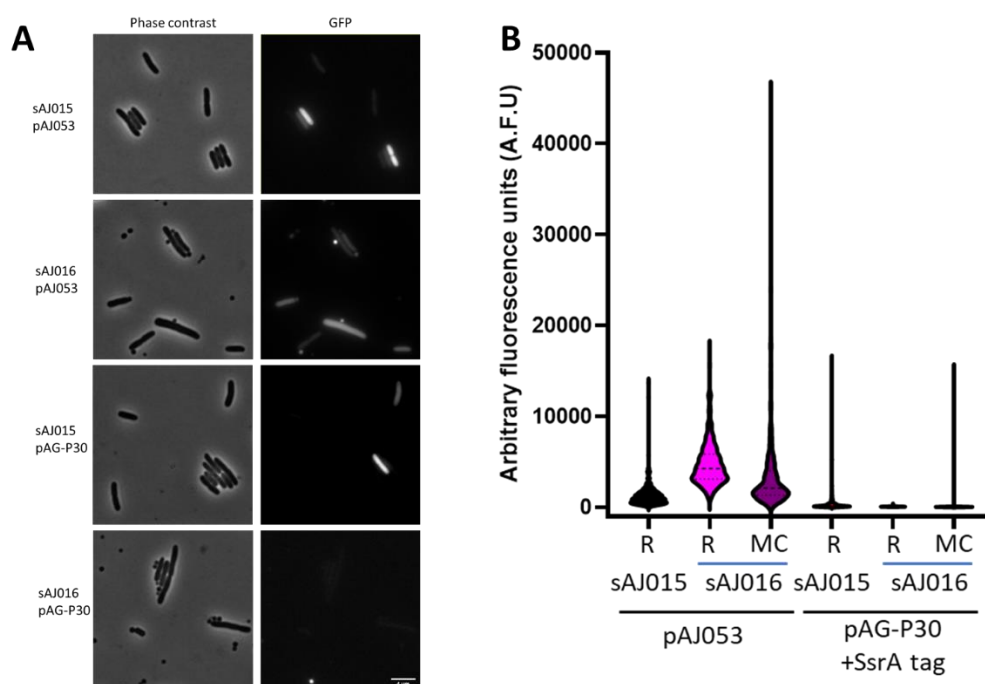




**Supplementary figure 10.5. Time-lapse analyses of *E. coli* with split universal replicon system.** The *E. coli* strain was diluted back from an overnight culture before being grown in fresh M9 minimal media for 2 hours. OD600 was ~0.1 before applying 1  $\mu$ l of cellular suspension to a M9 agarose pad and imaging using a Nikon Ti eclipse with cool LED light source. The scale bar is representative of 4  $\mu$ m and is applicable to all images. Time in the top left represents hours and minutes (HH:MM).

### 10.4 Addition of a degradation system

As it had been suggested that minicells were not gaining plasmids, it was hypothesised that rod cells GFP intensity may have been masking minicells independently expressing GFP. Therefore, to see if exploiting the degradation of cytosolic GFP would reveal more minicells independently expressing, an SsrA tag was added. The replicate of the pAJ053 minimal replicon showed the same phenomenon as previously described where minicells were significantly lower in fluorescence compared to the rod cells. The SsrA tag showed a dramatic decrease in GFP intensity with a minimal amount of minicells seemingly above this threshold (6%), this indicated that the amount of minicells independently expressing was only around 6% of the population. This supports the hypothesis that the universal replicon did not segregate into minicell efficiently.



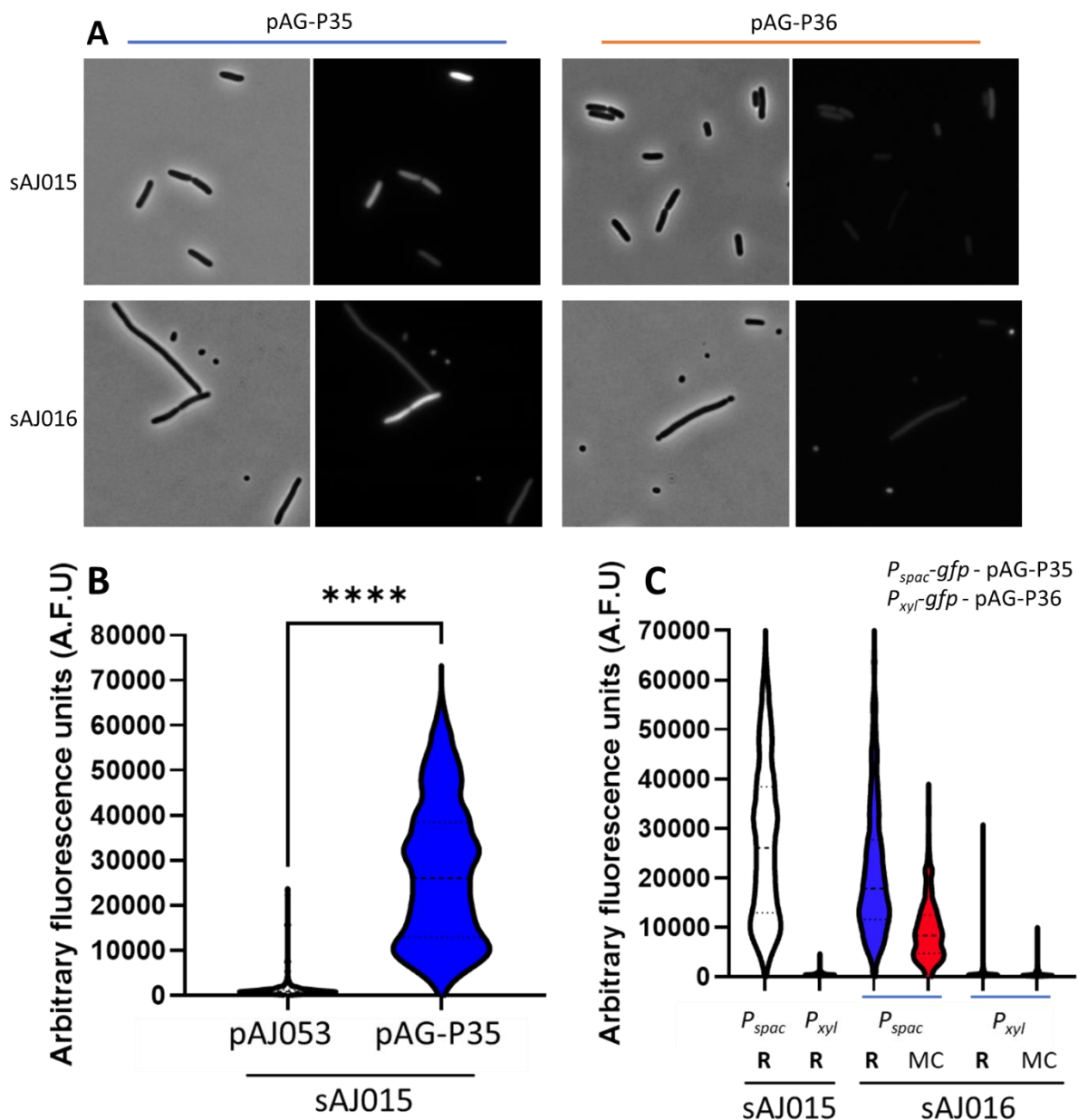
**Supplementary figure 10.6. Reporter degradation to reveal minicell expression.** The strains with and without the addition of SsrA tagged GFP were grown in M9 minimal media and imaged during mid-exponential growth. Images were analysed using ImageJ software and plots were made using GraphPad prism 9. Scale bars are

representative of 4  $\mu\text{M}$  and applicable to all images. **A)** Images of cells for phenotypic comparisons **B)** comparisons of plasmids with and without SsrA tags on GFP reporters.

### 10.5 Segregation system addition

To see if Alp7AR segregation system would increase minicells gaining a plasmid the operon was integrated onto the minimal replicon. The microscopy images (fig.10.7A) did not show an effect on the  $P_{spac}$  inducible variant. However, closer investigation of rod cell fluorescence in pAJ053 and pAG-P35 showed a large increase in heterogeneity and expression with Alp7AR (pAG-P35; fig.10.7B). This was probably a result of stress induction in the strain which could be caused by Alp7AR overexpression causing the strain to be sick, as published by Dermain *et al*, 2012, as it negatively effects chromosome segregation and division.

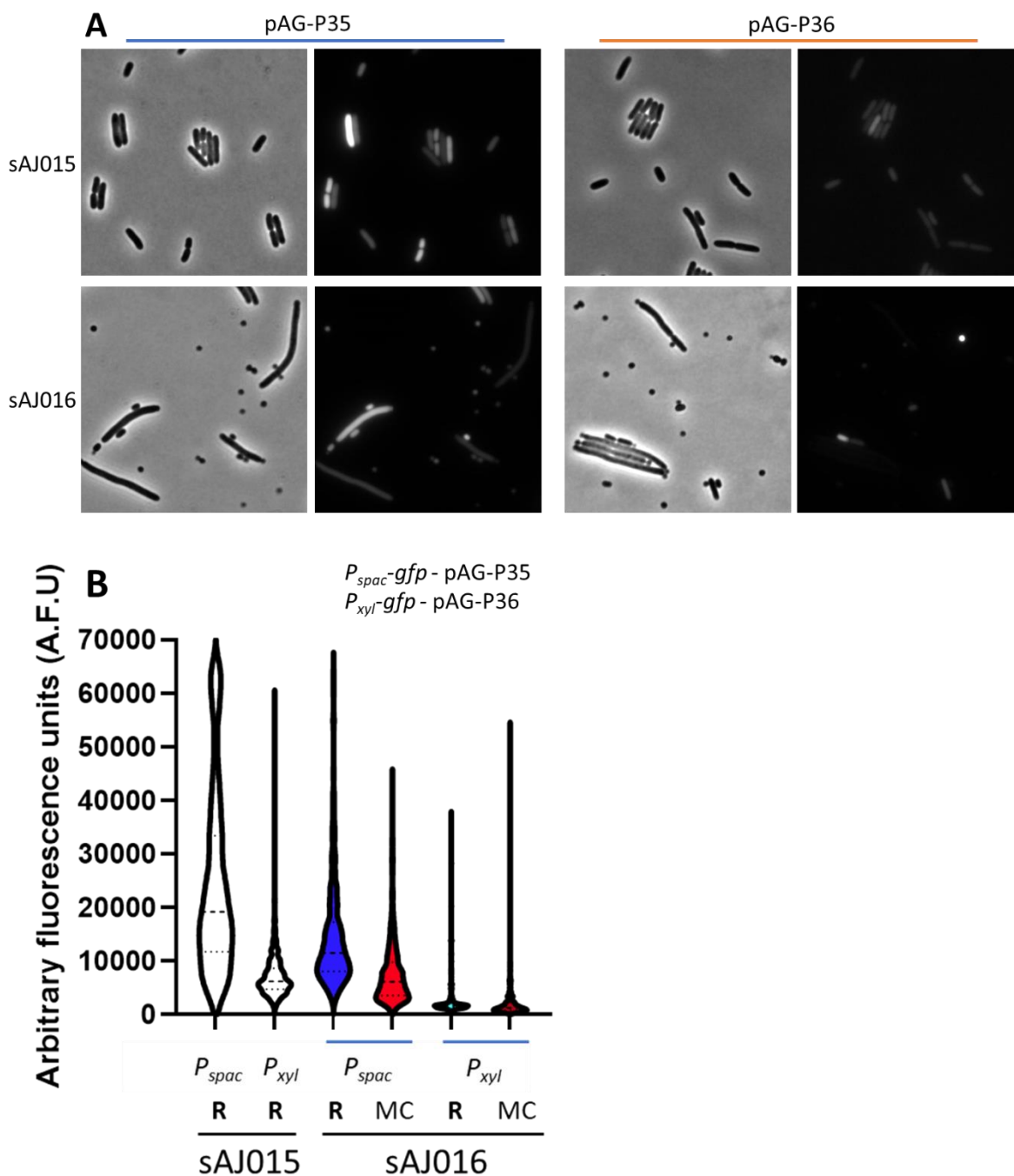
A plasmid alternative was made to replace the  $P_{spac}$  inducible promoter to see if  $lacI^q$  was responsible for the lack of repression as the plasmid copy number was too great to regulate (pAG-P35). The plasmid was made and tested by A. Guyet (unpublished) and found that the repression was too tight as rod cells were not fluorescent, but neither were any minicells which indicated that the plasmid was not entering cells. The Alp7AR operon was inserted into this plasmid to see if minicells expressing would be more prominent with the segregation system. The Alp7AR operon was controlled under the promoter intended for *B. subtilis* derived from pLS20 and the same as what was used in pGGR01 (Chapter 5). To see if the system would benefit from Alp7AR, a time course experiment was performed again looking at mid-exponential and late-exponential growing cells to see how fluorescence changes in rods and minicells. From microscopy images it was immediately evident that pAG-P36 ( $P_{xyl}$  promoter) GFP expression was greatly repressed in rods compared to pAG-P35 ( $P_{spac}$  promoter), from both vectors it was obvious that minicells do have some fluorescence. Nevertheless, the minicells expression from the vector with  $P_{xyl}$  controlled reporter (pAG-P36) did not show a large increase in independent minicell expression.



**Supplementary figure 10.7. Segregation system addition and promoter change.** Strains were grown in M9 minimal media and imaged after 4 hours of growth, during mid-exponential phase. Cells were applied to a 1.2% agarose pad to image using a Nikon Ti eclipse with cool LED light source. **A)** Images of cells for phenotypic comparisons **B)** comparisons of the pAJ053 vector without and with the Alp7AR operon **C)** comparisons of plasmids with Alp7AR and experimenting with different GFP inducers.

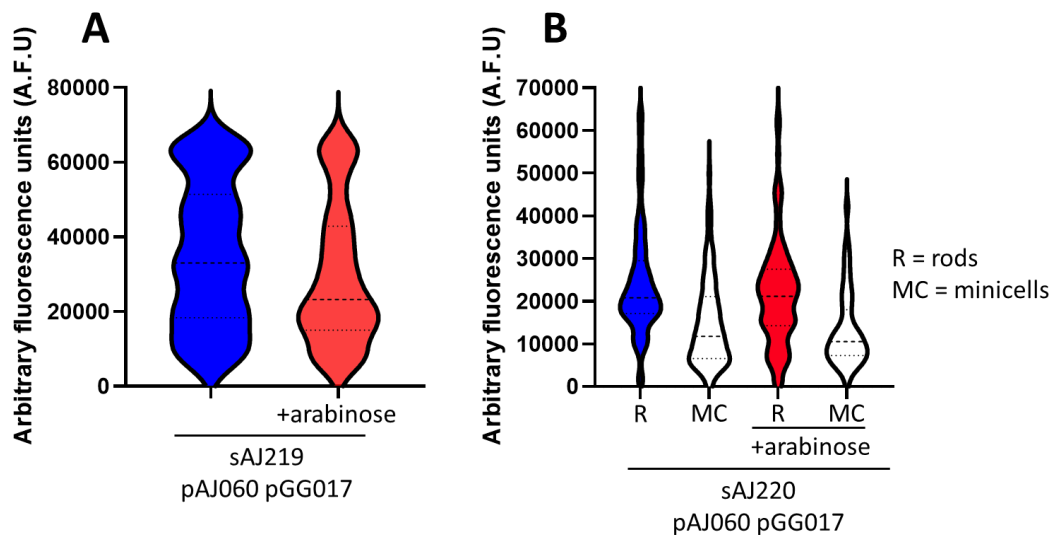
After 6 hours of growth, the average of rod cells fluorescence from sAJ016 with pAG-P35 ( $P_{spac}$ -*gfp*) was significantly lower than rod cells ( $P < 0.0001$ ) and was lower than minicells at the 4-hour time point. The rod population from sAJ016 fluorescence decreased significantly after a further 2 hours of growth (fig.10.8 blue plot). In contrast, the pAG-P36 ( $P_{xyl}$ -*gfp*) had increased fluorescence significantly in all populations after 6 hours of growth, noticeable in figure 10.8.

In the WT strain with pAG-P36 ( $P_{xyl}$ -*gfp*), there was a large increase in fluorescence after a further 2 hours growth as the population was not as tightly grouped ( $P < 0.0001$ ; fig.10.8B). A similar effect was seen in sAJ016 where the rods were not as dim or tightly grouped and more of the population was heterogeneous, indicating that repression of the  $P_{xyl}$  promoter is not as tight over time. A change in the minicell populations of the strain with pAG-P36 ( $P_{xyl}$ -*gfp*) was also observed where more minicells are brighter with a single outlier brighter than any of the rod cells. This indicates that there was independent GFP expression from a minicell, however, it is extremely inefficient as ~7% of the minicell population and 10% of the rods were counted GFP positive. Therefore, the Alp7AR system did not aid in minicells gaining a plasmid but rather seemed to cause more disruption to the system. The  $P_{xyl}$  promoter has proven to be too tight for expression in minicells, the Alp7A filament caused an over expression phenotype with the  $P_{spac}$  driven plasmid which was not emulated in the  $P_{xyl}$  implying that the promoter is so tightly repressed that filament disruption did not cause an effect. The repressor of this  $P_{xyl}$  promoter is not known so screening tests are being performed from strains in the Keio knockout collection to see if this can be identified and potentially altered to increase sensitivity within the system (Aur lie Guyet, unpublished).



**Supplementary figure 10.8. Later growth phase analyses of segregation system addition and promoter change.** Strains were grown in M9 minimal media and images after 6 hours of growth during mid-exponential phase. Cells were applied to a 1.2% agarose pad to image using a Nikon Ti eclipse with cool LED light source. **A)** Images of cells for phenotypic comparisons **B)** comparisons of plasmids with A17AR and experimenting with different GFP inducers.

Another segregation system was designed to address the potential issues of Alp7A overexpression. This system was derived from the native *B. subtilis* system where it was found that RacA is a chromosome anchorage protein which aids in segregation during sporulation (Ben-Yehuda *et al*, 2003). The binding sites of RacA were identified using Chromatin immunoprecipitation (ChIP) and showed that RacA binds to a 105 bp area of the *ywjI* coding sequence (Ben-Yehuda *et al*, 2005). RacA then interacts with DivIVA which is associated with the curved membranes at the poles to anchor the chromosome (Ben-Yehuda *et al*, 2003). This system had been shown to work within *E. coli* demonstrated by Lenarcic *et al*, 2009. Therefore, two plasmids were integrated into RepA<sup>+</sup> strains. The universal replicon had the 106 bp region cloned for RacA binding, whereas the second plasmid, pGG017, had arabinose inducible DivIVA/RacA to move the plasmids to the poles. However, this system did not increase the number of minicells gaining a plasmid as there was a large increase in expression from the sGG219 which may be a result of stress from harbouring 2 plasmids. Minicells from sGG220 did not increase the amount of independent expression seen and the average minicell fluorescence was still significantly lower than that of the rod cells ( $P < 0.001$ ).



**Supplementary figure 10.9. DivIVA/RacA segregation of the universal replicon.** Strains were all co-transformed with DivIVA/RacA plasmids and the universal replicon. Arabinose inductions included the addition

of 0.02% arabinose to the media. For imaging, the strains were grown in M9 minimal media and mounted onto 1.2% agarose pads after 4 hours of growth. Fluorescence was analysed using ImageJ and plots were constructed using GraphPad prism 9. N=100 cells per population.

### 10.6 Conclusions and discussion

In this chapter, an integration within the Portabolomics project was attempted within the universal replicon strand and minicell compartmentalisation. The universal replicon did not seem to work as efficiently as vectors tried earlier within this study (Chapter 5), and there did not seem to be efficient minicell plasmid gain. The universal replicon was not well understood but was known to replicate via rolling circle, as opposed to the theta-type replication methods studied in Chapter 5. There was clearly a discrepancy between the two types of replicon and how they work/segregate in the minicell producing strain.

As mentioned within the introduction, the plasmid replicated through a rolling circle mechanism. This produced single stranded intermediates which was hypothesised to be diluting out LacI within the cytoplasm and acting as a higher copy number variant. There was no upregulation in minicell expression with the  $P_{xyl}$  promoter which showed that it was unlikely many minicells were gaining a plasmid. However, it was unknown which repressor was acting upon the  $P_{xyl}$  promoter. As both the RSF1010 driven plasmid used within Chapter 5 and pAJ053 had been described as 10-copies per genome, it was hypothesised they should act similarly, however, the rolling circle mechanisms clearly disrupted the system and showed these two plasmids were not comparative.

It was clear in this strain that there were regulation issues but also very inefficient segregation which would require more work to understand why the vector performed the way this study had seen. Future work within the strains would be useful to localise the plasmid, this could help to understand why the yield of minicells with a plasmid was so low. It was clear that minicells could independently express from the plasmid but this was very inefficient seen by time-lapse



analyses (see section 10.3). More work on copy number would be required throughout growth as it could be that there was an increase at certain points throughout growth, southern blot analyses would also be useful for detection of single stranded intermediates and their frequency.