



**The Application of Antisense Silencing for the  
Characterisation of Essential Gene Stringency  
and for the Development of Species–Specific  
Antimicrobials**

**Ashley John Chessher (BSc, MSc)**

A thesis submitted for the degree of Doctor of Philosophy (PhD) at  
Newcastle University

School of Biology  
Newcastle Institute for Research on Sustainability (NIReS)

October 2013

## Abstract

The emergence of multiple-antibiotic resistance among clinical pathogens has created an urgent requirement for the development of new antibiotics. The current lack of new antibiotics has not only renewed interest in traditional natural product screening approaches, but also prompted efforts to develop alternate antimicrobial strategies.

Antisense RNA based silencing provides a strategy for developing whole cell screening assays, whereby antisense RNA induction leads to target protein depletion and subsequently the increased sensitivity of test organisms to target specific inhibitors. The development of synthetic derivatives to expressed antisense RNA such as peptide Nucleic Acids (PNA), has also been explored for use as bacterial inhibitors. This thesis aims to examine two novel antimicrobial strategies, firstly by comparing mRNA and protein based techniques to evaluate essential gene requirement in bacteria, to identify novel targets for antibiotic screening assays. Secondly, to evaluate the potential use of peptide peptide-PNA's as antimicrobials capable of targeting individual bacterial species.

To successfully develop either approach requires the identification and validation of suitable gene encoded molecular targets. Essential genes may provide potential candidates, yet a suitable system is necessary for characterisation to enable genes to be ranked, so that the most suitable targets can be prioritized. A disproportionate growth requirement (stringency) is known to exist among essential genes, which provides a means to delineate between essentially required targets, yet is based upon the measurement of mRNA abundance. Due to post-transcription and translation mechanisms, mRNA does not provide a reliable indicator of expressed protein, which represents the ultimate output of gene expression.

This study demonstrates the use of a quantitative proteomics strategy for evaluating essential gene stringency at the protein level, using the *E.coli* gene *fabI*. Using expressed antisense RNA silencing to deplete target protein concentration and to reduce normal growth rate to 50%, absolute protein determinations were used to define a Minimum Protein Level (MPL<sub>50</sub>), for the quantitative characterisation of essential gene stringency. To support the

justification of evaluating gene stringency using expressed protein abundance, the stringency of operon based genes *fusA* and *rpIE* using antisense RNA silencing was investigated and revealed transcript profiles that contradict the use of Minimum Transcript Level (MTL<sub>50</sub>) previously used to define gene stringency.

Finally, to demonstrate a potential application that would benefit from the characterisation of essential gene stringency, the species-specificity of a peptide-PNA targeting the essential gene *ftsZ* was evaluated. Exposing a mixed culture of *S.typhimurium* and *E.coli* to a peptide-PNA conjugate, incorporating a 2 base pair mismatch demonstrated the capacity to inhibit translation of *ftsZ* in *S.typhimurium* but not *E.coli*.

This study highlights how characterising essential genes using the MPL<sub>50</sub> can be used to delineate stringently required gene targets to support antimicrobial screening and the development of species specific antimicrobials. Furthermore the applications of evaluating gene stringency may be extended further, to provide a tool for standardising genetic components in synthetic biology approaches.

## Acknowledgements

This thesis was in many ways a collaboration, since it was completed with the extensive support and advice from many individuals, for which I am eternally grateful.

I would like to thank both my supervisors Dr Jem Stach and Dr Liam Good, who provided me with a rare opportunity to contribute to an important scientific field, but also my own personal growth. It has been a once in a life-time experience.

I wish to thank the BBSRC for providing the generous financial support throughout my four years of study and to Newcastle University, especially the School of Biology. Also thanks to the Society of General Microbiology for providing assistance in attending conferences. Special thanks to Ros Brown for all the help and support she provided, and thanks also the technicians Miriam, Sue and Anne, for turning a blind eye to all the reagents and consumables I “borrowed” during my PhD.

My thanks to Dr Shan Goh for her patience and time in providing the training essential to the thesis. Special thanks to Dr Achim Treumann, for the abundance of aid provided in interpreting Mass spectra. Thanks also to Dr Martin Edwards for providing training and help, especially during my first few months in the Devonshire.

Thanks to everyone in the Devonshire building, especially everyone in the 4<sup>th</sup> floor Lab. Special thanks to Shreya for all the support she has provided, conferences will never be the same again.

I would like to thank my sister Marina for her support and my parents John and Lesley Chessher for their support; in particular the 5 star quality of the accommodation and hospitality of their “hotel” throughout the duration of my PhD (may it never end).

Lastly I would like to thank my long suffering Maddie (Boobaa) for putting up with the mood swings, frustration and the myriad of difficulties associated with individuals undertaking a PhD.

*Ashley Chessher, September 2013*

## List of Abbreviations and Symbols

°C	Degree Celsius
a, A	Adenine
ACP	Acryl Carrier Protein
Amp	Ampicillin
ATCC	American Type Culture Collection
ATP	Adenosine-5'-triphosphate
AUG	Translation start codon
bp	Base pair
BSA	Bovine Serum Albumin
c, C	Cytosine
cDNA	Complementary DNA
Cfu	Colony forming unit
CoA	Coenzyme A
DHF	7,8-dihydrofolate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dTMP	Deoxththaminemonophosphate
DTT	Dithiothreitol
dUMP	Deoxyuracilmonophosphate
EDTA	Ethyldiaminetetraacetic acid
ESBL	Extended-spectrum $\beta$ -lactamases
GFP	Green Fluorescent Protein

g, G	Guanine
HGT	Horizontal Gene transfer
IPTG	Isopropyl $\beta$ -D-1-thiogalactopyranoside
$^{15}\text{N}$	Isotopically labelled nitrogen
kb	Kilobase
kDa	Kilodalton
l	Litre
LB	Luria-Bertani
LC-MS/MS	Tandem mass spectrometry
LPS	Lipopolysaccharride
M	Molar
MCS	Multiple Cloning Site
MDR	Multi-drug Resistance
mg	Milligram
MHB	Mueller-Hinton Broth
MIC	Minimum Inhibitory Concentration
ml	Millilitre
mM	Millimolar
mmol	Millimole
mRNA	Messenger RNA
MW	Molecular Weight
MRSA	Methicillin Resistant <i>Staphylococcus aureus</i>

n/a	Not Applicable
NADH	Nicotinamide Adenine Dinucleotide
ng	Nanogram
nM	Nanomolar
nt	Nucleotide
OD	Optical Density
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PMO	Phosphorodiamidate Morpholino Oligomers
PNA	Peptide Nucleic Acid
PT	Paired Termini
PTasRNA	Paired Termini antisense
RBS	Ribosome Binding Site
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
R	Resistance
RPM	Revolutions Per Minute
RT	Reverse Transcriptase
RT-PCR	Reverse Transcriptase PCR
SD	Standard Deviation
SE	Standard Error

t,T	Thymine
TBE	Tris-Borate-EDTA
TCA	Tricarboxylic Acid Cycle
THF	5,6,7,8-tetrahydrofolate
$\mu\text{l}$	Microlitre
$\mu\text{m}$	Micrometer
$\mu\text{M}$	Micromolar
u,U	Uracil



## Table of Contents

<b>Abstract</b>	<b>i</b>
<b>Acknowledgements</b> .....	<b>iii</b>
<b>List of Abbreviations and Symbols</b> .....	<b>iv</b>
<b>List of Figures</b> .....	<b>xii</b>
<b>List of Tables</b> .....	<b>xv</b>
<b>Chapter 1 Introduction</b> .....	<b>1</b>
1.1 Antibiotics overview .....	1
1.1.1 Antibiotic origins, function & resistance .....	1
1.1.2 Antibiotic mode of action .....	3
1.1.3 The emergence of antibiotic resistance .....	5
1.1.4 The antibiotic discovery paradigm .....	9
1.1.5 Possibilities to exploit new sources of antibiotics .....	10
1.1.6 Antibiotic screening approaches.....	14
1.2 Searching for new antibiotics .....	17
1.2.1 Identification of novel antibiotic targets.....	17
1.2.2 Antisense RNA .....	22
1.2.3 Antisense RNA quantitative effects .....	26
1.3 The use of mass spectrometry in antibiotic discovery.....	30
1.3.1 Tandem mass spectrometry .....	30
1.3.2 Proteomic strategies.....	32
1.3.3 Mass spectrometry quantification strategies.....	33
1.4 Gene silencing using synthetic antisense structures .....	36
1.5 Project outline .....	40
1.6 Specific aims and objectives.....	45
1.7 Thesis Outline.....	45
<b>Chapter 2 Methods &amp; Materials</b> .....	<b>47</b>

2.1 Selection of essential gene targets for silencing.....	47
2.1.1 Bacterial growth conditions and strains .....	48
2.1.2 Bacterial genomic DNA and plasmid extraction.....	48
2.1.3 Primer preparation and annealing temperature optimization .....	49
2.1.4 Agarose gel electrophoresis.....	50
2.1.5 Cloning of antisense and ORF sequences into pHN678 and pBAD plasmids.....	50
2.1.6 Restriction digest of PCR inserts and plasmids.....	51
2.1.7 Ligation of PCR inserts into plasmids.....	51
2.1.8 Transformation of antisense and transcomplementation plasmids into E.coli .....	52
2.2 Evaluation of antisense specificity of <i>fusA</i> and <i>rplE</i> constructs .....	52
2.3 Validation of antisense specificity by transcomplementation .....	53
2.4 Delineation of the inhibitory effects of silencing essential gene targets <i>fusA</i> and <i>rplE</i> .....	54
2.5 Evaluation of essential gene stringency by quantitative assessment of mRNA transcripts .....	54
2.5.1 Isolation and clean-up of total RNA .....	54
2.5.2 cDNA synthesis of extracted RNA.....	56
2.5.3 Relative quantification of mRNA transcripts by QPCR .....	56
2.6 Evaluation of essential gene stringency by protein quantification.....	62
2.6.1 Development of a <sup>15</sup> N labelled protein internal standard .....	62
2.6.2 Extraction of <sup>15</sup> N labelled protein internal standard from E.coli Lysate using IMAC.....	62
2.6.3 SDS-PAGE of extracted <sup>15</sup> N labelled protein internal standards .....	63
2.6.4 Purification and concentration of <sup>15</sup> N labeled protein internal standards .....	63
2.6.5 Antisense induction for protein and RNA quantification.....	64
2.6.6 Protein digestion and SDS-gel extraction from E.coli lysate.....	64

2.6.7 Evaluation of representative tryptic peptides to use for the identification and quantification of <sup>15</sup> N labelled protein internal standard and for the assessment of matrix effects .....	65
2.6.8 Quantification of FabI Internal Standard .....	66
2.6.9 LC-MS/MS Analysis of FabI protein in silenced samples .....	66
2.6.10 Whole cell screening assays using triclosan and phosphomycin ...	67
2.7 Assessment of target specificity using PNA.....	67
2.7.1 Design of Species-specific PNA .....	68
2.7.2 Transformation of <i>S. typhimurium</i> and <i>E.coli</i> with reporter gene plasmids .....	68
2.7.3 Mixed microbial assay .....	69
2.8 Statistics .....	69
<b>Chapter 3 Results .....</b>	<b>71</b>
3.1 Evaluation of gene silencing of <i>fusA</i> and <i>rplE</i> .....	71
3.1.1 Specificity of expressed antisense RNA constructs.....	71
3.1.2 Validation of antisense construct specificity by transcomplementation .....	74
3.1.3 Delineating inhibitory effects caused by gene silencing of <i>fusA</i> and <i>rplE</i> .....	79
3.1.4 Evaluation of gene stringency of <i>fusA</i> and <i>rplE</i> using Mean Transcript Level 50 (MTL <sub>50</sub> ).....	83
3.2 Development of LC-MS/MS protein quantification method .....	88
3.2.1 <sup>15</sup> N labelled protein internal standard preparation for LC-MS/MS .....	88
3.2.2 Confirmation of complete <sup>15</sup> N labelling of protein internal standards .....	92
3.2.3 Selection of peptides for quantification of protein internal standard by LC-MS/MS.....	100
3.3 Quantification and assessment of gene stringency using protein abundance.....	112
3.3.1 Quantification of <sup>15</sup> N labelled FabI protein internal standard .....	112

3.3.2 Quantification of FabI protein per cell following gene silencing of <i>fabI</i> in <i>E.coli</i> .....	114
3.3.3 MTL <sub>50</sub> and MPL <sub>50</sub> for evaluating stringency of the essential <i>E.coli</i> gene <i>fabI</i> .....	121
3.3.4 Antisense RNA based whole-cell screening assays using triclosan and phosphomycin .....	124
3.4 Species-specificity of peptide-PNA conjugates.....	127
3.4.1 Evaluation of PNA to discriminate between bacterial species in a mixed culture .....	127
<b>Chapter 4 Discussion .....</b>	<b>129</b>
4.1 Introduction.....	129
4.2 Specificity of antisense constructs .....	129
4.3 Validation of <i>fusA</i> and <i>rplE</i> expressed antisense RNA specificity.....	131
4.4 Growth inhibitory effects of silencing <i>rplE</i> and <i>fusA</i> in <i>E.coli</i> .....	133
4.5 Evaluation of stringency in essential genes <i>rplE</i> and <i>fusA</i> .....	136
4.6 Evaluation of gene stringency in essential gene <i>fabI</i> by proteomics.....	140
4.7 Species specificity of peptide-PNA conjugates in mixed bacterial cultures .....	145
<b>Chapter 5 Conclusion and Future Work.....</b>	<b>146</b>
<b>References .....</b>	<b>149</b>
<b>Appendix A: Growth Media &amp; Buffer compositions.....</b>	<b>188</b>
<b>Appendix B: Publications associated with this study .....</b>	<b>190</b>

## List of Figures

Figure 1.1. Cellular targets of current antibiotics in clinical use (adapted from Walsh, 2003).....	3
Figure 1.2. Whole cell screening approach to antibiotic discovery.....	16
Figure 1.3. Mechanisms of antisense RNA inhibition.....	24
Figure 1.4. Overview of the LC-MS/MS based quantification.....	36
Figure 3.1 Relative growth rate of <i>E.coli</i> following expressed antisense RNA silencing of essential gene targets <i>fusA</i> and <i>rpIE</i> .....	72
Figure 3.2 Relative growth rates of <i>E.coli</i> following expressed antisense RNA silencing of essential gene targets <i>acpP</i> , <i>ftsZ</i> , <i>fabI</i> , and <i>murA</i> . ....	73
Figure 3.3 Transcomplementation of expressed antisense RNA silencing effects of the essential gene <i>rpIE</i> in <i>E.coli</i> . ....	75
Figure 3.4 Transcomplementation control mimicking the expression conditions used for the rescue of <i>rpIE</i> .....	76
Figure 3.5 Transcomplementation of expressed antisense RNA silencing effects of the essential gene <i>fusA</i> in <i>E.coli</i> . ....	77
Figure 3.6 Transcomplementation control mimicking the expression conditions used for the rescue of <i>fusA</i> . ....	78
Figure 3.7 Delineation of growth effects following expressed RNA silencing of the essential gene <i>rpIE</i> in <i>E.coli</i> . ....	80
Figure 3.8 Delineation of growth effects following silencing of the essential gene <i>fusA</i> in <i>E.coli</i> . ....	81
Figure 3.9 Viable cell counts of <i>E.coli</i> expressing empty pHN678 construct.....	82
Figure 3.10 Relative growth rate and mRNA abundance profiles following expressed antisense RNA silencing of the essential gene <i>rpIE</i> in <i>E.coli</i> . ....	84
Figure 3.11 Relative growth rate and mRNA abundance profiles following expressed antisense RNA silencing of the essential gene <i>fusA</i> in <i>E.coli</i> . ....	85
Figure 3.12 MTL <sub>50</sub> for essential gene <i>rpIE</i> . ....	86
Figure 3.13 MTL <sub>50</sub> for essential gene <i>fusA</i> .....	87

Figure 3.14 Purification of <sup>15</sup> N labelled FabI and MurA protein from <i>E.coli</i> whole cell lysate. ....	89
Figure 3.15 Purification and concentration of <sup>15</sup> N labelled FabI and MurA.....	91
Figure 3.16 GPM Results for unlabelled FabI and MurA.....	93
Figure 3.17 GPM Results examining <sup>15</sup> N Incorporation in FabI and MurA.....	95
Figure 3.18 Mass spectrum of tryptic peptide ILVTGVASK used for establishing the level of <sup>15</sup> N incorporation in FabI.....	96
Figure 3.19 Mass spectrum of tryptic peptide SMLNPGSALLTSYLGAER used for establishing the level of <sup>15</sup> N incorporation in FabI.....	97
Figure 3.20 Mass spectrum of tryptic peptide MGAHAEIESNTVICHGVEK used for establishing the level of <sup>15</sup> N incorporation in MurA. ....	98
Figure 3.21 Mass spectrum of tryptic peptide LQGEVTISGAK used for establishing the level of <sup>15</sup> N incorporation in MurA. ....	99
Figure 3.22 Tryptic peptides identified for the quantification of FabI internal standard.....	102
Figure 3.23 LC-MS/MS spectra for unlabelled EGAELAFTYQNDK.....	104
Figure 3.24 LC-MS/MS spectra for <sup>15</sup> N labelled peptide EGAELAFTYQNDK. ....	105
Figure 3.25 LC-MS/MS characteristics of peptide EGAELAFTYQNDK. ....	106
Figure 3.26 LC-MS/MS characteristics of peptide EGAELAFTYQNDK. ....	107
Figure 3.27 LC-MS/MS spectra for unlabelled and <sup>15</sup> N labelled peptide ILVTGVASK.....	108
Figure 3.28 LC-MS/MS spectra for <sup>15</sup> N labelled peptide ILVTGVASK. ....	109
Figure 3.29 LC-MS/MS characteristics of peptide ILVTGVASK.....	110
Figure 3.30 LC-MS/MS characteristics of peptide ILVTGVASK.....	111
Figure 3.31 Tryptic peptide standard curves for quantification of <sup>15</sup> N FabI internal standard. ....	113
Figure 3.32 FabI protein molecules per cell following expressed antisense silencing of the cognate essential gene. ....	115
Figure 3.33 FabI protein molecules per cell in <i>E.coli</i> MG1 ME5305 and Wild-type <i>E.coli</i> . ....	116

Figure 3.34 mRNA transcript and protein abundance in <i>E.coli</i> following expressed antisense RNA silencing of the essential gene <i>fabI</i> .....	118
Figure 3.35 Relative growth rate correlation with FabI molecules per cell following expressed antisense RNA silencing of the essential gene <i>fabI</i> in <i>E.coli</i> . .....	119
Figure 3.36 Relative mRNA abundance correlation with FabI per cell following expressed antisense RNA silencing of the essential gene <i>fabI</i> in <i>E.coli</i> . .....	120
Figure 3.37 Determining MTL <sub>50</sub> value for the essential gene <i>fabI</i> . .....	122
Figure 3.38 Determining MPL <sub>50</sub> values for the essential gene <i>fabI</i> . .....	123
Figure 3.39 Triclosan sensitivity in <i>E.coli</i> following expressed antisense RNA silencing of <i>fabI</i> . .....	125
Figure 3.40. Phosphomycin sensitivity in <i>E.coli</i> following expressed antisense RNA silencing of <i>murA</i> . .....	126
Figure 3.41 Effects of species-specific PNA on <i>E.coli</i> and <i>S.typhimurium</i> . .....	128

## List of Tables

Table 1.1. The targets and inhibitory modes of action of antibiotics in clinical use .....	4
Table 1.2. Resistance mechanisms associated with current antibiotics.....	8
Table 1.3. Evaluation of antibiotic screening approaches.....	15
Table 2.1 Strains used in this study .....	57
Table 2.2 Paired termini antisense expression plasmids used in this study.....	58
Table 2.3 Over expression vectors used in this study.....	59
Table 2.4 PCR primers used in this study .....	60
Table 2.5 QPCR primers used in this study .....	61



# Chapter 1 Introduction

## 1.1 Antibiotics overview

### 1.1.1 Antibiotic origins, function & resistance

The discovery and derivatization of antibiotics from natural products has provided the foundations of modern chemotherapy, while pioneering techniques in isolation, characterisation, purification and systematic screening have contributed to the development of the modern pharmaceutical industry. The study and elucidation of antibiotic modes of action on bacterial physiology, has not only advanced the understanding of cellular mechanisms underlying replication, transcription and translation, but also provided selection tools for cloning strategies (Fabbretti *et al.*, 2011).

The origins of naturally derived antibiotics can be traced to organisms within the plant and animal kingdoms, primarily unicellular bacteria and eukaryotic fungi. Of particular interest are the filamentous gram-positive bacteria belonging to the order Actinomycetales (Lechevalier and Lechevalier, 1967), typified by the genera *Streptomyces*, which exist ubiquitously within the terrestrial environment, fulfilling a saprophytic role (Goodfellow and Williams, 1983). *Streptomyces* are among the most prolific producers of naturally derived antibiotics that have entered clinical practice, with a speculative capability of producing up to 100,000 potential antimicrobial compounds (Watve *et al.*, 2001).

Antibiotics are characterized as low molecular weight compounds (MW<3000) which are synthesized via complex secondary metabolic pathways, divorced from the primary metabolic functions concerned with cell growth (Bérdy, 2005). Secondary metabolic pathways are a consequence of distinct biosynthetic gene clusters that encode metabolic enzymes, regulatory genes and resistance mechanisms, implying functional evolution as a unit (Maplestone *et al.*, 1992). The secondary metabolites produced, possess intricate molecular scaffolds with unique functional group configurations (Silver, 2008). These properties enable specific interactions with molecular targets (cellular components or enzymes) (Figure 1.1), through specific binding sites either on the target surface, or at sites that are exposed following structural re-arrangement, when the target is assembled as part of a macromolecular complex with other cellular constituents

(Lange *et al.*, 2007). The subsequent binding alters the ordered structure of the target causing impairment or complete abolition of normal function. Essential cellular function is subsequently effected, which depending on the degree of target inhibition, is characterized as either inhibitory (bacteriostatic) or capable of initiating cell death (bactericidal) (Walsh, 2003).

Impaired cellular function and cell death can also occur via a secondary pathway that is activated following primary antibiotic target binding, which initiates a universal mechanism of cell death through oxidative damage (Kohanski *et al.*, 2010). Interaction between the antibiotic and respective cellular target, alters metabolic feedback to the Tricarboxylic Acid Cycle (TCA), through increased oxidation of the reduced form of the co-enzyme nicotinamide adenine dinucleotide (NADH). Hyper-activation of the electron transport chain ensues, causing an increase in the formation of superoxide ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl (OH) free radicals, which react readily with enzymes containing iron complexes. Iron is subsequently lost following oxidation and becomes freely available to interact with  $H_2O_2$  via the Fenton reaction to produce OH radicals, which are highly reactive and capable of readily oxidising DNA and proteins (Imlay, 2003).

The role of antibiotics was deemed to be an evolved survival strategy in the guise of a chemical defence mechanism, for minimising competition from other bacteria (Fajardo *et al.*, 2009) and providing protection against bacterivorous predators such as protozoa and nematodes (Jousset, 2012). This concept was supported by widespread antibiotic production among organisms subject to Darwinian competition, in addition to the structural complexity of natural products encoded by a significant proportion of the genomes of Actinomycetes and the sophisticated interaction between the antibiotic and its cognate receptor (Williams *et al.*, 1989). More recently it has been suggested that, antibiotics may fulfil a role as autoregulators in intracellular communication, since they are known to induce secondary metabolism and aerial mycelium formation in Actinomycetes, in addition to cellular differentiation in other species (Beppu, 1992). The dual role of defence and communication has been suggested to be concentration dependant, with antibiotics behaving as autologous signals at low concentrations, whereas at high concentrations they

act in the traditional sense of inhibiting bacterial growth (Fajardo and Martinez, 2008).

### 1.1.2 Antibiotic mode of action

Antibiotics are broadly grouped according to the cellular pathways and targets they affect (Table 1.1) peptidoglycan assembly in cell wall synthesis, disruption of the outer cytoplasmic membranes, protein synthesis machinery such as the ribosome and associated elongation factors, enzymes responsible for replication and transcription and the biosynthesis of folate, tRNA and fatty acids (Lange *et al.*, 2007) .

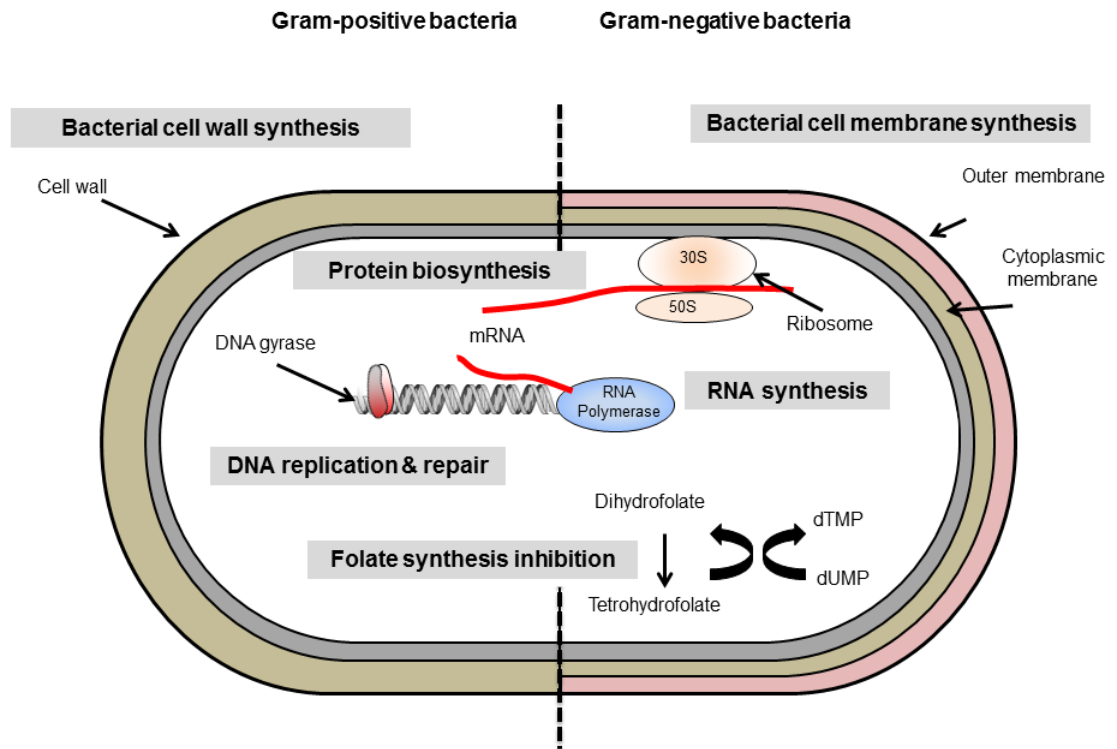


Figure 1.1. Cellular targets of current antibiotics in clinical use (adapted from Walsh, 2003).

**Table 1.1. The targets and inhibitory modes of action of antibiotics in clinical use**

<b>Pathway</b>	<b>Antibiotic class</b>	<b>Primary Target</b>	<b>Mechanism of action</b>
Cell wall synthesis	β-Lactams and derivatives: Cephalosporins Carbenems Monobactams	DD-transpeptidases (penicillin binding proteins)	Pencilloylation of the penicillin binding protein active site, preventing the catalysis of peptide bond formation between peptidoglycan units.
	Glycopeptide	Peptidoglycan units	Binds peptidoglycan units (at D-alanyl-D-alanine-dipeptide), thereby Inhibiting transglycolase activity and preventing cross linking of peptidoglycan.
DNA replication	Quinolones/fluoroquinolones	A-subunit of DNA gyrase or A-subunit of Topoisomerase IV	Interferes with the maintenance of chromosome topology, through the Inhibition of strand cleavage and re-joining, resulting in double strand breaks and replication fork arrest.
Protein synthesis	Aminoglycosides	16S rRNA 30S ribosome	tRNA mismatching resulting in mistranslation.
	Tetracyclines	30S ribosome	Blocks access of aminoacyl t-RNA to the ribosome.
	Macrolides	50S ribosome	Inhibiting the initiation phase of translation and translocation of peptidyl tRNA.
Transcription	Rifamycins	B-subunit of DNA-dependent RNA polymerase	Stable binding to the DNA/RNA tunnel of the B-subunit subsequently blocks RNA elongation.
Folate biosynthesis	Sulfonamide	Dihydropteroate synthase	Acts as a competitively inhibitor
	Trimethoprim	Dihydrofolate reductase	Prevents recycling of folate coenzymes 7,8 dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF)

### **1.1.3 The emergence of antibiotic resistance**

Decades of anthropogenic activity in biopharmaceuticals, medicine and agriculture, have been speculated to increase the capacity for bacterial evolution. Antibiotic exposure promotes a transient increase in rates of mutation, recombination and Horizontal Gene Transfer (HGT), which underlie genetic diversity. Over time lineages displaying higher mutation rates predominate, leading to the directional selection of antibiotic resistance among bacteria (Gillings and Stokes, 2012). Although the probability of antibiotic resistance developing was initially thought to be low, subsequent advances in microbial genetics have increased understanding regarding the precursors to resistance and its dissemination amongst other species (Davies, 1994). Within a clinical context, persistent antibiotic use has prompted the emergence of three predominant classes of resistant pathogens; gram positive organisms such as Methicillin Resistant *Staphylococcus aureus* (MRSA), and gram negative organisms such as multidrug resistant (MDR) *Escherichia coli*, *Enterococcus faecium*, *Enterobacter cloacae*, Extended-spectrum  $\beta$ -lactamases (ESBL) *Klebsiella pneumoniae*, MDR *Actinobacter baumannii* and *Pseudomonas aeruginosa*. In addition a third class consisting of multidrug resistant (XDR) *Mycobacterium tuberculosis* has also been observed (Fischbach and Walsh, 2009). It is now accepted that antibiotic exposure creates a Darwinian selection pressure which promotes an adaptive response, through the development of survival mechanisms that enable continued growth in cytotoxic antibiotic concentrations (Wright, 2007). Those microorganisms best adapted for survival persist with the capability of disseminating resistance traits amongst other species (Allen *et al.*, 2010).

Antibiotic resistance mechanisms are considered artefacts arising from a comprehensive and evolving natural reservoir of resistance genes, among pathogenic and environmental bacteria. This compendium encompasses cryptic resistance elements within chromosomes and precursor genes encoding proteins with alternate functions, that serve as prototypes to bona fide resistance mechanisms, collectively referred to as the antibiotic resistome (Wright, 2007). The resistome is thought to have originated from the co-evolution of proteins with alternate biochemical function which undergo convergence, whereby a single functional transcriptional unit develops from the

merging of contiguous genes sets coding for structural, regulatory and resistance elements into a single bio-synthetic cluster (Fischbach, 2009). The source of antibiotic resistance mechanisms has been attributed to Actinomycetes, which require a resistance strategy to compensate for antibiotic production. Indeed antibiotic resistance in Actinomycetes is known to increase in conjunction with synthesis. In *Streptomyces coelicolor*, elucidation of the production of the antibiotic actinorhordin, revealed a coupling between biosynthesis and intracellular export. Pathway intermediates were identified and found to be capable of binding to a transcriptional repressor (ActR), responsible for regulating the expression of a trans-membrane efflux pump (actA). As a result of this mechanism, *S.coelicolor* was primed in preparation for the toxic build-up of antibiotic (Tahlan *et al.*, 2007).

Resistance mechanisms may be classified as either active (stimulated by an evolutionary selective pressure) or passive (a consequence of innate cell function or general adaptive process) (Wright, 2005). In these cases, the expression of intrinsic resistance mechanisms can arise following spontaneous chromosomal mutation or a series of cooperative mutations within genes that dictate the synthesis and structural assembly of the antibiotic target. Mutations may cause structural re-orientation of the active site (target structural mutations), enhance access to the target (target access mutations), or shield the target from antibiotic action (target protection mutations) (Martinez and Baquero, 2000).

A prerequisite for spontaneous mutation is pre-mutagenic damage to chromosomal DNA as a consequence of native replication errors following DNA synthesis, characterised as base substitution, frame-shift, or sequence substitutions. Alternatively, mutations may arise from DNA lesions caused by adverse chemical reactions (Oxidation, methylation, depurination, deamination and hydrolytic decomposition of nucleotide bases), in conjunction with failures in cell repair mechanisms (Maki, 2002). The probability of a spontaneous mutation event creating resistant phenotypes (mutability) is dependent on gene structure, length and surrounding sequences, which may be prone to genetic alteration. Environmental cues such as nutrient limitation are also known to increase the rate of mutation by initiating an adaptive response, involving the expression of contingency genes, linked to the SOS response (Martinez *et al.*,

2009). Such factors underlie the conditions for mutator phenotypes to arise within bacterial populations, which provide a survival mechanism for bacteria to adapt and respond to environmental changes. Alternatively resistance genes may be acquired from other species through horizontal gene transfer (Mazodier and Davies, 1991), encompassing the transmission of mobile genetic elements from bacteriophages (transduction), plasmids and conjugative transposons (conjugation) and integration of exogenous DNA within chromosomal DNA (transformation) (Alekshun and Levy, 2007). Ultimately such mutations support the development of common resistance mechanisms enabling mutant bacteria to compete with wild-type counterparts. The outcome is subject to the relative fitness conveyed by the mutation, which may present a significant biological cost in terms of additional metabolic requirements, arising from the expression and maintenance of resistance genes (Andersson and Levin, 1999). Although resistance is often viewed as reducing fitness, it can be compensated by additional mutations in other chromosomal loci, restoring fitness equivalent to wild-type strains (Martinez and Baquero, 2000).

Despite the extensive documentation of emerging resistance mutations, only three new classes of antibiotic have been developed in the last few decades, consisting of mutilins, lipopeptides and oxazolidinones (Fischbach and Walsh, 2009). Efforts at reversing resistance within a clinical context, through reducing antibiotic treatments have had limited effects (Andersson and Hughes, 2010). The primary means to treat resistant strains may lie in the discovery of antibiotics possessing novel modes of action capable of negating established resistance mechanisms.

**Table 1.2. Resistance mechanisms associated with current antibiotics.**

<b>Resistance Mechanism</b>	<b>Mode of action</b>	<b>Antibiotic example</b>	<b>Mutation description</b>
Structural modification of the molecular target	Alterations in target structure reduce interactions / disrupt recognition sites for antibiotic binding	Quinolones	Mutations in <i>gyrA</i> (Ser83 – Leu), <i>gyrB</i> (Asp426-Asn), which encode the subunits of DNA gyrase and subunit of topoisomerase IV encoded by <i>parC</i> (Ser80-Arg)
		Aminoglycosides	Mutation in nucleotide A1408G of Helix 44 of decoding site of 30S subunit
Alteration in cell membrane permeability	Restricts entry of the antibiotic to intracellular targets	Vancomycin	Thickened cell wall in <i>S.aureus</i>
		Polymyxin	Modification of lipopolysaccharide and lipid A composition in <i>S.enterica</i> by 2 component regulatory system PmrAB
Expression of active efflux pumps	Removes antibiotic from the cytoplasm and across inner and outer membrane of the cell envelope	Tetracycline	Tet transmembrane efflux transmembrane proteins A-E (gram-) and K, L (gram+) protein
		Macrolides	MsrA ATP binding cassette (ABC) efflux protein
Expression of enzymes	Enzymatic modification or degradation of antibiotic structure rendering it inactive	Aminoglycosides	Acetyltransferase(AAC), Nucleotidyltransferases(ANT), Phosphotransferases (APH)
Overproduction of molecular target	Excess target saturates antibiotic levels, increasing	Isoniazid	Mutations in the promoter region of <i>inhA</i> encoding enoyl transferase in <i>M.tuberculosis</i>



#### **1.1.4 The antibiotic discovery paradigm**

The paradigm of antibiotic discovery can be traced to the convergence of two separate approaches; systematic chemical synthesis and natural product discovery. Systematic chemical synthesis aimed to utilise reaction mechanisms to generate variant chemical structures with antibacterial properties. This culminated in the development of the first true antibacterial prontosil rubrum, which demonstrated *in vivo* activity against streptococcal infection. Further investigation attributed antibacterial activity to the metabolite sulfonilamide, which heralded the process of rational design and screening of antimetabolites leading to the development of antifolates. The origins of natural product discovery began with the serendipitous observation and subsequent isolation of penicillin from the mould *Penicillium notatum*. This was followed by the discovery of streptomycin, which provided a template for the screening fermentation broths derived from soil Actinomycetes. This became the prevailing approach which facilitated the discovery of the majority of antibiotic classes, referred to as “golden age of antibiotics” (Silver, 2012).

Over recent decades traditional discovery programs within the pharmaceutical industry have declined, due to a loss in profitability associated with developing new antibiotics. This has been attributed to the short duration of antibiotic therapy which generates a lower return on capital invested in comparison to other treatments (e.g.: hypertensives). Profitability has been further exacerbated by the conservative pricing of antibiotics and efforts to limit their prescribing to minimise resistance (Tillotson, 2008). A substantial proportion of the economic costs incurred, have been attributed to the reliability of natural sources and the characterisation necessary to identify active components in often complex extracts (Li and Vederas, 2009). The most significant factor in the decline of traditional antibacterial discovery occurred following the introduction of combinatorial chemistry. CombiChem refers to the systematic assembly of diverse chemical structures by the connection of individual components, through biological, chemical or biosynthetic means (Gallop *et al.*, 1994). Using this approach it is possible to construct structurally diverse compounds through the addition of functional groups to a primary scaffold element, often derived from a natural product template (Koehn and Carter, 2005). Unlike traditional natural product libraries, sufficient quantities can be synthesized economically

and undesirable chemical properties that may cause unwanted side effects can be avoided at the design stage.

The introduction of combichem subsequently prompted a paradigm shift in screening strategies that saw empirical screening from natural sources, superseded by High Throughput Screening (HTS) using synthetic libraries (Li and Vederas, 2009). Yet despite extensive efforts, synthetic libraries have failed to make a significant impact. The evidence for this limited success has been exemplified by the screening of synthetic polyketides libraries, which have demonstrated a hit rate of <0.001% compared to a 0.3% hit rate achieved with natural product libraries (Weissman and Leadlay, 2005). Even the use of genomics to identify and select essential genes for target based screening has failed to deliver new antibiotic entities. In a landmark study, a genomics based approach was used to identify 70 essential genes, which proceeded to generate only 5 leads from subsequent HTS campaigns (Payne *et al.*, 2007). Similar results were identified in 127 antibacterial screening programs covering 69 essential targets (Chan *et al.*, 2004).

Conclusions drawn from these studies revealed the limitations of synthetic libraries, specifically an inherent lack of chemical diversity in terms of steric complexity, unique functional groups, chiral centres, and broad molecular properties associated with natural products. Furthermore, synthetic compounds often lack evolved target specificity, often against subtle features such as protein domains and binding motifs (Koehn and Carter, 2005). The lack of chemical diversity may have arisen from an inherent bias towards compounds that fulfil Lipinski's rule of 5, a set of parameters associated with solubility and permeability, in terms of molecular weight, Log p and number of hydrogen acceptors and donors (Lipinski *et al.*, 2012). In comparison natural products typically display higher MW and polarity compared to other drug classes (O'Shea and Moser, 2008). With increasing rates of antibiotic resistance and a lack of novel antibiotics in development, there has been a renaissance in natural product discovery.

### **1.1.5 Possibilities to exploit new sources of antibiotics**

Current efforts in natural product discovery have focused on identifying new antibiotic producing microorganisms on the rationale that taxonomic diversity is

a proxy for the chemical scaffold diversity, associated with natural products (Bull *et al.*, 2000). New microorganisms have been sought by surveying previously unexplored geographical locations, where extreme environmental conditions of temperature pH and salinity are encountered. Considerable focus has been expended on the marine environment including sea ice, the ocean subsurface, hydrothermal vents and the ocean floor (Keller and Zengler, 2004).

Marine sediments in particular have shown large diversity of microorganisms in particular Actinomycetes (Stach and Bull, 2005; Pathom-aree *et al.*, 2006). Currently members covering 50 genera of Actinomycetes have been isolated and cultured from marine habitats including the discovery of new genera; *Demequina*, *Lamia*, *Marinactinospora*, *Marisediminicola*, *Miniimonas*, *Paraoerskovia*, *Phycococcus*, *Phycicola*, *Salinibacterium*, *Salinispora*, *Aciscionella* and *Serinicoccus* (Goodfellow and Fiedler, 2010). Akin with their terrestrial counterparts, marine Actinomycetes possess a significant coding capacity reserved for numerous biosynthetic clusters encoding secondary metabolites (Baltz, 2008). Evidence has emerged suggesting that marine secondary metabolites are synthesised via unique halogenation mechanisms, absent in terrestrial Actinomycetes (Butler, 1998). Marine halogenated metabolites are speculated to possess novel properties which arise from the incorporation of halogen atoms within aromatic, heterocyclic rings and carbon centres, that influence steric effects and electrophilic reactivity of the antibiotic scaffold, thereby dictating biological activity (Vaillancourt *et al.*, 2006). Recently a novel enzymatic pathway that utilises vanadium-dependant haloperoxidases was identified within the biosynthetic gene cluster of a marine *Streptomyces* (Lane and Moore, 2011). Consequently, natural products derived from rare marine Actinomycetes display desirable characteristics that may be exploited to treat antibiotic resistant pathogens. Screening for such products is therefore a priority for antibiotic discovery programs.

The search for Actinomycete derived natural products has been supported by advances in genomics and chemical biology, which have aided the elucidation of biosynthesis mechanisms, and subsequently defined new biodiscovery approaches (Kurtboke, 2012). The exploitation of whole Actinomycete genomes such as *Streptomyces avermitilis* (Omura *et al.*, 2001) and use of bioinformatic analyses to interrogate sequences, has provided the basis for numerous

genome mining strategies (Van Lanen and Shen, 2006; Zerikly and Challis, 2009).

One such strategy has been founded on manipulating the synthesis of novel secondary metabolites such as polyketides (PK) and non-ribosomal proteins (NRP). It is known that the synthesis of PK and NRP antibiotics proceeds in an assembly line sequence, where the construction of unique scaffolds arises from the coordinate assembly of monomer units by multi-modular enzymes encoded in biosynthetic clusters. The structural organization of the multimodular protein domains function as a template to direct the order of monomer units within a linear oligomer, while catalytic domains govern the activation and incorporation via covalent tethering (Fischbach and Walsh, 2006). Using experimentally derived data, it has been possible to ascribe product structure to subunit composition thereby enabling characterization of individual subunits (McDaniel *et al.*, 1995). Functional information for individual subunits can be utilized in predicting metabolic products, including their putative physicochemical properties, as exemplified by the polyene marolactam Salinilactam A from *Salinispora tropica* (Udwary *et al.*, 2007). The re-arrangement of monomer units within the assembly sequence can also enable the synthesis of novel metabolic products. In one example the loading module of Avermectin polyketide synthase was substituted with a cyclohexanecarboxylic loading module, enabling the synthesis of the novel analogue doramectin (Wang *et al.*, 2011).

Alternative genome mining approaches have combined bioinformatics analysis with assay guided fractionation in a genomisotopic approach. Analysis of the genome of *Pseudomonas fluorescens* Pf-5 revealed three unknown biosynthetic clusters for non-ribosomal peptide synthases, the nucleotide sequence of which enabled the prediction of amino acid assembly of the product. By using a isotopically labelled amino acid, incorporation into the unknown product could be identified by NMR, resulting in the discovery of the novel lipopeptide orfamide A (Gross *et al.*, 2007).

Biosynthetic clusters may also be isolated and expressed in host organisms followed by the purification of proteins, which are then incubated with co-factors in an *in vitro* reconstitution approach, as performed with the biosynthetic cluster of lantibiotic haloduracin produced by *Bacillus halodurans* C-125 (McClerren *et al.*, 2006). In cases where biosynthetic gene clusters are not constitutively

expressed or dictated by specific environmental conditions, expression may be induced by modulating transcriptional regulatory factors. In one example, overexpression of the transcriptional activator *apdR* under an inducible promoter in *Aspergillus nidulans* lead to the identification of aspyridones A and B (Bergmann *et al.*, 2007). Alternatively cryptic biosynthetic clusters can be cloned and expressed in laboratory and industrial strains of *Streptomyces* with characterized metabolomes (Baltz, 2010).

An alternative biodiscovery perspective is to examine microbial communities directly within their ecological habitats in order to provide an assessment of functional diversity. This has been achieved using metagenomics whereby DNA is isolated directly from environmental sources and cloned into a model organism such as *E.coli*. Transformants are then screened for functional activity in a high-throughput assay, in order to assess metabolic potential (Handelsman *et al.*, 1998). The comparison of metagenomic data from microbial communities can not only reveal trends that suggest functional diversity, but also reveal spatio-temporal characteristics. Metagenomic data can therefore reflect environmental factors that promote the distribution and evolution of antibiotic biosynthetic gene clusters that contribute towards speciation and diversification (Kurtboke, 2012). The detection of rare Actinomycetes can also be pursued using taxonomical studies to devise selective isolation procedures. Refinements of Actinomycetes systematics and the development of selective isolation techniques within culture dependant bio-prospecting strategies have also been investigated (Goodfellow and Fiedler, 2010).

In addition to the identification of new taxa, existing Actinomycetes can be evaluated with greater scrutiny to reveal their secondary metabolite producing capacity. It has been documented that a discrepancy exists between the number of compounds produced and potential synthesis capability of many Actinomycetes. An attempt to explore this facet is exemplified by the One Strain Many Compounds (OSMAC) approach, whereby cultivation conditions are systematically modified to influence key steps in biosynthesis and thereby generate diverse metabolic profiles (Bode *et al.*, 2002). Successful bio-discovery requires not only consideration of ecological habitat, selective isolation procedures and bacterial systematics for the identification of rare

Actinomycetes, but also the implementation of an appropriate screening strategy to assess the bioactivity of secondary metabolites that are synthesized (Kurtboke, 2012).

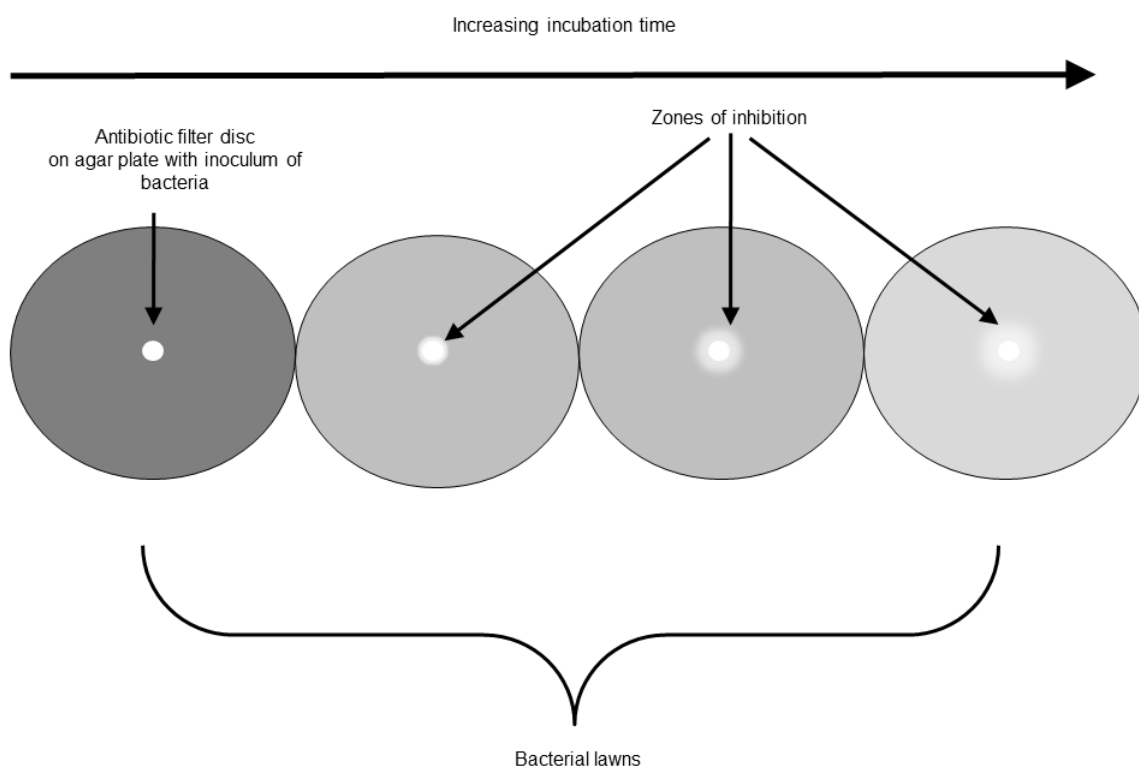
### **1.1.6 Antibiotic screening approaches**

A key requirement for an antibacterial screening assay is the capacity to discriminate between known antibiotics and novel undiscovered compounds. Within the terrestrial biosphere, antibiotic biosynthetic clusters are not distributed uniformly. Random genome sampling has revealed that 1% of Actinomycetes present in the soil produce streptomycin compared to 0.1% that synthesize tetracycline, with other antibiotics isolated at even lower frequencies of  $1 \times 10^{-7}$  (Baltz, 2007). Consequently antibiotics that occur at high frequencies are easily isolated, thereby potentially masking the presence of novel antibiotics that exist in low abundance. The consistent rediscovery of known compounds therefore represents a significant technical challenge to screening approaches (Williams, 2009).

Conventional screening strategies have utilised whole cell screens, cell free or modular assays to evaluate inhibitors. Whole cell screening involves seeding a target organism onto media to which a natural product extract is added (Figure 1.2). Antibacterial activity is then assessed by observing zones of inhibition (Mills and Dougherty, 2012). Refinements to this assay format have led to the development of mechanism based screening that targets pathways such as the cell wall, using hypersusceptible mutant strains to identify  $\beta$ -lactam antibiotics (Gadebusch et al., 1992) and the anucleate blue cell assay to detect Type II topoisomerase inhibitors of DNA synthesis (Oyamada et al., 2006). Cell free assays differ in that a single target or multiple targets encoding enzymes representing a pathway are reconstituted *in vitro*; inhibition is monitored directly using biochemical assay to examine enzyme kinetics. Modular assays are similar to cell free systems except that a target is identified *in silico*. The target sequence is cloned into *E.coli* or other suitable host organisms, expressed with a Histag and purified. The isolated target is then screened against natural product extracts, and signs of inhibition are evaluated in a binding assay.

**Table 1.3. Evaluation of antibiotic screening approaches.**

<b>Assay type</b>	<b>Advantages</b>	<b>Disadvantages</b>
Wholecell antibacterial activity	Identifies <i>in vivo</i> antibacterial activity	Unable to discriminate between secondary mechanisms related to cytotoxicity and actual antibacterial activity
	Selects compounds capable of transversing the cell wall	Compounds lacking cell penetration, but, which would otherwise inhibit the target with suitable modification are overlooked
	Enables multi-protein interactions to be studied without the need to reconstitute complex cell systems	Secondary biochemical assay required to identify the mode of action
Target based cell free systems	High sensitivity	Low sensitivity compared to cell free systems
	Validates mode of action in addition to identifying target	Requires prior knowledge of the target Complex reaction or substrates make enzymatic tests difficult Constrained to targets with enzymatic function



**Figure 1.2. Whole cell screening approach to antibiotic discovery.**

Using whole cell screening provides a number of advantages over cell free systems (Table 1.3) and prior to the development of synthetic libraries has been the pre-eminent system for evaluating natural products. Rational Drug Design (RDD) represents a further approach that has benefited from advances in biophysical characterization techniques, to enable the derivatization of sufficient structural data from molecular targets. This information can then be used for the construction of putative inhibitors (Donadio *et al.*, 2002).

Considerable effort to overcome the inability of whole cell assays to delineate between cellular targets have been pursued using recombinant organisms as the basis for target-based screening strategies, in which the expression or activity of a specific cellular target is modulated. For example the Keio collection, consisting of approximately 4000 individual essential gene knockouts strains of *E.coli* (Baba *et al.*, 2006), which was subsequently screened to identify mutants with increased susceptibilities to antibiotics (Tamae *et al.*, 2008).



An alternate approach has been to delete chromosomal copies of essential genes in *E.coli*, replacing them with plasmids containing a complementary copy of the gene, under the control of an inducible promoter, to enable gene expression modulation. Using this system it is possible to reduce target protein expression in order to generate hypersensitivity to target specific inhibitors. Screening strains against cognate inhibitors demonstrates increased susceptibility, by decreasing the MIC (DeVito *et al.*, 2002). Conversely strains have been engineered to overexpress essential proteins from inducible plasmids, thereby rendering strains hyposensitive to target protein inhibitors (Xu *et al.*, 2006).

Of the target based assays developed to date many have used known targets for which resistance had already been demonstrated. The discovery of novel antibiotics therefore requires the identification and validation of either molecular targets or components of cellular pathways possessing unique modes of action, not currently subject to resistance mechanisms (Black and Hodgson, 2005). Potential targets should be evaluated for their capacity to form multiple interactions to reduce the likelihood of generating endogenous resistance according to the multi-target hypothesis (Silver, 2007).

## **1.2 Searching for new antibiotics**

### **1.2.1 Identification of novel antibiotic targets**

Despite the conserved range of cellular targets exploited by current antibiotics, the number of potential targets may be larger than anticipated, due to the multiple co-factors and accessory components that cooperate with essential targets within biosynthesis pathways (Donadio *et al.*, 2002). A suitable example of this multiple-target approach is the peptidoglycan biosynthesis pathway that culminates in assembly of the bacterial cell wall. Numerous cytoplasmic intermediates have previously been unavailable due to the inability to reconstitute them *in vitro*. Efforts to synthesise key cytoplasmic precursors such as UDPMurNAc-pentapeptide, have aided biochemical characterization of the peptidoglycan pathway necessary for the development of assays and screens for lipid-linked steps. To date screening for Lipid I and II intermediates revealed that MraT a integral membrane translocase, was found to be the target of uridine-based nucleoside antibiotics (Bugg *et al.*, 2011). Additional targets have

been identified in other metabolic pathways including Aminoacyl t-RNA synthetases, Polypeptide deformylase and fatty acid biosynthesis (McDevitt and Rosenberg, 2001), enzymes responsible for NAD(P) biosynthesis (Bi *et al.*, 2011) and component enzymes of cell division (Lock and Harry, 2008).

Irrespective of novelty, there is a requirement for candidate targets to fulfil the following criteria; (i) they must demonstrate an essential function, (ii) the target should be conserved across a range of species for broad-spectrum activity, (iii) the target should be specific to bacteria with no human homolog to limit toxicity and (iv) the target should exist as a single gene copy to minimise potential resistance development. Ideally the target should also display desirable physiochemical properties to enable inclusion into a high-throughput screening format (Brown and Warren, 1998).

In recent years a paradigm shift in the identification of potential antibiotic targets has emerged following advances in genomics and bioinformatics (Bull *et al.*, 2000), specifically following the advent of high-throughput whole genome shotgun sequencing, pioneered with *Haemophilus influenzae* (Fleischmann *et al.*, 1995). The number of bacterial genomes sequenced has since increased dramatically following the development of new sequencing technologies such as pyrosequencing, DNA ligase-mediated sequencing, and the Illumina platform (McLeod *et al.*, 2012). Accompanying the progress in genome sequencing technologies have been parallel developments in bioinformatics. Algorithm based programs such as TIGR Assembler, Glimmer and Genemark have enabled the assembly of contiguous sequences, identification of open reading frames and revealed putative function of genes on the basis of protein structural motif homology or operon structure (McDevitt and Rosenberg, 2001; McLeod *et al.*, 2012). Critically, bioinformatics programs such as BLAST and FASTA have permitted the development of comparative genomics *in silico*, enabling the identification of conserved orthologues (homologous genes derived from a common ancestral gene) across bacteria species. Following the genome comparison of *H. influenzae* and *Mycoplasma genitalium*, approximately 256 genes with purported essential functions common to both organisms were identified. From this data was established the concept of the minimal genome, a core set of genes required for sustaining cellular existence (Mushegian and Koonin, 1996). Subsequent genome studies have assigned essential functions

to 620 genes in *E.coli* (Gerdes *et al.*, 2003), 478 genes in *H.influenzae* (Glass *et al.*, 2006) 658 genes in *S.aureus* (Forsyth *et al.*, 2002) and 271 genes in *B.subtilis* (Kobayashi *et al.*, 2003b).

Within core gene sets a number of ubiquitous cell functions have been identified consistently, representing elementary cell functions of transcription, translation, replication, membrane transport and energy conversion (Koonin, 2003). From an antibacterial search and discovery perspective, these genes may serve as potential targets since they are evolutionary conserved among closely related bacteria species (Jordan *et al.*, 2002). Critically, the majority of genes identified within minimal gene sets are also proportionally biased towards enzymes (Gao and Zhang, 2011), representing ideal targets for potential inhibitors, since they possess an active site, which is predisposed towards ligand interaction and proves highly amenable for biochemical assays to validate target function (Bumann, 2008).

The pursuit of inhibitors with specific activity for an individual essential gene target has been questioned due to the high frequency of spontaneous resistance mutation ( $10^{-6} - 10^{-9}$ ), that occur in single genes (Silver, 2011). The use of hybrid pharmacophores, combinations of single target inhibitors and structure based drug design have been suggested as potential methods for minimising resistance in individually encoded targets. However, a more viable approach would be to identify a range of essential genes amenable to polypharmacology, whereby a single inhibitor has the capacity to engage with multiple molecular targets (Brötz-Oesterhelt and Brunner, 2008). According to the multi-target hypothesis, endogenous resistance development is minimised where antibiotics bind with multiple molecular targets, since mutations would be required in each gene encoded target to render an antibiotic completely ineffective (Silver and Bostian, 1993).

Indeed the majority of antibiotics discovered using whole cell screening approaches, act on multiple encoded targets or on individual structures assembled from the products of multiple genes (Silver, 2007). The targeting of Penicillin Binding Proteins (PBP's) by  $\beta$ -lactams and topoisomerase GyrA/ParC subunits by quinolones represents two such examples. More recently it has been suggested that the bacterial proteolytic complex may also prove a suitable target (Raju *et al.*, 2012). Consequently new targets have been proposed

including the MurA ligases (D and E), involved in the catalysis of peptidoglycan, and Lpx acyltransferases (A and D), which are involved in lipidA biosynthesis (East and Silver, 2013).

Defining core gene sets however has proved problematic, with varying estimates that obscure consensus as to the number of essential genes a set comprises (Juhas *et al.*, 2011). A phylogenetically balanced study that analysed 147 bacterial and archaea genomes, found the core set to consist of 38 essential genes (Charlebois and Doolittle, 2004). More recently a comprehensive analysis comparing the genome of *M.genitalium* to 92 gram negative and 93 gram positive bacteria revealed a core set of 151 genes (Huang *et al.*, 2012). The discrepancy in the core set suggests that not all essential genes are conserved among species (Koonin, 2003). It has been determined that the number of core genes decreases as the number of phylogenetically diverse groups used in comparative analysis increases. This reflects Non-orthologous gene displacement (NOGD), whereby non-orthologous genes replace orthologous counterparts encoding the same essential cellular function. Consequently alternative biochemical pathways arise, which provide a capacity for metabolic redundancy rendering many gene targets dispensable and therefore unsuitable as target candidates for screening (Becker *et al.*, 2006). A known example of this phenomenon is the *fabI* homologue *fabK* in *Streptococcus pneumoniae*, which is not the subject to the same degree of inhibition by triclosan (Heath and Rock, 2000).

Further discrepancies arise depending on the environmental conditions under which a minimal gene set is defined (Koonin, 2003). The requirements imposed by an organism's lifestyle and environment conditions may actually abolish the function of some essential genes. Notably the type II fatty acid synthesis pathway can be negated by gram positive pathogens capable of utilising C18 unsaturated fatty acids from human serum (Brinster *et al.*, 2009). Further variability among core gene sets arises from the experimental strategy used to validate gene essentiality. The majority of techniques used are based upon the generation of mutants, grown as clonal populations or as part of mixed populations. Clones grown as part of a mixed population are selected against by competitive outgrowth, thus demonstrating reduced fitness rather than true essentiality which can result in false assignment (Gerdes *et al.*, 2006).

Common experimental techniques for determining gene essentiality are founded upon random mutagenesis and targeted gene disruption (Miesel *et al.*, 2003; McLeod *et al.*, 2012). Random mutagenesis strategies include Transposon Saturation Mutagenesis and variants such as Genomic Analysis and Mapping By *In vitro* Transposition (GAMBIT) (Akerley *et al.*, 1998), Genome scanning (Reich *et al.*, 1999) and Transposon-Mediated Differential Hybridisation (TMDH) (Chaudhuri *et al.*, 2009). A common theme to these techniques is the use of transposons (mobile genetic elements), which are engineered into plasmids containing a conditional replicon and antibiotic selection marker. Once introduced into a cell the transposon inserts randomly in the chromosome with its location mapped by sequencing. Transposition into an essential gene coding sequence disrupts the gene function resulting in cell death thereby confirming essential function (McLeod *et al.*, 2012). Targeted gene disruption techniques involve plasmid insertion mutagenesis, whereby the central sequence of a target gene is cloned into a suicide vector. Integration of the vector via a single crossover recombination event involving the target gene and homologous sequence on plasmid results in allelic replacement of the target gene with a resistance marker (McLeod *et al.*, 2012).

Mutagenesis based techniques pose the risk of generating polar effects, that involve gene disruption arising from non-sense mutations in translation regions, reducing the synthesis of distal proteins in operons (Zipser, 1969). The gradient of polarity is evident due to the distance between the mutant and distal genes, often with strongest effects at the proximal end (Epstein and Beckwith, 1968). Integration of external DNA sequences within a single operon gene may disrupt the transcription of genes downstream to the insertion event that may also be essential, subsequently essentiality may be attributed in error (McLeod *et al.*, 2012). Characteristic of all techniques is the definition of essentiality through abolition of function, whereby all genes that cannot tolerate replacement or disruption are deemed essential. Such techniques are incapable of assessing the contribution to fitness of individual essential genes, which may be conserved due to small advantage presented under some growth conditions. This difficulty has been addressed using gene down regulation, whereby an inducible promoter is inserted upstream of the target gene. One variant technique in particular has proved useful and is founded on natural phenomenon of Antisense RNA based regulation.

### **1.2.2 Antisense RNA**

Despite the recognized and accepted concepts of gene organization, It was suggested that gene regulation could be coordinated by genes that encode a transitory intermediate composed of RNA (Jacob and Monod, 1961). Regulation via RNA was later confirmed in prokaryotes, following investigation into the replication of plasmid colE1 (Tomizawa *et al.*, 1981), where it was determined that a complementary RNA blocks primer RNA formation required by DNA polymerase I to initiate synthesis (Eguchi, 1991). Although many fundamental principles have been ascertained through the study of bacterial plasmids (Simons and Kleckner, 1988), RNA based regulation has also been observed in eukaryotes (Almeida and Allshire, 2005) and plants (Baulcombe, 2004).

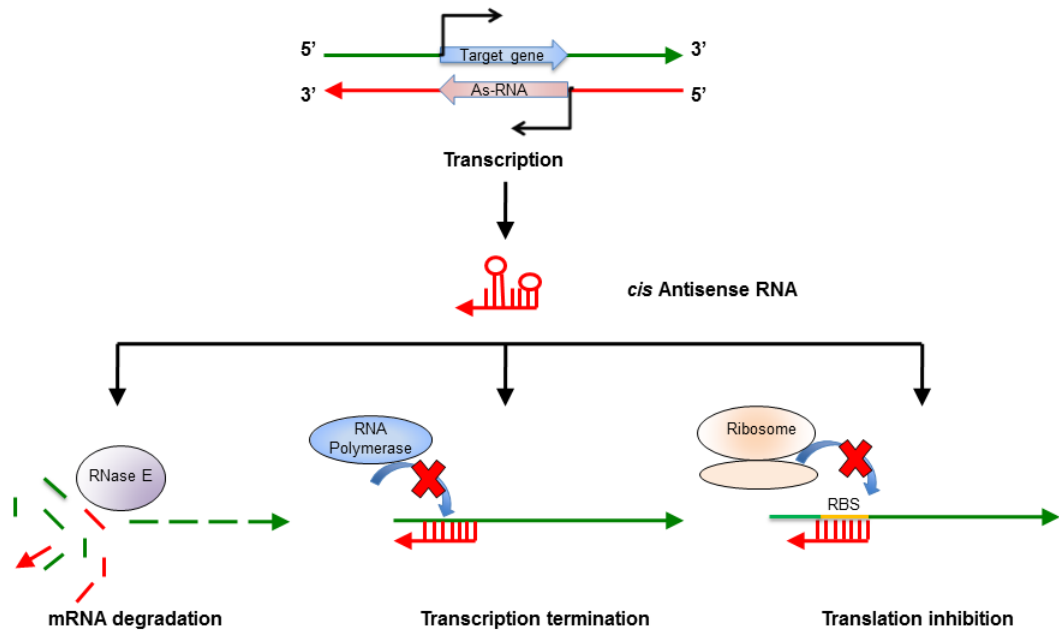
RNA based gene regulation has been associated with heterogeneous RNAs that coordinate gene expression through diverse mechanisms and can be classified into four broad groups (Waters and Storz, 2009). Riboswitches are multidomain RNA's capable of binding metabolites which initiates a global conformational change enabling the riboswitch to bind within the 5' untranslated region (UTR), where it occludes the Shine Dalgarno sequence (Montange and Batey, 2008). Protein binding small RNA's possess innate enzyme activity, or antagonize binding (Waters and Storz, 2009). Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) represents the most recent addition to the RNA regulatory family and comprises a defensive system against phages (Sorek *et al.*, 2008). The most significant group consists of small RNAs encoded in *cis* (or Antisense) and *trans* form (Waters and Storz, 2009). Trans-encoded RNA's are transcribed at distant genomic locations in relation to the target mRNA they regulate, in contrast to *cis*-encoded RNA which are transcribed parallel to their respective target mRNA on the sense DNA strand and therefore share full sequence complementarity (Thomason and Storz, 2011).

Antisense RNA are of particular interest since they represent a subset of small RNA entities (sRNA's) in prokaryotes, responsible for the coordination of gene expression through mechanisms that influence transcription and translation. Following advances in transcriptome analysis, in particular the use of Deep RNA sequencing (RNA-Seq) (Wang *et al.*, 2009) the number of antisense RNA transcripts identified has increased. In *E.coli* alone over 1000 antisense

transcripts have been identified (Dornenburg *et al.*, 2010). From this wealth of data has emerged an array of regulatory roles including the inhibition of transposition, repression of toxic protein synthesis, regulation of transcription factors and metabolic enzymes (Thomason and Storz, 2010). Roles in bacterial virulence (Gripenland *et al.*, 2010) and more recently the restriction modification system in prokaryotes have also been observed (Mruk *et al.*, 2011).

The regulatory functions performed by (*cis*-encoded) antisense RNAs is founded upon complementary hybridization with cognate mRNA sequences at the 5' > 3' end, internally or 3' and 5' untranslated regions (UTRs) (Georg and Hess, 2011). The capacity for target hybridization is attributed to the characteristic secondary structural features of antisense RNA, consisting of a ubiquitous YUNR motif (Y= pyrimidine, R=purine). The motif forms a U-turn loop which serves as a recognition element and provides a scaffold for facilitating helix formation with the target mRNA (Franch *et al.*, 1999). The process of sequence hybridization is thought to proceed via two independent binding pathways, that are initiated by a recognition event between loop structures on both the antisense and target mRNA, before progressing towards the formation of duplex RNA (Brantl, 2007).

A multistep pathway elucidated from the CopT/CopA antisense system of plasmid R1 (Kolb *et al.*, 2000), is initiated with the binding of stem loops to form an unstable kissing complex that is extended via a single stranded region to form a four-helix junction intermediate. The intermediate is transformed into a stable inhibitory complex which converts to a stable RNA duplex. An alternative single step binding pathway was reported for the RNA-IN/RNA out antisense system responsible for regulating the transposition of transposase IS10 (Kittle *et al.*, 1989). In this particular system, an initial interaction occurs between stem loop and single stranded target that permits extension via the loop and stem domain. Following complete RNA duplex formation between antisense RNA and its cognate mRNA target, gene expression can be modulated, by altering transcript stability and either transcription or translation efficiency of the target mRNA via distinct mechanisms (Figure 1.3. Mechanisms of antisense RNA inhibition.) (Georg and Hess, 2011).



**Figure 1.3. Mechanisms of antisense RNA inhibition.**

The stability and half-life of target mRNA is known to decrease following antisense duplex formation through a degradation pathway. In the cyanobacterium *Synechocystis* 6803, the expression of iron stress induced protein (Isia) is known to decrease following over expression of its antisense counterpart IsrR (Iron stress repressed RNA) (Duhring *et al.*, 2006). Conversely stability may also be increased as exemplified with the glutamate decarboxylase transcriptional activator/repressor (*gadX/gadW*) of the *E.coli* acid response system. Glutamate decarboxylase contains an intergenic region between *gadX* and *gadW* that encodes the antisense RNA GadY, complementarily to the 3' UTR of *gadX*. Overexpression of GadY increases accumulation of *gadX* mRNA which increases expression of downstream decarboxylases (Opdyke *et al.*, 2004).

The alteration in mRNA stability is thought to involve a common degradative pathway that regulates mRNA decay. The process is dictated by the structural organization of prokaryote mRNA transcripts. Bacterial mRNA's possess a 5'-terminal tri-phosphate, while the 3'-terminus typically forms a stem loop structure, which is subject to processing events that effect stability and initiate



the process of mRNA degradation (Belasco, 2010). The 3' terminal can be destabilized by polyadenylation by Poly(A)polymerase (Dreyfus and Régnier, 2002), while RNA pyrophosphohydrolase (RppH) is known to convert 5' triphosphate to 5' monophosphates (Deana *et al.*, 2008). Both events initiate mRNA degradation via two distinct pathways, involving the RNA degradosome, a multi-protein assembly responsible for mRNA processing and degradation (Carpousis, 2007). The principle component of the degradosome is RNaseE, a multi-domain endoribonuclease, which cleaves polyribosomal RNA to fragments. The C-terminal of RNaseE serves as a molecular scaffold for assembly of accessory proteins including; Polynucleotide phosphorylase (PNP) a endoribonuclease with 3' activity, RhlB a DEAD-box RNA helicase with ATPase dependent activity for unwinding RNA, and enolase, a glycolytic enzyme which has a purported role as a metabolite sensor (Carpousis, 2007). Degradation may occur via the 5'-dependant pathway, whereby the 5' single stranded regions are subject to hydrolysis of 5'-terminal triphosphate to 5'-terminal monophosphate by RppH. The 5' terminal monophosphate facilitates the binding of RNaseE via subdomains, thereby enabling the correct orientation for initiating internal cleavage (Bouvier and Carpousis, 2011). Alternatively endonucleolytic degradation by RNaseE is initiated by a 5' independent mechanism that generates multiple fragments which are then subject to polyadenylation by polyA polymerase. This permits 3' exonucleases such as PNPase, to attempt to remove structural features such as stem loops (Carpousis *et al.*, 1999).

In some cases, antisense RNA exerts its modulatory effects at the level of gene transcription. The hybridization between antisense RNA and it's cognate mRNA target can initiate the formation of secondary structure which prevents RNA polymerase proceeding, thereby inhibiting transcription. A known example of this mechanism has been observed in *Vibrio anguillarum*, where transcription of the Iron uptake biosynthesis operon was due to a 427 nucleotide antisense transcript that formed a terminator structure consisting of a stem loop (Stork *et al.*, 2007).

An alternate mechanism of antisense RNA based regulation referred to as transcriptional interference has been noted, which arises from the proximity and transcriptional direction of promoters. Convergent promoters facilitate the

collision of independent RNA polymerases, causing displacement of one polymerase and generating inverse correlations of mRNA and antisense RNA, similar to those observed following analysis of the *ubiGmccBA* operon in *Clostridium acetobutylicum* (Andre *et al.*, 2008). Alternatively the collision event may stall elongation or promote backtracking of the polymerase (Crampton *et al.*, 2006). If the strength of converging promoters differs, then the weaker promoter may be occluded or RNA polymerase and associated transcriptional apparatus may be displaced (Sitting Duck interference) (Georg and Hess, 2011).

Antisense RNA based regulation also prevails at the post-transcriptional level, where the hybridization of antisense RNA and cognate mRNA target, directly modulates ribosome binding efficiency. In *E.coli* mutation of *SymR*, a *cis* encoded antisense regulator complementally to the promoter sequence of the SOS-induced antitoxin gene *symE*, causes a differential increase in protein compared to RNA (Kawano *et al.*, 2007). Post-transcriptional control by antisense RNA may be influenced by Hfq, an RNA binding protein, with a multimeric pore structure known to interact with small RNAs by facilitating base-pairing between *cis*-encoded and target mRNA. Roles of Hfq have been postulated in translation repression via binding of sRNA's that sequester the RBS, thereby preventing ribosome binding and subsequently inhibit protein translation. Conversely antisense RNA binding may also expose translation initiation regions and enhance translation. (Vogel and Luisi, 2011).

### **1.2.3 Antisense RNA quantitative effects**

The widespread conservation of antisense RNA based regulation among species, suggests a significant evolutionary role, which complements existing control mechanisms responsible for regulating both transcription and translation (Thomason and Storz, 2010). From a cellular perspective RNA based regulation presents an efficient regulatory mechanism, since the energy costs associated with transcription are considerably less in comparison to the process of protein translation and assembly. In addition, due to speed of synthesis and degradation, mRNA expression can be rapidly modulated unlike protein regulation (Gripenland *et al.*, 2010). Indeed, it was shown that the rate of synthesis is up to two magnitudes faster for antisense RNAs compared to mRNA (Shimoni *et al.*, 2007). Unlike protein based regulation, antisense RNA

has the capacity to minimise temporal fluctuations and filters transient signals in gene expression arising from stochastic events in transcription and translation, thereby minimising surges in protein abundance (Kaern *et al.*, 2005). The regulatory capacity of antisense RNA provides a highly responsive system, capable of adapting to diverse and often sudden stimuli. Consequently antisense regulation is often associated with genes that require expedient expression in response to environmental changes such as regulators of the cellular stress response. Such genes include the outer membrane porin protein F (ompF) in *E.coli*, which is regulated by the antisense RNA MicF. Expression of MicF causes a reduction in protein expression, thereby limiting extracellular uptake, especially in the presence of antibiotics (Delihias and Forst, 2001). Antisense based regulation is also prevalent where the accumulation of toxic proteins would be detrimental to cell viability. The hok/sok system for regulating plasmid stability by post-segregation killing involves the expression of hok (host killing), a membrane associated toxin that causes irreversible damage to cell membranes and is regulated via a 64 nucleotide antisense RNA sok (suppression of killing). Inhibition occurs indirectly as sok inhibits translation of mok (modulation of killing), which in turn mediates the translation of hok (Thisted and Gerdes, 1992). Metabolism represents further cellular activity requiring a rapid response to variations in nutrient levels, and is represented by such systems such as the *lac* operon of *E.coli* which is disordinately regulated by a 109 nucleotide antisense RNA Spot 42 (Møller *et al.*, 2002). Virulence is also reliant on antisense regulation for the expression of key proteins, when particular environmental conditions are encountered. In pathogenesis antisense regulation provides the appropriate degree of responsiveness to host immune threats as observed in *Salmonella enterica*, where a 1.2kb antisense RNA AmgR was found to be complementary to MgtC, which encodes an inner membrane protein essential for intracellular survival within macrophages (Lee and Groisman, 2010).

Quantitative studies of antisense RNA regulation have suggested that the equilibrium between the transcription of antisense and target mRNA dictates gene expression via a threshold linear response (Levine *et al.*, 2007; Levine and Hwa, 2008). Through the modulation of gene expression, characteristic expression profiles are generated for both mRNA and antisense transcripts that infer the regulatory mechanism in addition to advocating the physiological

effect. (Lapidot and Pilpel, 2006). The fine modulation and temporal effects of antisense RNA based regulation, have been exploited for numerous experimental purposes (Rasmussen *et al.*, 2007). Primarily, antisense RNA has been developed for gene silencing techniques and applied to the identification of essential genes in both gram positive and negative organisms, such as *Staphylococcus aureus* (Forsyth *et al.*, 2002), and more recently *E.coli* (Meng *et al.*, 2012). Adaption of the same antisense RNA silencing strategy has also enabled the delineation of bacteriostatic and bactericidal targets in mycobacteria (Kaur *et al.*, 2009). Antibiotic discovery strategies have also benefited from the implementation of antisense RNA silencing in whole cell target-based assays, to elucidate inhibitor mode of action using a differential assay format (Singh *et al.*, 2007). Success with this approach has been demonstrated, with the identification of novel fatty acid synthesis inhibitors platencin and platensimycin (Wenzel *et al.*, 2011) and the novel protein synthesis inhibitor peptidyltransferase (Bandow *et al.*, 2003).

More recently antisense RNA silencing has been used to evaluate the individual contributions of essential genes in maintaining cell viability. Using a combination of insertion mutagenesis and DNA microarrays revealed that the fitness contribution of essential genes under specified growth conditions varies significantly (Badarinarayana *et al.*, 2001). Further efforts have attempted to quantitate essential gene requirement for cell viability termed stringency, by examining mRNA transcript abundance. In a novel study the titration of *E.coli* growth rates using antisense RNA silencing of essential gene targets *fabI*, *murA*, *acpP* and *ftsZ*, established a differential transcript requirement for each gene to maintain cell growth. By defining the minimum transcript level required to maintain 50% cell viability, a hierarchy in stringency was revealed in the order of *acpP* > *ftsZ* > *fabI* > *murA* (Goh *et al.*, 2009). This concept was supported by a recent study that examined cell tolerance to degradation of essential protein and its subsequent effect on cell growth in determining cell vulnerability (Wei *et al.*, 2011).

The concept of quantifying essential gene stringency represents a significant asset to antibiotic discovery strategies; as it would permit the prioritization of essential gene targets for inclusion into screening assays. However, despite the apparent relationship between mRNA reduction and decline in cell growth,

mRNA abundance provides only a surrogate for expressed protein. Functional protein epitomizes the culmination of gene expression, and is viewed as accurate reflection of the physiological state of a bacterial cell. Current estimates of essential gene stringency based upon mRNA abundance may therefore be inaccurate, since the correlation between mRNA and protein expression is often non-linear (Lee et al, 2003). Even across different species, steady state protein abundance displays a higher correlation than corresponding mRNA abundances (Laurent *et al.*, 2010)

The lack of concurrence between mRNA and protein expression is attributed to regulatory mechanisms that operate during transcription and translation (Vogel and Marcotte, 2012). The efficient transcription of mRNA is dependant upon the complementarity of its Shine Dalgarno sequence, which dictates ribosome binding efficiency and therefore translation. The complex secondary structure of RNA represents a further influence since it can either expose or sequester the ribosome binding site under different environmental conditions. At the translational level small antisense RNA affects post-transcription levels via translation inhibition, interference, or degradation of mRNA transcripts, while ribosome density affects translation efficiency. Ensuing translation, protein half life is influenced by factors such as intrinsic stability, rates of degradation, modification by phosphorylation and cell localization (Maier *et al.*, 2009).

Consequently the evaluation of essential gene stringency may be reflected by measuring protein abundance. Protein quantification has previously been performed using western blotting, whereby proteins are transferred from SDS-PAGE gel to an absorbent membrane (e.g.: nitrocellulose) and probed with an antibody specific for the protein under investigation. Quantification is achieved by chemiluminescence using a secondary antibody conjugated to an enzyme, that catalyses the breakdown of a substrate to a coloured product (Kurien and Scofield, 2006). However western blotting lacks quantitative capacity and is incapable of distinguishing between post-translational modifications. Critically only those proteins targeted are subject to analysis, at the exclusion of the global proteome (Mann, 2008). To perform accurate quantification at the protein level requires the use of sensitive techniques such as liquid chromatography tandem mass spectrometry (LC-MS/MS).

## 1.3 The use of mass spectrometry in antibiotic discovery

### 1.3.1 Tandem mass spectrometry

Technological advances in LC-MS/MS have permitted global proteomic analyses, enabling the systematic identification of primary sequences, structural characterization of post-translational modifications, elucidation of protein-protein interactions and quantification of expressed proteins in complex matrices (Aebersold and Mann, 2003).

The Proteomics based LC-MS/MS process, initiates with the pre-separation of individual proteins from a whole cell lysate using either 1D or 2D gel electrophoresis, with the aim of removing low molecular weight contaminants capable of interfering with mass analysis. The protein bands of interest are exercised from polyacrylamide gels and subject to destaining and in-gel digestion. During this process samples are reduced to covert disulphide bonds to free sulfhydryl groups which are then alkylated to a S-carboxymethyl derivative, to facilitate disruption of three-dimensional structure and expose cleavage sites for heterophase enzymatic digestion (Shevchenko *et al.*, 2006). The protease trypsin is frequently utilized, since it predicatively cleaves C-terminal residues at arginine and lysine, generating an appropriate mass range of peptides required for fragmentation during LC-MS/MS (Olsen *et al.*, 2004).

According to peptide fragmentation models, the N-terminus is protonated, but the charge is capable of migrating along the backbone by internal solvation, becoming randomly localized on amide bonds. Cleavage along the peptide backbone results in cleavage at either the alkyl carbonyl bond (CHR-CO), peptide amide bond (CO-NH) or amino alkyl bond (NH-CHR). Following fragmentation, the charge can be retained at either the N terminus producing *a,b,c* ions or at the C terminus producing *x y z* ions. The peptides generated by trypsin are typically doubly charged structurally informative *y* ions, providing a high degree of predictability with respect to their fragmentation patterns (Covey *et al.*, 1991).

Digested peptides are eluted from the polyacrylamide matrix in solvent and subject to further separation using High Performance Liquid Chromatography (HPLC) to minimise sample complexity. By reducing complexity the dynamic range can be increased to permit low abundance peptides to be detected in the

presence of peptides of higher abundance, thereby increasing the confidence of protein identification. Typically a Reversed Phase (RP-HPLC) format is employed, involving the differential separation of peptides between a stationary (nonpolar matrix) and mobile phase (polar solvent) according to the degree of hydrophobic interaction. During this stage hydrophobic peptides interact with the stationary phase, while hydrophilic peptides are eluted earlier in the mobile phase (Dass, 2007).

Eluted peptides are then introduced into an ion source and converted to charged species by introduction or removal of an electron using electrospray ionization, whereby ions are desorbed under a high electric field (Fenn *et al.*, 1989). During this process the sample flows through a capillary with high potential that generates an electrostatic charge that dispenses solution into charged droplets for transportation, via a pressure gradient to a mass analyser (Dass, 2007). The mass analyser of choice is often the orbitrap mass spectrometer, which has provided an unprecedented level of sensitivity required for high resolution tandem mass spectrometry techniques (Makarov and Scigelova, 2010). Using this form of mass analyser provides the high mass resolution, accuracy (2-5 ppm), mass charge ratio and dynamic range ( $10^3$ ) required for protein characterization and quantification. The orbitrap operates on the principle of dynamic ion trapping in electrostatic fields (Makarov, 2000) and constitutes a central spindle electrode encased by a coaxial outer electrode. Following electrospray ionization, ions are transported through a series of quadrupoles that guide, transport and store ions prior to delivery via an optical deflection lens system into the orbitrap. The ions become trapped about the central spindle and undergo harmonic ion oscillations, the frequency of which provides the basis for mass charge values (Hu *et al.*, 2005). The mass charge values of the ions generate a peptide mass fingerprint, which can then be compared to an *in silico* generated mass within a protein sequence database. The database is constructed from theoretical spectra, derived from peptide fragmentation models. Comparisons between the experimental and theoretical spectra is scored to assess the probability of a match and validated using algorithm based descriptive, interpretative, stochastic probability or statistical and probability models (Sadygov *et al.*, 2004).

### **1.3.2 Proteomic strategies**

The characterization of functional differences between biological systems requires comprehensive proteome coverage, if system-wide variations are to be identified and quantified accurately (Cox and Mann, 2007). To date partial proteome coverage has been achieved for 60% of open reading frames in the minimal genome bacteria *Mycoplasma pneumonia* (Kühner *et al.*, 2009), which is marginally higher than that of multicellular organisms such as *Caenorhabditis elegans* (54%) (Schimpf *et al.*, 2009) and *Arabidopsis thaliana* 50% (Baerenfaller *et al.*, 2008). The challenges in obtaining complete proteome coverage can be attributed to a number of technical and biological factors (Beck *et al.*, 2011). The amenability of proteins to solubilisation and digestion dictates whether appropriate tryptic peptides and by extension, characteristic ion spectra can be generated. In the event of obtaining quality spectra, identification of proteins by matching the MS dataset to known sequences is dependant on the quality of annotated open reading frames held within genome databases. Inaccuracies may arise due to experimental conditions which may unknowingly influence transcription and translation parameters, thereby altering protein abundance. Furthermore, sequence coverage is subject to a sufficient fraction of peptides being detected, each of which may vary in terms of ionization and fragmentation properties. The number of peptides arising from enzymatic digestion may also be limited by the size of the protein, with smaller proteins generating fewer unique peptides capable of unambiguously identifying the protein under investigation (Beck *et al.*, 2011). In order to provide consequential data, experimental workflows must be consistently reproducible and accurate for reliable quantification across multiple samples. To this end shotgun (discovery), directed and targeted MS-based strategies that differ in terms of how prior information is utilised and analysed, have been introduced (Domon and Aebersold, 2010).

The shotgun approach utilizes ionized peptides arising from electrospray ionization (precursor ions) to generate an initial mass spectrum known as a survey scan. Data-dependant analysis (DDA) using heuristics is used to perform product ion scanning, whereby precursor ion signal intensity is used to determine  $m/z$  ratio and subsequent peptide mass. A suitable precursor ion is then selected for fragmentation by Collision Activated Dissociation (CAD), that



generates fragments via collision with inert atoms (Hunt *et al.*, 1986). The resulting fragment-ion masses from the product-ion spectra are used for protein identification, while the precursor ion signal intensity is used as the basis for quantification. Since no prior protein knowledge is required, the shotgun approach is suited to open discovery. The method however suffers from bias towards the selection of precursor ions, which represent the most abundant proteins present in a complex sample. Furthermore different samples of peptide ions in each experiment arise from the heuristics used. A variation to this approach is a directed proteomics strategy which entails two independent LC-MS analyses. An initial LC-MS/MS run generates survey scans to identify precursor ions relating to a peptides of interest, and records their mass spectrum characteristics (elution time,  $m/z$  ratio, charge), which are formed into a master inclusion list as part of the data dependant analysis. The LC-MS/MS is repeated in product ion mode using the inclusion list to limit CAD to specific fragments identified in the survey scan. Further improvements can be obtained by adopting a targeted proteomics strategy, which requires the use of triple quadruple instruments operating in Selective Reaction Monitoring (SRM) mode. Unlike previous strategies target proteins are preselected and their precursor ion  $m/z$ , retention time and unique fragment ions formed by CAD are defined to form a selected reaction monitoring (SRM) transition, which functions as assay parameters for the protein of interest (Picotti and Aebersold, 2012). Ultimately the aim of each strategy is achieve consistent detection of individual or subsets of protein.

### **1.3.3 Mass spectrometry quantification strategies**

The accurate differential (relative) or quantitative analysis of protein is paramount and requires the implementation of suitable methods that compare individual peptides between experiments. Quantitation is not integral to the mass spectrometric process, since instrument response is dependant upon the physiochemical properties of proteolytic peptides, which are often influenced by matrix effects (Patterson and Aebersold, 2003). A range of techniques have been introduced based on the isotope dilution principle, in which isotope labelled peptides serve as proxies for their native equivalents. Since isotope labelled peptides possess the same physiochemical properties as native peptides, they generate the same chromatographic and mass spectrometric

profiles. However due to the difference in mass, a differentiation between experimental samples can be made.

Differential (Relative) quantification aims to examine fold changes in protein expression between experimental conditions and can be determined using label free methods (Spectral counts and MS ion intensity) and stable isotope labelling techniques. Spectral counting is founded on an observed linear relationship between protein abundance and the number of precursor ions selected for MS/MS fragmentation (Liu *et al.*, 2004). The generation of MS/MS spectra associated with a particular peptide can therefore be counted and compared relative to the spectral counts of the same peptide under different experimental conditions, enabling relative quantification. Variations to this approach have lead to the estimation of protein expression using the Protein Abundance Index (PAI), derived from the ratio of sequenced peptides and total number of predicted tryptic peptides of a protein (Rappsilber *et al.*, 2002) and absolute Protein Expression profiling (APEX) (Lu *et al.*, 2007) .

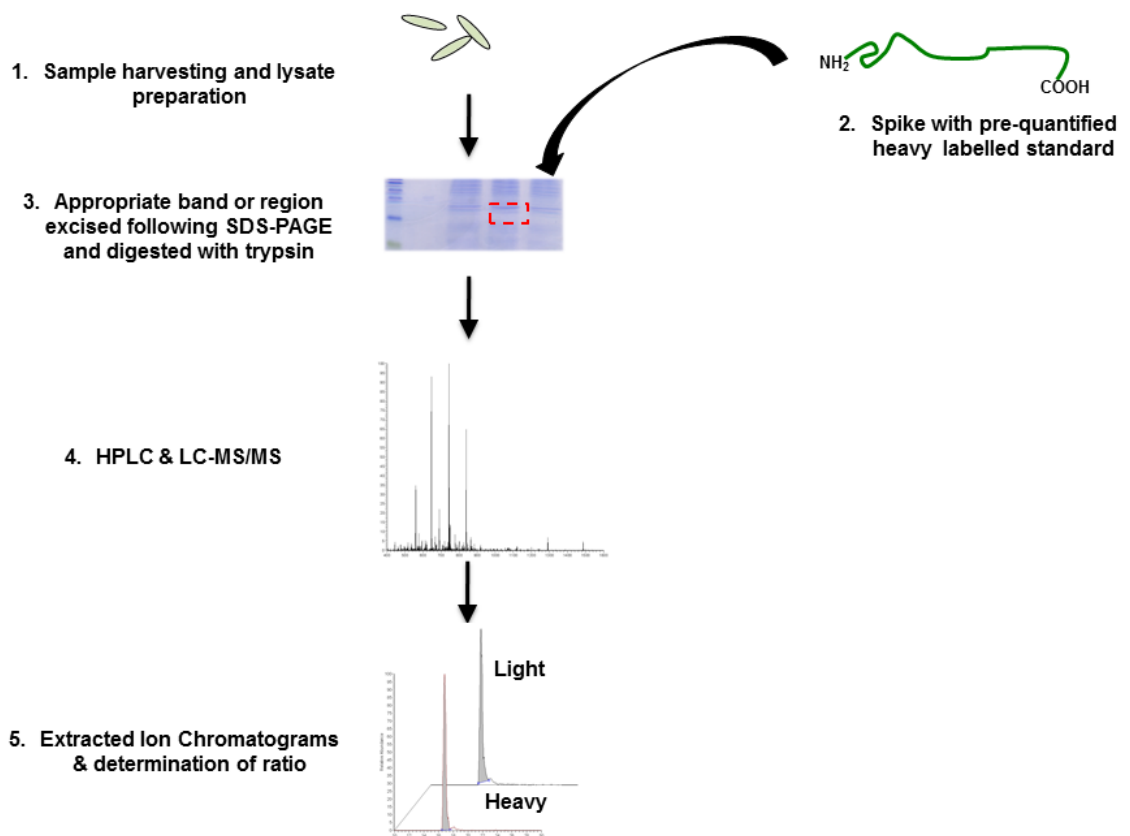
Alternatively the peak area or ion intensity of all identifiable tryptic peptides including their associated parameters of elution time and  $m/z$  ratio can be integrated over a time chromatographic scale. The subsequent data generates an extracted ion chromatogram (XIC) profile that is subsequently normalized to the peak area of an internal protein standard (Bondarenko *et al.*, 2002). Although both techniques provide cost-effective approaches to compare changes in protein expression for multiple experiments, across higher dynamic range, accuracy is unreliable. Systematic and non systematic variations relating to ionization efficiency, sample preparation and instrumental drift are emulated in data, requiring normalization to correct for variation. Furthermore, the linearity of response for each protein is problematic due to variations in saturation effects for individual proteins (Bantscheff *et al.*, 2007; Xie *et al.*, 2011).

Stable isotope labelling provides an alternative to label free methods, by providing greater accuracy for relative quantification. Conceptually, the approach involves the incorporation of low abundance heavy isotopes ( $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^{18}\text{O}$ ) into proteins and peptides, where they function as a label. Differences in ion signal intensities for heavy and light forms of the peptide are therefore taken to reflect quantitative changes in protein abundance (Becker, 2008). Heavy isotopes may be incorporated into proteins metabolically during growth on

labelled media, exemplified using the Stable Isotope Labelling with Amino acids in Cell culture (SILAC) approach, where culture media deficient in essential amino acids is supplemented with isotope labelled equivalents (Ong *et al.*, 2002).

A variation of this approach is to introduce heavy isotopes of  $^{18}\text{O}$  directly into peptides via the C-terminal of proteins following protease digestion. Alternatively isotope containing tags may be introduced via chemical modification of specific amino acid residues. This approach is utilised by Isotope-coded Affinity tags (ICAT), which react with sulfhydryl groups of cysteine residues (Gygi *et al.*, 1999); isobaric Tags for Relative and Absolute Quantification (iTRAQ) which link a carbonyl and reporter group (Wiese *et al.*, 2007) and Tandem Mass tags (TMT), which react with amine groups (Thompson *et al.*, 2003).

Despite providing adequate means to examine changes in protein expression, precise determination of actual protein molecules is not possible without reference to a standard of pre-determined quantity. The requirement for accurate quantification of protein *per se*, has been met by the development of the Absolute Quantification strategy (AQUA) (Kirkpatrick *et al.*, 2005). In this approach, the target protein under investigation is initially analysed by LC-MS/MS to identify a suitable unique tryptic peptide representative of the target protein, for use as an internal standard (Figure 1.4). The standard is synthesised with an appropriate heavy label and evaluated using LC-MS/MS to determine parameters of retention time and product ion intensities. A quantified amount of standard is then introduced to the sample during tryptic digestion and analysed by LC-MS/MS. Quantification is performed by integrating peaks for light and heavy ions corresponding to the sample and standard respectively and analysing the ratio of the peak area, which over a linear range enables extrapolation of the sample concentration. Using this strategy has enabled the quantification of low abundance yeast proteins including phosphorylated forms (Gerber *et al.*, 2003).



**Figure 1.4. Overview of the LC-MS/MS based quantification.**

The use of isotope labelled peptides as internal standards has been developed further to enable multiplex protein quantification, using artificially constructed proteins known as quantification concatamers (QconCAT), which can contain multiple proteolytic peptides of the target protein (Brun *et al.*, 2007) . A further augmentation has been the development of Protein Standard Absolute Quantification (PSAQ), which utilises a full length isotope labelled protein equivalent to the target protein under investigation. (Brun *et al.*, 2009).

#### **1.4 Gene silencing using synthetic antisense structures**

Antisense based silencing has not been constrained exclusively to sequences derived biologically. Attempts to create synthetic compounds capable of binding DNA and RNA in a sequence specific manner have culminated in the development of numerous nucleic acid analogues. Initial efforts comprised dideoxynucleotide bases interconnected by various chemical linkers such as phosphotriester, methylphosphonate, phosphororthiolate, and carbamate (Summerton and Weller, 1997). Subsequent improvements in target specificity

and biostability were achieved through the structural alterations of dideoxynucleotides to generate non-ionic backbones. The derivatization of morpholine subunits from ribonucleosides, formed the basis for phosphorodiamidate morpholino oligonucleotides (PMO's), with significantly improved properties for biological applications (Stirchak *et al.*, 1989). Alternate substitutes for nucleobases precipitated in the synthesis of Peptide Nucleic Acids (PNA) (Nielsen *et al.*, 1991). Structurally, PNA's are comprised of noncyclic peptides (polyamides of N-2-aminorthyl glycine linked via carbonyl linkers to nucleobases), in substitution of the sugar phosphate backbone (Lundin *et al.*, 2006). This arrangement creates structural flexibility with a neutral charge that negates electrostatic repulsion, thereby increasing hybridization efficiency and sequencing specificity with nucleic acid counterparts (Good and Nielsen, 1997). The high affinity, discriminatory capacity and biostability of PNAs, has made them pre-eminent for antisense applications where short sequences are encountered (Summerton, 2006).

Recognition between PNA and complementary sequences elicits the formation of duplex structures via Watson-Crick hydrogen bonding, when the PNA is composed of purine /pyrimidine nucleobases. If the PNA composition exclusively incorporates homopyrimidines, then triplex structures are assembled via Watson-Crick and Hoogsteen hydrogen bonds (Larsen *et al.*, 1999). To date, complexes representing triplex, triplex Invasion, duplex and double duplex invasion are been observed (Nielsen, 2010). The formation of these alternate complexes, contributes to variant silencing mechanisms. Triplex forming PNA's significantly distort RNA structure that precipitates steric hindrance, causing inhibition of both translation initiation and ribosome elongation, contrary to duplex structures which prevent translation initiation (Knudsen and Nielsen, 1996) and possibly ribosome read-through (Kulyté *et al.*, 2005). In contrast to expressed antisense, translation inhibition of mRNA appears to be the primary silencing mechanism, since PNA complexes show a significant degree of biostability towards RNaseH (Knudsen and Nielsen, 1996), thereby preventing RNase mediated mRNA degradation.

The biological stability PNA's have exhibited in bacterial extracts (Demidov *et al.*, 1994), and in addition to their hybridization properties, PNA's makes them a novel research tool for synthetic RNA silencing applications in bacteria (Good

and Stach, 2011). Central to this development have been efforts to address bacterial entry and accumulation of PNAs to an effective intracellular concentration. These factors are critical since growth inhibition is attributed to PNA accumulation and slow efflux resulting in potent antibactericidal activity (Nikravesh *et al.*, 2007).

The primary obstacle opposing cellular entry is the bacterial membrane, which in gram negative bacteria comprises a cell wall consisting of an outer membrane, a thin peptidoglycan layer, a layer of periplasm, and a plasma membrane (Beveridge, 1999). The outer membrane consists of lipopolysaccharide (LPS), which provides an effective barrier due to its rigid lipid interior and strong interaction between the peptidoglycan layers. The cellular accumulation of PNA is opposed by transmembrane porins within the outer membrane, which possess the capacity to remove certain peptides (Nikaido, 2003). However in a recent study, one such membrane transport protein Smba was speculated to be involved in actively transporting peptide PNA conjugates into cells (Ghosal *et al.*, 2013).

The significance of cellular entry and accumulation was previously evaluated by exposing *E.coli* strains A19 and D22 deficient in cell walls and specific membrane pumps AcrAB and Emr to a PNA targeting the *lac* repressor to enable a correlation between susceptibility and  $\beta$ -galactosidase activity following permeabilisation. Although the absence of membrane pumps showed little effect on activity, the lack of LPS was found to be a significant factor in increasing  $\beta$ -galactosidase activity, suggesting increased membrane permeabilisation promotes greater susceptibility (Good *et al.*, 2000a).

Consequently numerous strategies have been deployed to improve delivery of PNA's across the membrane. Initial attempts utilized microinjection, electroporation or co-transfection with cationic lipids (Koppelhus and Nielsen, 2003). A pioneering approach has been to conjugate PNA's to a synthetic cell wall permeabilising peptide KFFKFFKFFK possessing cationic, hydrophobic and amphiphilic properties (Good *et al.*, 2001b). The presence of a peptide promotes uptake across the membrane by receptor independent mechanism, through interaction with complementarity groups in the LPS (Vaara and Porro, 1996). Divalent cation binding sites of the LPS in particular, may have a role in compromising membrane integrity through the formation of transient rents that

enable PNA entry (Hancock, 1997). The effectiveness of this strategy was demonstrated by conjugating permeabilising peptide to a PNA targeting the essential gene *acpP* in *E.coli*, resulting in growth inhibition (Good *et al.*, 2001b). Further evidence was obtained using chromogenic reporter systems to monitor permeabilisation of both the outer and inner membrane, which showed an increase in fluorescence in the presence of PNA peptide conjugates compared to free PNA (Eriksson *et al.*, 2002). Although the majority of studies examining PNA-conjugates have utilised to gram negative bacteria, their effectiveness has also been established in gram positive species such as *S.aureus*, where the targeting of essential genes also causes growth inhibition (Nekhotiaeva *et al.*, 2004).

Due to the specificity and bactericidal activity displayed *in vitro* and *in vivo*, there has been intense interest into developing PNA's into species specific antimicrobials, that avoid compromising natural microbiota defences (Good and Stach, 2011). Alternative PNA based approaches have focused on re-introducing antibiotic susceptibility using PNA's to target resistance mechanisms. One study demonstrated that PNA targeting of the multidrug efflux transport in CmeABC in *Campylobacter jejuni*, was found to increase sensitivity to ciprofloxacin and erythromycin (Jeon and Zhang, 2009a). Additional roles for PNA's have also been explored including targeted gene repair, whereby PNAs are constructed with a corrected sequence of a mutated gene. The formation of triplex PNA structure with the target gene sequence perturbs the regular helical structure, initiating DNA repair mechanisms that result in homologous recombination. Subsequently the repaired sequence is exchanged with the mutated gene (Vasquez *et al.*, 2001). A viable demonstration of this potential was shown by single base pair modification of  $\beta$ -globin intron using pseudocomplementary PNAs (Lonkar *et al.*, 2009). Further roles for PNA's have been demonstrated such as artificial restriction DNA cutters that enable high fidelity site selective scission of DNA (Miyajima *et al.*, 2009), probes for microarrays (Brandt and Hoheisel, 2004), and investigation of microbial communities (Hatamoto *et al.*, 2010).

## 1.5 Project outline

The central aim of this project is to investigate the application of antisense silencing in two distinct roles. Firstly, as a technique for determining the stringency of essential genes in *E.coli* and how stringency differs between individually transcribed and operon based genes. Secondly as a species specific silencer, by evaluating the capacity of synthetic antisense sequences to discriminate between essential gene homologues and thereby enable selective growth inhibition of a specific organism.

Expressed antisense RNA silencing using inducible paired termini vectors, has been employed to determine essential gene stringency, as defined by a Minimum Transcript Level ( $MTL_{50}$ ) (Goh *et al.*, 2009). Determinations of essential gene stringency have been limited to individually transcribed genes (Goh *et al.*, 2009), which are not subject to the complex regulation associated with operon based genes (Ames and Martin, 1964). This raises the question of whether the  $MTL_{50}$  is applicable to operon based genes. Furthermore, stringency is based upon the relationship between growth rate decline and mRNA abundance, which is assumed as a proxy for expressed protein. However post-transcriptional and translation regulation mechanisms, account for uncorrelated abundancies between mRNA and protein (Maier *et al.*, 2009). Consequently measurements of mRNA abundance may be unsuitable for determining essential gene stringency requirements. Determinations of gene stringency would therefore have more relevancy if the relationship between growth rate decline and protein abundance was examined. From this a Minimum Protein Level ( $MPL_{50}$ ) could be derived to replace  $MTL_{50}$ , as a more accurate measure of gene stringency. A method capable of accurately quantifying expressed protein following gene silencing is therefore essential.

Historically, protein quantification has been performed using biochemical assays (Sapan *et al.*, 1999), immunoassays and more recently protein microarrays (MacBeath, 2002). However such techniques lack sensitivity and are subject to cross reactivity, which complicates quantification (Patterson and Aebersold, 2003). Recent developments in the quantitative capacity of LC-MS/MS provide a number of advantages over such methods. LC-MS/MS provides a greater sensitivity over a large dynamic range, enabling low abundance proteins to be detected. This is crucial for antisense silencing



experiments, where target protein may be depleted to unpredictable levels. Crucially, LC-MS/MS permits the identification of individual proteins from complex matrices, such as whole cell lysate. If required parallel identification and quantification of more than one protein can also be accomplished, which supports the evaluation of operon based genes, where the monitoring of more than one protein may be required. Furthermore LC-MS/MS possess the capacity to discriminate between proteins subject to subtle post-translation modifications that can be used to estimate rates of degradation and synthesis. (Bantscheff *et al.*, 2007; Bronsema *et al.*, 2012). The viability of determining protein abundance has been demonstrated with LC-MS/MS using label free methods a modified protein abundance index method (Ishihama *et al.*, 2005), revealing proteins to exist over a dynamic range from 100 to  $10^5$  copies per cell (Ishihama *et al.*, 2008).

In this study, an absolute quantification strategy using LC-MS/MS that incorporates the use of a stable isotope ( $^{15}\text{N}$ ) labelled protein internal standard for absolute quantification, will be used to examine protein expression in *E.coli* subject to antisense silencing. Although LC-MS/MS strategies that use protein internal references for absolute quantification are not widely employed, they have nonetheless been demonstrated in bacteria such as *Leptospira interrogans* (Malmström *et al.*, 2009).

The essential *E.coli* genes *fabI* and *murA* have been selected as candidate targets for developing protein internal standards, since both genes are individually transcribed and have been previously evaluated with regards to gene stringency. Furthermore, both genes have well characterised functions and are subject to inhibition by antibiotics with known modes of action. In *E.coli* *fabI* encodes the enzyme enoyl-acyl carrier protein reductase, which functions as part of the dissociated (type II) fatty acid synthase system, that regulates membrane lipid synthesis (Magnuson *et al.*, 1993). The broad spectrum biocide triclosan (trichloro derivative of 2-hydroxy-phenyl ether) specifically targets FabI in *E.coli*, in addition to gram positive bacteria, fungi and mycobacteria (Saleh *et al.*, 2011). Triclosan inhibits lipid synthesis, through direct targeting of *fabI* as demonstrated in mutants, which show an approximate 300-fold increase in resistance (McMurry *et al.*, 1998). The established mode of action arises from irreversible binding of triclosan to the enoyl substrate site, leading to an

increase in affinity for NAD<sup>+</sup> (Heath *et al.*, 1999). Triclosan binds non-covalently via face to face stacking of the phenol ring and nicotinamide rings hydrogen bonding. Consequently a stable ternary complex is formed that is incapable of catalysis between phenolic hydroxyl and hydrolysis of 2' nicotinamide ribose, causing enzyme inhibition. The binding efficiency of triclosan is enhanced by its ability to induce a closed conformation of a flexible loop structure located above the enoyl active site (Qiu *et al.*, 1999).

The essential gene *murA* encodes enolpyruvyl transferase, which catalyses the transfer of an enolpyruvate moiety from phosphoenolpyruvate (PEP) to uridine diphosphate. This represents the initiating step in the synthesis of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) glycan units that comprise peptidoglycan, of the bacterial cell wall (Lovering *et al.*, 2012). The antibiotic fosfomycin ((1R,2S)-1,2-epoxypropylphosphonic acid) Inhibits MurA through the formation of covalent interactions between a thiol group of cysteine115 residue within the active site of the enzyme, which prevents a conformational change in the enzyme required for the dissociation of the product UDP-N-glucosamine (UNAG) (Eschenburg *et al.*, 2005).

Once validated, LC-MS/MS will be applied to investigating gene stringency in operon based genes in *E.coli* by examining *fusA* and *rplE*. The essential genes *fusA* which is encoded in the *str* operon (Dean *et al.*, 1981b) and *rplE* encoded in the *spc* operon (Cerretti *et al.*, 1983). The essential gene *fusA* encodes elongation factor G (EF-G), a translational GTPase that binds to the ribosome complex and induces a conformational change involving hydrolysis of GTP, which initiates translocation of tRNA and mRNA (Rodnina *et al.*, 1997). A functional role has also been determined in ribosome recycling, where EF-G in conjunction with the essential protein Ribosome Recycling Factor (RRF) induces dissociation of the 70S ribosome into its component subunits in preparation for next round of protein synthesis (Hirokawa *et al.*, 2005). In *E.coli* *rplE* encodes the 5S rRNA ribosome accessory protein L5, which has been demonstrated to be essential for cell survival (Korepanov *et al.*, 2007). The L5 protein is one of 21 accessory proteins that in conjunction with 16S rRNA constitute the small ribosome subunit (30S), that interacts with tRNA and mRNA (Steitz, 2008) The exact role of *rplE* was unknown until recently, when it was

revealed that the L5 protein forms a prominent part of the central protuberance (CP) of the large ribosomal subunit (Korepanov *et al.*, 2012).

In addition to quantifying expressed protein for the purposes of measuring gene stringency, the use of LC-MS/MS can also be applied to support the development of an antisense RNA based screening assay for detecting novel target specific inhibitors. To date antisense based target assays have demonstrated potential, with the discovery of novel antibiotics such as platensimycin a FabF inhibitor (Wang *et al.*, 2006), platencin a FabH /F inhibitor (Wang *et al.*, 2007) and Phaeosphenone, which targets the encoded product of *rpsD* (Zhang *et al.*, 2008). By sensitizing *E.coli* to target specific inhibitors and then measuring expressed protein abundance at particular MIC's, would enable suitable levels of IPTG induction required for antisense silencing to be defined. Consequently assay sensitivity could be optimised for screening target specific compounds that are synthesized in low abundance.

Furthermore the knowledge of the expressed protein abundance following antibiotic exposure could be used generate a characteristic proteomic signature (Brotz-Oesterhelt *et al.*, 2005). This data can then be used to identify candidate cell targets for antibiotic screening and infer potential modes of action. In addition, characterisation of proteomic signatures provides a basis for constructing reference compendia for the purposes of identifying novel antimicrobial compounds (Freiberg *et al.*, 2004). Using candidate targets *fabI* and *murA*, this study intends to use a validated LC-MS/MS approach to measure protein abundance in *E.coli* at different inhibitory concentrations of triclosan and phosphomycin, which aims to provide sufficient data for a mathematical model for estimating assay sensitivity

An alternate to using expressed antisense RNA from plasmids, for the purpose of evaluating essential gene stringency or fine tuning the sensitivity of screening, are peptide PNA's. The capacity of peptide PNA conjugates to induce gene silencing, has been evaluated against expressed antisense RNA and found to be highly comparable (Goh *et al.*, 2009). In addition, the inherent stability of PNA's *in vivo*, has facilitated their development as species-specific antimicrobials in an effort to address antibiotic resistance (Good and Stach, 2011). Indeed bactericidal activity has previously been demonstrated against individual gene targets in *E.coli* (Good *et al.*, 2001b). To date however

no effort has been made to ascertain if PNA's can be designed to differentiate between a genes in separate bacterial species. This study intend to evaluate species-specificity, by designing a PNA that targets an essential gene homologue (*ftsZ*) in *Salmonella typhimurium*, when grown in a mixed microbial culture with *E.coli*.

## 1.6 Specific aims and objectives

1. Identify essential genes for silencing using bioinformatics strategy.
2. Construct antisense RNA expressing strains for *E.coli* essential genes *fusA* and *rplE*.
3. Validate target specificity of antisense RNA constructs by constructing over expression vectors to be used in a transcomplementation strategy.
4. Delineate whether bactericidal or bacteriostatic inhibition occurs following silencing of essential genes *fusA* and *rplE* using viable counts.
5. Design and validate a QPCR assay for the relative quantification of mRNA abundance, of *fusA*, *rplE*, *fabI* and *murA*.
6. Determine essential gene stringency of *fusA* and *rplE*, using expressed antisense RNA silencing to achieve a titration of growth rate in *E.coli*, and measure associated mRNA abundance. From this data MTL<sub>50</sub> values for *fusA* and *rplE* will be derived.
7. Synthesise heavy labelled protein standards for *fabI* and *murA* and evaluate suitable tryptic peptides for identification and quantification of respective proteins
8. Quantitate heavy labelled standards using a standard curve constructed from commercial prequantified peptide using LC-MS/MS.
9. Determine gene stringency of *fabI*, using expressed antisense RNA silencing to achieve a titration of growth rate *E.coli*. Protein abundance will be measured using LC-MS/MS and FabI molecules per cell will be calculated to derive a MPL<sub>50</sub> value for measuring gene stringency.
10. Assess discriminatory capacity of species-specific PNA in mixed bacterial culture.

## 1.7 Thesis Outline

### Chapter 1. Introduction

This Introduction describes the significant features of antibiotics and the challenges posed by multidrug resistance. New strategies for antibiotic discovery are outlined, with particular reference to the use of antisense RNA and quantitative LC-MS/MS. An argument is presented for the assessment of essential gene stringency based upon expressed protein.

### Chapter 2. Methods and Materials

The materials and methods details the construction of antisense RNA and transcomplementation expression vectors, in addition to an LC-MS/MS quantitative assay, using a <sup>15</sup>N labelled internal protein standard, for the evaluation of essential gene stringency. In addition a method used to assess the species specificity of a PNA in a mixed culture of *S.typhimurium* and *E.coli* is also described.

### Chapter 3. Results

The experimental findings regarding target specificity of antisense RNA expression constructs, their validation by transcomplementation, and characterization of inhibitory action of essential gene targets is presented. Analysis of essential gene stringency using mRNA abundance of candidate genes is given. This chapter also reports the validation of a quantitative LC-MS/MS assay for measuring protein molecules per cell following expressed antisense silencing, and subsequent deriving of the MPL50 value as a measure of essential gene stringency. The analysis of species-specific PNA effects on bacteria in a mixed culture is also presented.

### Chapter 4. Discussion

An evaluation of the experimental findings obtained in this study is made. Explanations to account for the observed data including anomalies are proposed, with support from appropriate literature.

### Chapter 5. Conclusions and Future Work

This chapter draws on the discussion to frame the findings of the study in the wider context of antibiotic discovery, in addition to extending the scope of the research to support applications in other fields of biological enquiry. Recommendations to address the limitations highlighted during the course of this study are made.

### Appendix

Additional experimental detail is presented where appropriate.

### References

Literature used to provide a theoretical background is presented.

## Chapter 2 Methods & Materials

### 2.1 Selection of essential gene targets for silencing

A hierarchy of gene stringency (Jem .Stach, personal communication) was generated by obtaining transcript abundance, presence in an operon and number of protein-protein interactions for the essential protein. Transcript abundance for *E. coli* K12 genes was averaged from microarray experiments conducted in LB media at 37<sup>0</sup>C (RNA extracted at mid-exponential phase,  $n = 50$  (Glasner *et al.*, 2003). Presence of the gene in an operon, and location within the operon was obtained from the RegulonDB database (Gama-Castro *et al.*, 2008), and the number of protein interactions was taken from the experimental dataset of the Bacteriome.org database (Su *et al.*, 2008). For each category, the essential gene was ranked, in the case of location within an operon; weighting was assigned prior to ranking, such that genes that were present at the start of large operons, containing other essential genes downstream, were scored highly. Weightings of 20:4:2 were applied for the number of essential genes downstream, the number of genes in the operon, and the number of genes downstream, respectively. The weighted score for each gene was calculated as sum product of these categories, multiplied by the sum of the weights for each category. The weights were arbitrarily assigned considering the mechanism of antisense gene silencing i.e. silencing of the first gene in an operon is more likely to affect genes downstream. The estimated transcript number and number of protein interactions, were given simple rank scores (higher numbers in each giving a higher rank scores). Finally, for each essential gene an overall rank score was applied by weighting (6:6:3) for transcript copy number, number of interactors and presence in an operon, respectively. The final weightings were arbitrarily applied considering that genes with high transcriptional abundance, producing products which are highly interactive, are more likely to be stringently required. Weighting were also applied such that the final rankings for genes *acpP*, *fabI*, *ftsZ* and *murA* were consistent with previous reported MTL<sub>50</sub> values (Goh *et al.*, 2009). The genes *rplE* and *fusA* were chosen for further study, as they were predicted to be stringently required (ranked 1 and 8, respectively) and were also present in operons. This latter fact was considered important for assessing the utility of the MTL<sub>50</sub> score for genes present in operons, where downstream affects will likely

result from antisense RNA silencing. Nucleotide sequences complementary to the ribosome binding region of mRNA transcribed for *fusA* (-93 +86) and *rpIE* (-95 +60 ) were identified and cloned into plasmid pHN678, which contains flanking inverted repeats that form secondary structure in the form of dsRNA termini. The termini increase the stability of antisense transcripts thereby improving silencing efficiency (Nakashima *et al.*, 2006). To validate the specificity of silencing constructs, transcomplementation plasmids expressing the entire ORF of *fusA* and *rpIE* were also constructed using pBAD (Guzman *et al.*, 1995)). For the purposes of comparison, an evaluation was made against antisense RNA expression vectors and associated transcomplementation vectors for essential gene targets *acpP*, *ftsZ*, *fabI*, and *murA* (kindly supplied by Shan Goh, Royal Veterinary College, U.K).

### **2.1.1 Bacterial growth conditions and strains**

Bacterial growth was performed in liquid and on solid media using Muller Hinton Broth (MHB) (Melford) for both *E.coli* and *S.typhimurium*. Growth conditions were 37°C, with aeration at 200-250 rpm for broth cultures. Where maintenance of plasmids was required, media was supplemented with antibiotics (Melford) at a final concentration of 30µg/ml chloramphenicol, 100µg/ml ampicillin, 50µg/ml kanamycin. Detailed recipes or growth media and buffer solutions can be found in Appendix: A. All strains used in this study are listed in Table 2.1.

### **2.1.2 Bacterial genomic DNA and plasmid extraction**

Both antisense and complete ORF sequences for *fusA*, *rpIE*, *fabI* and *murA* were amplified from genomic DNA, which was isolated from *E.coli*-K12 using the GenElute™ Bacterial Genomic DNA kit (Sigma). Briefly 1.5 ml of overnight bacterial culture was harvested by centrifugation at 16,000 x g for 2 minutes. The supernatant was removed and pelleted cells were resuspended in 180 µl Lysis Solution T with 20 µl of RNase A and incubated for 2 minutes at room temperature. After incubation, 20 µl of proteinase K solution was added and samples were incubated for 30 minutes at 55°C. A volume of 200 µl of Lysis Solution C was added to samples which were then incubated at 55°C for a further 10 minutes. GenElute Miniprep Binding columns were prepared by addition of 500 µl of Column Preparation solution. Columns were centrifuged at



12,000 x g for 1 minute and the flow through was discarded. Prior to loading, 200 µl of ethanol (95-100%) was added to lysate and vortexed, before loading onto columns and centrifuged at 6,500 x g for 1 minute. The spin column was placed in a fresh collection tube and 500 µl of Wash Solution I was added, samples were then centrifuged at 16,000 x g for 3 minutes. The spin column was placed in a fresh collection tube and a further 500 µl of Wash Solution I was added, samples were centrifuged at 6,500 x g for 1 minute. The column was placed in a fresh collection tube and centrifuged at 16,000 x g for 1 minute. DNA was eluted by addition of 200 µl of Elute Solution followed by an incubation of 5 minutes at room temperature and centrifugation at 16,000 x g for 1 minute. Genomic DNA was quantified in triplicate using a NanoDrop™ (Thermo Scientific).

Plasmid DNA was extracted using the QIAprep® Spin kit (Qiagen). Briefly 5 ml of overnight bacterial culture was pelleted by centrifugation at 2000 x g for 5 minutes. Pelleted cells were resuspended in 250 µl Buffer P1 containing RNase A, to which 250 µl of Buffer P2 was added and the sample mixed by inversion. Buffer N3 (350 µl) was added and samples were centrifuged at 17,900 x g for 10 minutes. The supernatant was added to a QIAprep spin column and centrifuged at 17,900 x g for 1 minute. The flow through was discarded and 500 µl of Buffer PB was then added to the spin column. Samples were centrifuged at 17,900 x g for 1 minute, and the flow through was discarded. A second wash using 750 µl of Buffer PE was added to the spin column, prior to centrifugation at 17,900 x g for 1 minute. The wash through was removed and the sample was centrifuged at 17,900 x g for 1 minute. Plasmid DNA was eluted addition of 30 µl of Buffer EB directly to the centre of the spin column, which was incubated at room temperature for 1 minute, prior to centrifugation at 17,900 x g for 1 minute. Plasmid DNA was quantified in triplicate using a NanoDrop™ (Thermo Scientific).

### ***2.1.3 Primer preparation and annealing temperature optimization***

To ensure optimal amplification of antisense and ORF sequences, a primer validation study was performed. Lyophilized Primers (Sigma/Eurogentec) (Table 2.4 and Table 2.5), were re-dissolved to a concentration of 100 µM, and then diluted to a working concentration of 10 µM in molecular grade water (Melford). Initial gradient Polymerase Chain Reaction (PCR) was performed by preparing

a 25 $\mu$ l reaction, consisting of 2 x PCR master Mix (0.05 units/ $\mu$ l Taq DNA polymerase, 4 mM MgCl<sub>2</sub>, 0.4 mM each of dATP, dCTP, dGTP, dTTP) (Fermentas), diluted with molecular grade water to a final concentration of 1 x and mixed with forward and reverse primers at a final concentration of 0.2  $\mu$ M. A total amount of 100ng of template DNA was added. The thermal Gradient was devised by using a range of 3°C either side of the average calculated primer pair T<sub>m</sub>. The PCR thermal profile was as follows: denaturing 95°C for 2 minutes, 30 cycles of denaturing at 95°C for 30 seconds, annealing temperature for 30 seconds, extension at 72°C for 2 minutes and a final extension of 72°C for 5 minutes. All gradient PCR were performed on a DYAD™ DNA Engine (MJ Research). Optimal primer annealing temperatures were evaluated by visual inspection of amplified product using gel electrophoresis (see methods section: 2.1.4).

#### **2.1.4 Agarose gel electrophoresis**

Analysis of PCR products was performed by agarose gel electrophoresis to ensure correct size of amplified products and ascertain the optimal primer annealing temperature. Agarose gels were prepared by dissolving an appropriate amount of agarose (Invitrogen) to achieve a final concentration of 0.5-2% as required for optimal resolution of DNA fragments, in 10 x Tris-borate-EDTA buffer (1.3 M TRIS, 450 mM boric acid, 25 mM EDTA.Na<sub>2</sub> in H<sub>2</sub>O) (Sigma), diluted to 1 x concentrate. Agarose gels were stained with ethidium bromide (Sigma) at a final concentration of 0.5 $\mu$ g/ml. Gels were ran at 8v/cm for 1 hour and photographed using a Uvitec gel doc systems with Essential v12.6 imaging software (UVITECH). Samples were prepared by mixing 5-10  $\mu$ l of DNA with 6 x loading dye (Fermentas) to achieve 1 x final concentration. For size estimation 5  $\mu$ l of 100Kb ladder and Hind III digest (Fermentas) were used as required.

#### **2.1.5 Cloning of antisense and ORF sequences into pHN678 and pBAD plasmids**

Antisense sequences for essential genes, *fusA* and *rpIE* and the ORF of target genes *fabI*, *murA*, *fusA* and *rpIE* were amplified from *E.coli* K12 genomic DNA, using sequence specific primers (Table 2.4), Antisense sequences were designed with restriction sites for *XhoI* and *NcoI*, to permit placement of PCR

insert in reverse orientation in the multiple cloning site (MCS) of pHN678, in order to generate antisense transcript. Transcomplementation vectors were designed with restriction sites for *XhoI* and *EcoRI* (*fabI*, *murA*, *rpIE*) and *XhoI* and *SacI* (*fusA*). PCR amplification of target sequences was performed using Phusion® High fidelity DNA polymerase (New England Biolabs). A 50µl PCR reaction was prepared with final concentrations of 1 x reaction buffer, 0.2 µM primers with exception of *fabI* (1 µM), 200 µM dNTP, 0.02 units and 4 µl cDNA template. Thermal cycling was performed on 9700 geneamp thermal cycler (Applied Biosystems) using the following conditions: 98°C 30 seconds, 98°C for 10 seconds, optimized annealing temperature for 15 seconds, 72°C 15 seconds, and final extension of 72°C for 1 minute. Subsequent products were purified using QIAquick PCR purification kit (Qiagen) and quantified using a NanoDrop™ Spectrophotometer and associated software ND1000 v3.2.1 (Thermo Scientific). Confirmation of PCR products was performed by agarose gel electrophoresis (see methods section: 2.1.4).

#### **2.1.6 Restriction digest of PCR inserts and plasmids**

To prepare PCR inserts for cloning into their respective vectors, double restriction digests were performed using *XhoI* and *NcoI* for antisense sequences *fusA* and *rpIE*. Amplicons of complete ORF sequences were double digested with *XhoI* and *EcoRI* (*fabI*, *murA*, *rpIE*) and *XhoI* and *SacI* (*fusA*) in addition to pBAD. Restriction digests were performed according to manufacturers' instructions (Fermentas) and consisted of 10 x Tango buffer (330 mM Tris-acetate, 100 mM Mg-Acetate, 660 mM K-acetate, 1 mg/ml bovine serum albumin (BSA)) diluted to a final 2 x concentration. For double digests, 10 units of appropriate restriction enzyme (Fermentas) was added per 1µg of DNA, molecular grade water was then added to bring the final reaction volume up to 40 µl. Samples were incubated at 37°C for 2 hours followed by a thermal inactivation of restriction enzymes by incubating at 80°C for 20 minutes. Digested products were purified using QIAquick PCR purification kit (Qiagen) and quantified in triplicate using a NanoDrop™ (Thermo Scientific).

#### **2.1.7 Ligation of PCR inserts into plasmids**

Digested antisense and ORF sequences were ligated into pHN678, and pBAD respectively. Sticky-end ligation was performed using 100 ng of linear vector

DNA according to manufacturers' instructions. The molar ratio of insert DNA was calculated using the Ligations: Molar ratio of insert to vector calculator (Promega). Vector and insert DNA were mixed in a ratio of 1:3 with 10 x T4 DNA ligase buffer (Fermentas) (400 mM Tris-HCl, 100 mM MgCl<sub>2</sub>, 100 mM DTT, 5 mM ATP), which was diluted to 1 x concentration. For each reaction, 5 units of T4 DNA ligase (Fermentas), was added. Molecular grade water was then added to bring final reaction volume to in a 20 µl. Samples were incubated for 10 minutes at 22°C followed by thermal inactivation of T4 ligase by incubating at 65°C for 10 minutes. Confirmation of ligation insertion was performed by restriction digestion and agarose gel electrophoresis (see methods section: 2.1.4).

### **2.1.8 Transformation of antisense and transcomplementation plasmids into *E.coli***

Ligated plasmids containing pHN678 or pBAD with antisense sequences or complete ORF sequences respectively were introduced into *E.coli* by chemical transformation. For each plasmid, 5 µl of ligated product was added to one vial of One Shot TOP10 Chemically competent *E.coli* (Invitrogen). The competent cells were then incubated on ice for 30 minutes, prior to heat shocking for 30 seconds at 42°C without shaking. Competent cells were then left on ice to recover for 2 minutes. A total volume of 250 µl of pre-warmed SOC medium (see Appendix: A) was added to each vial, which were subsequently incubated at 37°C for 1 hour with aeration. A suitable volume of transformants (100 µl) was pipetted onto pre-warmed LB agar containing an appropriate antibiotic and incubated at 37°C overnight. Transformants carrying the correct plasmid with insert were screened using colony PCR with plasmid specific primers for pHN678 and pBAD respectively (Table 2.4).

## **2.2 Evaluation of antisense specificity of *fusA* and *rplE* constructs**

To examine the specificity of antisense RNA constructs, antisense growth assays were prepared according to the procedure used in a previous evaluation of essential gene targets (Goh *et al.*, 2009). A series of IPTG (Melford) concentrations were prepared to induce antisense RNA expression from pHN678 vectors, to generate a titration in *E.coli* growth rate. IPTG concentrations were prepared in molecular grade water at 10 x concentration,

20  $\mu\text{l}$  of which was pipetted in triplicate into a 96 well micro plate (Sarstedt). Overnight cultures of *E.coli* were standardized by OD<sub>550</sub> readings to  $2 \times 10^4$  cfu/ml, using a LibraS12 Spectrophotometer (Biochrom). Standardized culture volumes of 180  $\mu\text{l}$  were added to respective wells, to bring the final assay volume to 200  $\mu\text{l}$ . The 96 well microplate was incubated in a Powerwave HT plate reader (Biotek Instruments Inc) at 37°C with agitation every 5 seconds and readings at 550nm taken every 5 minutes for a period of 24 hours. Bacterial growth curves were plotted using Gen5 software v1.04.5 (BioTek). Raw absorbance data corresponding to the exponential phase of *E.coli* growth was exported to Excel (Microsoft). A trendline was applied and the gradient of the growth curve recorded. Relative growth rate was derived by normalizing the trendline gradient of induced cells to the trendline gradient of uninduced controls.

### **2.3 Validation of antisense specificity by transcomplementation**

To validate the target specificity of antisense constructs, rescue assays were performed by transforming both antisense and corresponding transcomplementation vectors into *E.coli* TOP10. A matrix of IPTG and L-arabinose induction concentrations were assessed to determine the level of induction required to demonstrate the rescue of bacterial growth rate, in accordance with a previous study (Goh *et al.*, 2009). A dilution series of IPTG and L-arabinose (Melford) were prepared at 20 x concentration, 10  $\mu\text{l}$  of each substrate was added to respective wells, prior to the addition of 180  $\mu\text{l}$  standardised culture in MH media supplemented with 30 $\mu\text{g/ml}$  chloramphenicol and 100  $\mu\text{g/ml}$  ampicillin. Bacterial growth curves were plotted using Gen5 software v1.04.5 (BioTek). Raw absorbance data corresponding to the exponential phase of *E.coli* growth was exported to Excel (Microsoft). A trendline was applied and the gradient of the growth curve recorded. Relative growth rate was derived by normalizing the trendline gradient of induced cells to the trendline gradient of uninduced controls.

## **2.4 Delineation of the inhibitory effects of silencing essential gene targets *fusA* and *rplE***

To examine the inhibitory effects of antisense RNA silencing on growth, viable cell counts were performed in the presence of 50  $\mu$ M IPTG, which was double the concentration used to silence growth in the examination of specificity. As per the procedure for antisense and rescue assays, overnight bacterial cultures were standardized by OD<sub>550</sub> readings to  $2 \times 10^4$  cfu/ml. A 50 ml volume of MH broth containing chloramphenicol (30 $\mu$ g/ml) was inoculated and then incubated at 37°C with aeration. Sample volumes of 100  $\mu$ l were removed every hour over a period of 8 hours, serially diluted ( $10^{-5}$  and  $10^{-6}$ ), plated onto MH agar (in triplicate) and incubated 37°C overnight. A viable colony count was then performed on plates containing between 30 and 300 colonies.

## **2.5 Evaluation of essential gene stringency by quantitative assessment of mRNA transcripts**

To maintain consistency with a previous assessment of essential gene stringency (Goh *et al.*, 2009), QPCR was used to provide relative quantification of mRNA abundance in *E.coli*, following silencing of essential gene targets *fusA* and *rplE*. Normalisation was performed relative to two reference genes; 16S rRNA which forms part of the 30S subunit (Shajani *et al.*, 2011) and *zipA* which encodes an inner membrane protein involved in the formation of septal ring structure during cell division (Hale and De Boer, 1997). Both genes had previously been selected following microarray analysis of the *E.coli* transcriptome (Shan Goh personal communication).

### **2.5.1 Isolation and clean-up of total RNA**

*E.coli* was induced with IPTG and harvested once the OD<sub>550</sub> of the control (uninduced) culture reached 0.1, as it had previously been determined that optimal correlation between mRNA abundance and relative growth rate occurred at this point in the growth curve (Goh *et al.*, 2009). Total RNA was extracted from *E.coli* using PureLink™ RNA Mini Kit including on-column DNase treatment (Life Technologies), in accordance with manufacturer's protocol for isolating bacterial cell RNA. Briefly, bacterial cells were harvested at

500 x g for 5 minutes and resuspended in 100µl of lysozyme solution (Sigma) containing 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1 mg lysozyme from chicken egg white. In addition 0.5 µl of 10% SDS and 350 µl of lysis buffer containing 1% 2-mercaptoethanol was also added. Samples were homogenized by repeated expulsion through a 21 gauge needle. Cell lysate was centrifuged at 12,000 x g for 2 minutes to remove cellular debris, prior to addition of 250 µl 100% ethanol to precipitate RNA. Samples were vortexed and transferred to a spin column with collection tube and centrifuged at 12,000 x g for 15 seconds. The spin column containing bound RNA was placed in a fresh collection tube to eliminate carryover and DNase treatment was performed. The spin column was washed with 350 µl of Wash buffer I and centrifuged at 12,000 x g for 15 seconds, prior to the addition of 80 µl of DNase solution, containing 1 x DNase I reaction buffer and resuspended DNase I (~3 U/µl). Samples were incubated at room temperature for 15 minutes prior to the addition of 350 µl Wash buffer I and centrifugation at 12,000 x g for 15 seconds. The spin column was placed in a fresh collection tube and 500 µl of Wash buffer II was added. Samples were centrifuged at 12,000 x g for 15 seconds and the spin column was removed and placed in a fresh collection tube. Samples were centrifuged at 12,000 x g for 1 minute and the spin column was transferred to a clean 1.5ml microcentrifuge tube. To elute the RNA, 30 µl of RNase/DNase free water was added to the center of the column, which was then incubated at room temperature for 1 minute prior to centrifugation at 12,000 x g for 2 minutes. Additional RNA purification was performed using TRI reagent (Molecular Research Centre, INC). Briefly 1 ml of TRI reagent was added to the eluted RNA samples and left at room temperature for 5 minutes. Following dissociation of nucleoprotein complexes, 0.1 ml of 1-bromo-3-chloropropane (BCP) (Sigma) was added, samples were mixed and stored at room temperature for 15 minutes. Samples were centrifuged at 12,000 x g for 15 minutes at 4°C. The aqueous phase containing RNA was removed and precipitated with 0.5 ml of isopropanol at room temperature for 10 minutes before being centrifuged at 12,000 x g for 8 minutes at 4 °C. RNA was washed with 1 ml of 75% ethanol and centrifuged at 12,000 x g for 5 minutes at 4°C. The RNA pellet was air-dried for 5 minutes prior to solubilisation in 50 µl RNase-free water.

### **2.5.2 cDNA synthesis of extracted RNA**

The conversion of RNA template to cDNA was performed using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). A 20µl reaction was prepared consisting of 5 x reaction buffer (250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM MgCl<sub>2</sub>, 50 mM DTT), diluted to 1 x concentration. In addition, 20 units Ribolock RNase inhibitor, 1 mM dNTP mix, 5 µM random hexamer and 200 units of M-MuLV Reverse Transcriptase were added. RNA template was standardized to 100 ng for each reaction. Reactions were prepared in two steps; initially molecular grade water, random hexamers and RNA template were heated to 65°C for 5 minutes to remove secondary structure, prior to addition of the remaining reaction components. cDNA synthesis was performed by incubating samples at 25°C for 5 minutes, 42 °C for 1 hour, then 70°C for 5 minutes to terminate reverse transcriptase activity. Genomic DNA contamination was assessed using reverse transcriptase negative and blank reactions.

### **2.5.3 Relative quantification of mRNA transcripts by QPCR**

QPCR assay validation was performed according to established guidelines (Nolan *et al.*, 2006). Briefly primer matrices consisting of concentrations 0.1 – 0.3 µM for all primers with exception of *fabI* (1-2 µM) were prepared and analysed in duplicate. Optimal primer concentrations for forward and reverse primers were selected according to the primer pair displaying the lowest C<sub>T</sub> (Cycle threshold) value, which signifies the number of cycles required for the fluorescent signal to exceed background level. Reaction efficiency was assessed using duplicate reactions of six fold serial dilutions of target amplicon, with efficiency calculated from standard curve slopes according to the equation  $E = 10^{[-1/\text{slope}]} - 1 \times 100$ . Samples were prepared in final volumes of 25 µl consisting of 2 x Maxima® SYBR Green qPCR Master Mix (Fermentas) diluted to 1 x concentration and 2 µl of cDNA synthesis reaction or 5 µl positive control DNA. Analysis was performed on a Chromo 4 (DYAD) using Opticon Monitor v 3.1.32 (Biorad). Relative quantification was performed using the DD<sub>Ct</sub> method (Livak and Schmittgen, 2001).



**Table 2.1 Strains used in this study**

<b>Strain</b>	<b>Genotype</b>	<b>Function</b>	<b>Source</b>
<i>E.coli</i> TOP10	F- mcrA $\Delta$ (mrr-hsdRMS-mcrBC) $\phi$ 80lacZ $\Delta$ M15 $\Delta$ lacX74 nupG recA1 araD139 $\Delta$ (ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 $\lambda$ -	Host Strain for plasmids	Invitrogen
<i>E.coli</i> As- <i>fabI</i>	As TOP10, pHN682	Expression of antisense <i>fabI</i> RNA	(Nakashima <i>et al.</i> , 2006)
<i>E.coli</i> As- <i>acpP</i>	As TOP10, pHN682	Expression of antisense <i>acpP</i> RNA	(Goh <i>et al.</i> , 2009)
<i>E.coli</i> As- <i>murA</i>	As TOP10, pHN682	Expression of antisense <i>murA</i> RNA	(Goh <i>et al.</i> , 2009)
<i>E.coli</i> As- <i>ftsZ</i>	As TOP10, pHN682	Expression of antisense <i>ftsZ</i> RNA	(Goh <i>et al.</i> , 2009)
<i>E.coli</i> Ash01	As TOP10, pHN678- <i>rplE</i>	Expression of antisense <i>rplE</i> RNA	This study
<i>E.coli</i> Ash02	As TOP10, pHN678- <i>fusA</i>	Expression of antisense <i>fusA</i> RNA	This study
<i>E.coli</i> Ash03	As TOP10, pBAD- <i>rplE</i>	Overexpression of <i>rplE</i>	This study
<i>E.coli</i> Ash04	As TOP10, pBAD- <i>fusA</i>	Overexpression of <i>fusA</i>	This study
<i>E.coli</i> Ash05	As TOP10, pHN678- <i>rplE</i> , pBAD- <i>rplE</i>	Transcomplementation rescue of <i>rplE</i> antisense	This study
<i>E.coli</i> Ash06	As TOP10, pHN678- <i>fusA</i> , pBAD- <i>fusA</i>	Transcomplementation rescue of <i>fusA</i> antisense	This study
<i>E.coli</i> Ash07	As TOP10, pHN678, pBAD-HisA	Transcomplementation control	This study
<i>E.coli</i> AG1 ME5305	recA1 endA1 gyrA96 thi-1 hsdR17(rk- mk+) supE44 relA1	Overexpression of FabI in minimal media	National Bioresource Project

**Table 2.2 Paired termini antisense expression plasmids used in this study.**

<b>Plasmid</b>	<b>Characteristic</b>	<b>Features</b>	<b>Location and length</b>	<b>Source</b>
pHN678	IPTG-inducible promoter ( <i>P<sub>trc</sub></i> ) with paired termini (PT) flanking MCS CamR	<i>E.coli</i> stabilized antisense expression vector	N/a	(Nakashima <i>et al.</i> , 2006)
pHN682	<i>fabI</i> antisense insert	Inducible expression of <i>fabI</i> antisense RNA	-74 to +68 of <i>fabI</i> (160 nucleotides)	(Nakashima <i>et al.</i> , 2006)
pHNA	<i>acpP</i> antisense insert	Inducible expression of <i>acpP</i> antisense RNA	-42 to +85 of <i>acpP</i> (127 nucleotides)	(Goh <i>et al.</i> , 2009)
pHNM	<i>murA</i> antisense insert	Inducible expression of <i>murA</i> antisense RNA	-54 to +78 of <i>fabI</i> (130 nucleotides)	(Goh <i>et al.</i> , 2009)
pHNZ	<i>ftsZ</i> antisense insert	Inducible expression of <i>ftsZ</i> antisense RNA	-53 to +76 of <i>fabI</i> (129 nucleotides)	(Goh <i>et al.</i> , 2009)
pHN678- <i>rplE</i>	<i>rplE</i> antisense insert	Inducible expression of <i>rplE</i> antisense RNA	-95 +60 of <i>rplE</i> (155 nucleotides)	This study
pHN678- <i>fusA</i>	<i>fusA</i> antisense insert	Inducible expression of <i>fusA</i> antisense RNA	-93 +86 of <i>fusA</i> (179 nucleotides)	This study

**Table 2.3 Over expression vectors used in this study**

<b>Plasmid</b>	<b>Characteristic</b>	<b>Features</b>	<b>Location and length</b>	<b>Source</b>
pBAD-HisA	L-arabinose inducible promoter ( $P_{bad}$ )	Inducible expression of essential genes	N/a	Invitrogen
pBAD-HisA-rpIE	Complete ORF <i>rpIE</i>	Inducible expression of <i>rpIE</i>	N/a	This study
pBAD-HisA-fusA	Complete ORF <i>fusA</i>	Inducible expression of <i>fusA</i>	N/a	This study
pGlo	Complete ORF Green Fluorescent Protein (GFP)	Inducible expression of GFP	N/a	Biorad
pDs-Red Express	Complete ORF Red Fluorescent Protein (RFP)	Inducible expression of RFP	N/a	Clontech

**Table 2.4 PCR primers used in this study**

<b>Primer Name</b>	<b>Target Amplified</b>	<b>Sequence</b>	<b>Amplicon size (Bp)</b>	<b>Purpose</b>
fusA-XF1	Antisense of <i>fusA</i>	CGTCCATGGGTTCGATGTGCGCACTGATA	179	Cloning of <i>fusA</i> antisense into pHN678, resulting in pHN678- <i>fusA</i>
fusA-XR2		TGTCTCGAGCCCTTCGGAGTTTTAGTCACC		
rplE-XF5	Antisense of <i>rplE</i>	CGTCCATGGGGGACTTGCATGACAGAATTG	155	Cloning of <i>rplE</i> antisense into pHN678, resulting in pHN678- <i>rplE</i>
rplE-XR5		TGTCTCGAGGGCTGACCGTGTAGGCTTTA		
fablf	<i>fabI</i>	ATCCTCGAGATGGGTTTTCTTTCCGGT	827	Cloning of <i>fabI</i> ORF into pBAD, resulting in pBAD- <i>fabI</i>
fablr		ATCGAATTCCGATTATTTTCAGTTCGAGTTCGT		
murA-OF-His	<i>murA</i>	GAGCTCGAGATGGATAAATTTCTGTGTTTCAGGGGC	1282	Cloning of <i>murA</i> ORF into pBAD, resulting in pBAD- <i>murA</i>
murA-OR-His		CCGGAATTCACGATTATTCGCCTTTCACACG		
fusAf	<i>fusA</i>	ATCCTCGAGATGGCTCGTACAACACCC	2135	Cloning of <i>fusA</i> ORF into pBAD, resulting in pBAD- <i>fusA</i>
fusAr		ATCGGTACCGCTTATTTACCACGGGCTT		
rplEf	<i>rplE</i>	ATCCTCGAGATGGCGAAACTGCATGATTAC	564	Cloning of <i>rplE</i> ORF into pBAD, resulting in pBAD- <i>rplE</i>
rplEr		ATCGAATTCCTACCTTACTTGCGGAACG		
pHN678 forward	pHN678 Multiple cloning site	CTGCAGGTCGTAAATCACTGCA	N/a	Screening for antisense inserts
pHN678 forward		CTTCTCTCATCCGCCAAAAGTAG	N/a	
pBAD forward	pBAD multiple cloning site	ATGGCTAGCAAAGGAGAAGAAC	N/a	Screening for ORF inserts
pBAD Reverse		TAGAGCTCATCCATGCCATGTG	N/a	

**Table 2.5 QPCR primers used in this study**

<b>Primer Name</b>	<b>Target Amplified</b>	<b>Sequence</b>	<b>Amplicon size (Bp)</b>	<b>Purpose</b>
fabI QPCR Forward	<i>fabI</i>	CTGACCCTTTCCTACCTTG	66	<i>fabI</i> target
fabI QPCR Reverse		TTTTGCCAGACCCATAAC		
murA QPCR Forward	<i>murA</i>	CACTACCTGCGCGTTGTACGA	64	<i>murA</i> target
murA QPCR Reverse		TGTTGAGGCCAGAAATGTG		
fusA QPCR Forward	<i>fusA</i>	ACCTGGGTGGTGAAGAAGT	105	<i>fusA</i> target
fusA QPCR Reverse		TCTTGAACGCAGAACCACAG		
rplE QPCR Forward	<i>rplE</i>	AAAACCGCTGATCACCAAAG	111	<i>rplE</i> target
rplE QPCR Reverse		AAGAACTCCCACATGCGTTC		
16S QPCR Ref Forward	16S	AGGCCTTCGGGTTGTAAAGT	97	Reference gene
16S QPCR Ref Reverse		GTTAGCCGGTGCTTCTTCTG		
ZipA QPCR Ref Forward	<i>ZipA</i>	ATAAACCGAAGCGCAAAGAA	102	Reference gene
ZipA QPCR Ref Reverse		CCGCTTGTTGAATGCTGTTA		

## **2.6 Evaluation of essential gene stringency by protein quantification**

To address the limitation of using mRNA abundance as a proxy for protein expression in determining essential gene stringency, a method for absolute quantification of expressed protein was developed. Central to this approach is the use of an LC-MS/MS assay incorporating a pre-quantified heavy labelled ( $^{15}\text{N}$ ) protein as an internal standard for the quantification of expressed protein following expressed antisense RNA silencing of essential gene targets.

### **2.6.1 Development of a $^{15}\text{N}$ labelled protein internal standard**

To validate the LC-MS/MS approach, the *E.coli* essential genes *fabI* and *murA* were selected as targets, since both have previously been evaluated for essential gene stringency. To enable complete labelling of the target protein, a metabolic labelling strategy was implemented. *E.coli* expressing pBAD-*fabI* and pBAD-*murA* were grown in liquid M9 minimal media supplemented with  $^{15}\text{N}$  ammonium chloride (Cambridge Isotope Laboratories Inc) as the sole nitrogen source (see Appendix: A). Due to difficulties in cultivating these particular strains of *E.coli* with ampicillin as a selection agent, an alternative strain was sought. *E.coli* clones overexpressing His-tagged FabI and MurA from the ASKA collection (A Complete Set of *E.coli* K-12 ORF Archive), were obtained from the National Bioresource Project-*E.coli*, (National Institute of Genetics, Japan). Suitable growth was achieved in M9 minimal media supplemented with chloramphenicol using both these strains. Bacterial cultures were grown to an  $\text{OD}_{550}$  of 0.5, prior to the induction of protein expression by the addition of IPTG to a final concentration of 1 mM. Bacterial cultures were then grown for approximately 12 hours prior to harvesting.

### **2.6.2 Extraction of $^{15}\text{N}$ labelled protein internal standard from *E.coli* Lysate using IMAC**

Following protein expression in minimal media, His-tagged FabI and MurA were extracted from *E.coli* using Immobilised Metal Affinity Chromatography (IMAC), in conjunction with a nickel column (Invitrogen). All reagents used in the preparation of IMAC buffers were sourced from Sigma. Approximately 250 ml of bacterial culture was centrifuged in a sorvall RC5C Refrigerated Superspeed Centrifuge (Dupont) at 10,000 x g for 10 minutes. To each pellet 10 ml of Lysis buffer (20 mM Tris-Cl pH 7.6, 1 M NaCl, 0.1 mM PMSF, 20 mM Imidazole, 2%

Triton-X-100, 150 mg lysozyme) was added. The sample was then sonicated for 5 minutes at amplitude of 10 microns using a Soniprep 150 (MSE (UK) Ltd). Lysed samples were centrifuged at 20,000 x g for 10 minutes to remove cellular debris prior to the lysate being loaded onto a Nickel column (GE Healthcare Life sciences) coupled with AKta Prime (GE Healthcare Life sciences). Bound protein was washed and removed from the column using an elution buffer (20 mM Tris-Cl pH 7.6, 1 M NaCl, 0.1 mM PMSF, 300 mM Imidazole, 2% Triton-X-100). Binding and elution of his-tagged FabI and MurA was monitored by U.V spectrometry on a trace in PrimeView 5.0 (Amersham Biosciences).

### ***2.6.3 SDS-PAGE of extracted <sup>15</sup>N labelled protein internal standards***

Extracted protein was evaluated for approximate size using denaturing SDS-PAGE. All reagents used in the preparation of polyacrylamide gels were obtained from Melford, with the exception of 30% Acrylamide/0.8% Bisacrylamide which was sourced from Sigma. Poly-acrylamide gels (12-20% concentration) were prepared using Tris-Cl pH 8.8, containing final concentrations of 0.1% SDS, 0.375 M Tris-Cl pH8.8 (separating gel), (0.375 M Tris-Cl pH 6.8, for the stacking gel) 0.05% ammonium persulfate and 0.03% TEMED. A 5 x Tris-glycine running buffer was prepared (0.025M Tris, 0.192 M glycine and 0.1 M SDS) and used at 1 x concentration. Gels were photographed using Image-Scanner (Amersham Biosciences and Labscan v5.0 (GE Healthcare Life Sciences). Protein samples were prepared in 30 µl volumes using 5 x SDS Page loading dye (Fermentas) and 20 x Reducing agent (Fermentas) at final 1 x concentration. Samples were heated at 97°C for 4 minutes to denature protein samples, prior to loading. For size estimation of proteins, 5 µl of Page Ruler Pre-stained Protein Ladder (Fermentas) was used.

### ***2.6.4 Purification and concentration of <sup>15</sup>N labeled protein internal standards***

To remove contaminants associated with the IMAC elution buffer and thereby prevent interference with downstream LC-MS/MS analysis, extracted <sup>15</sup>N labelled protein (FabI and MurA) was purified using an acetone precipitation protocol (New England Biolabs). Briefly, 100 µl of <sup>15</sup>N labelled protein was mixed with 400 µl of ice-cold acetone (Sigma), vortexed and incubated for 1 hour at -20°C. The protein extract was centrifuged at 13,000 x g for 10 minutes

and the supernatant was decanted. The protein pellet was then redissolved in 100  $\mu$ l of molecular grade water. A small volume of redissolved protein (80  $\mu$ l) was mixed with 120  $\mu$ l of 5 x SDS loading buffer, 370  $\mu$ l of molecular grade water and 30  $\mu$ l of 20 x reducing agent. The protein sample was heated to 97°C for 3 minutes and placed in a sonibath (VWR) for 5 minutes, followed by a further heating step of 97°C for 3 minutes. The sample was centrifuged at 16,000 x g for 10 minutes and the supernatant was removed to a fresh tube. Protein purity was confirmed by SDS-PAGE, using a 12% polyacrylamide gel.

### **2.6.5 Antisense induction for protein and RNA quantification**

An overnight culture of *As-fabl* was standardized by OD<sub>550</sub> readings to approximately  $2 \times 10^4$  cfu/ml in a final volume of 200 ml in MH broth containing chloramphenicol (30 $\mu$ g/ml). An IPTG dilution series was prepared using final concentrations of 50, 55 and 70  $\mu$ M corresponding to growth inhibition of approximately 75%, 50% and 25% respectively. Cultures were incubated at 37°C with aeration until the OD<sub>550</sub> of a control culture reached 0.1. Bacterial cells were harvested at 12,000 x g for 5 minutes, resuspended in 21 ml fresh MH broth which was subsequently split into two 10 ml aliquots for protein and RNA isolation (see methods section: 2.5.1) for analysis by QPCR respectively. The Protein aliquot was centrifuged and resuspended in 1.5 ml MH broth and sonicated at 10 microns for 45 seconds five times.

### **2.6.6 Protein digestion and SDS-gel extraction from *E.coli* lysate**

For LC-MS/MS, there is a requirement for protein to be digested into its respective peptides, to render it amenable to electrospray ionization. Consequently *E.coli* lysates were separated by SDS-PAGE to reduce sample complexity prior to LC-MS/MS analysis. All reagents with the exception of trypsin were purchased from Sigma. Proteins were excised from SDS-PAGE gels using a scalpel and placed into a 1.5 ml microcentrifuge tube containing 140  $\mu$ l of 200 mM ammonium bicarbonate (NH<sub>4</sub>HCO<sub>3</sub>) pH 7.8. Gel pieces were destained in a 37°C incubator for 15 minutes. Samples were centrifuged at 10,000 rpm for 10 seconds, followed by removal of the supernatant, 140  $\mu$ l of 200 mM NH<sub>4</sub>HCO<sub>3</sub> pH 7.8/acetonitrile (MeCN) 4:6 was then added, followed by the addition of 140  $\mu$ l of 50mM NH<sub>4</sub>HCO<sub>3</sub> pH 7.8. Samples were incubated at 37°C for 30 minutes. Following incubation, the supernatant was removed after a



brief centrifugation at 10,000 x g for 10 seconds after which 140 µl of MeCN was added, followed by incubation at room temperature for 5 minutes. The MeCN was removed following brief centrifugation and the entire destaining procedure was repeated until no coomassie dye was visible. The gel pieces were reduced by addition of 50 µl 10 mM DTT in 100 mM NH<sub>4</sub>HCO<sub>3</sub> and incubated at 56°C for 1 hour. Samples were briefly centrifuged and the supernatant was removed and 50 µl of 50 mM iodacetamide was added. Samples were incubated in the dark for 30 minutes. The supernatant was removed and gel pieces were washed with 200 µl 100 mM NH<sub>4</sub>HCO<sub>3</sub> pH 7.8 and incubated at 37°C for 15 minutes with shaking. The supernatant was removed and 200 µl 50 mM NH<sub>4</sub>HCO<sub>3</sub>/MeCN (50:50 v/v) was added, followed by incubation at 37°C for 15 minutes with shaking. Following removal of the supernatant, 140 µl of MeCN was added followed by incubation at 37°C for 5 minutes with shaking. The MeCN was removed following centrifugation and 140 µl of fresh MeCN was added until gel pieces turned white. Gel pieces were then digested with trypsin solution consisting of 10 µl of 0.5 µg/µl trypsin (Promega), 250 µl 50 mM NH<sub>4</sub>HCO<sub>3</sub> and 1 mM CaCl<sub>2</sub>. A 30 µl volume of digest solution was added to the gel pieces followed by incubation at room temperature for 5 minutes. A 30 µl addition of 50 mM NH<sub>4</sub>HCO was then added to the gel pieces which were then incubated at 37°C overnight. Peptides were extracted by a 10 µl addition of 5% trifluoroacetic acid (CF<sub>3</sub>CO<sub>2</sub>H), vortexed and then centrifuged at 10,000 x g for 10 seconds. The supernatant was removed to a fresh (non-autoclaved) 1.5 ml microcentrifuge tube. Gel pieces were covered with 2% CF<sub>3</sub>CO<sub>2</sub>H acid/60% MeCN solution, vortexed and centrifuged again followed by collection of the supernatant. A 20 µl volume of 100% MeCN was then added to the gel pieces, which were subsequently incubated at room temperature for 5 minutes until changing from colourless to white, at which point supernatant was removed. Extracted peptide solution was concentrated by in a speed vac (Eppendorf) and resuspended in 10 µl 1% CF<sub>3</sub>CO<sub>2</sub>H, 5% MeCN and stored at -80°C.

#### ***2.6.7 Evaluation of representative tryptic peptides to use for the identification and quantification of <sup>15</sup>N labelled protein internal standard and for the assessment of matrix effects***

To evaluate the potential of matrix effects, a post-extraction spike method was employed (Matuszewski *et al.*, 2003). A known volume (2 µl) of purified FabI

internal standard was spiked, prior to in-gel digestion with trypsin, into replicate samples of different volumes (10µl, 20µl, 30µl) of *E.coli* TOP10 cell lysate. The cell lysate originated from *E.coli* silenced for *fabI* expression to achieve a titration of the relative growth rate. Induction concentrations of IPTG (50 µM, 55 µM and 65µM) were examined and chosen to achieve an approximate 25%, 50% and 75% reduction in the relative growth rate respectively. Subsequent LC-MS/MS analysis identified 30 tryptic peptides which were evaluated according to established criteria (Han and Higgs, 2008). The absolute area under each extracted ion chromatogram corresponding to the <sup>15</sup>N labelled protein internal standard spike and unlabelled species from the *E.coli* lysate were compared, to ascertain if the signal of the <sup>15</sup>N labelled standard suffered significant variation.

#### **2.6.8 Quantification of *FabI* Internal Standard**

The *FabI* internal standard was quantified using a standard curve, constructed from commercially synthesized and pre-quantified peptide analogues of EGAELAFTYQNDK and ILVTGVASK peptides (JPT). Both peptides were redissolved in RNase/DNase free water (Melford) and serially diluted to generate individual standard curves spanning 6 orders of magnitude (0.5-50,000 fmol). Serial dilutions of each peptide were spiked into trypsin digests (in triplicate) containing a 2 µl volume of *FabI* internal standard and analysed by LC-MS/MS (see methods section: 2.6.9). The absolute area under each extracted ion chromatogram corresponding to the <sup>15</sup>N labelled protein internal standard and the unlabelled peptide analogue were averaged. Standard curves for each peptide were generated in Sigma plot and a straight line equation fitted. The concentration of the <sup>15</sup>N labelled internal standard was then determined from the straight line equation.

#### **2.6.9 LC-MS/MS Analysis of *FabI* protein in silenced samples**

LC-MS/MS was performed by the North East Proteome Facility (NEPAF) using an in-house protocol. Peptides were concentrated on a Pepmap C-18 trap column (300 µm ID x 5 mm) and separated on a Pepmap C18 reversed phase column (Dionex, UK) (3 µm particles, 75 µm ID x 250 mm), using a linear gradient over 42 min from 96% A (0.05% formic acid), 4% B (0.05% formic acid, 80% acetonitrile) to 35% A, 65% B and a flow rate of 300 nl/min. Using the

software package; Xcalibur 2.0. Intact peptides were detected between  $m/z$  400 and  $m/z$  1,600 in the Orbitrap XL (ThermoFisher) at a resolution of 30,000. Internal calibration was performed using the ion signal of  $(\text{Si}(\text{CH}_3)_3\text{O})\text{H}^+$  at  $m/z$  445.120025 as a lock mass. Parts per million mass accuracy on an Orbitrap mass spectrometer (via lock mass injection into a C-trap). Maximum ion accumulation time allowed on the LTQ Orbitrap was 1s for all scan modes. Automatic gain control was used to prevent over-filling of the ion trap. Collision induced dissociation (CID) spectra of the top 5 peptide ions (rejection of singly charged precursors) were acquired between  $m/z$  400 and  $m/z$  1,600 at normalized collision energy of 35. Dynamic exclusion was set with a repeat count of 1, a repeat time of 30s and an exclusion time of 3 min. The chromatography feature was enabled with a correlation area ratio of 1.0. Activation Q was set to 0.25 with 30 ms activation time.

#### **2.6.10 Whole cell screening assays using triclosan and phosphomycin**

To examine the potential of defining appropriate levels of sensitivity in antisense based whole cell screening assays using  $\text{MPL}_{50}$  Values, an MIC liquid assay was performed. The target specific antibiotics for *fabI* (triclosan) and *murA* (phosphomycin) were selected as proxies for natural compounds, to examine the level of antisense silencing required to reduce the MIC. MIC assays were performed as per antisense assays (see methods section 2.22.2). A dilution series of triclosan (Sigma) and phosphomycin (Sigma) were prepared in 100% Dimethyl sulfoxide (DMSO) (Sigma) and water respectively, based upon published MIC values for triclosan (Bailey *et al.*, 2009) and phosphomycin (Takahata *et al.*, 2010). To each well of a 96 well assay plate, 10  $\mu\text{l}$  of respective antibiotic and 10  $\mu\text{l}$  of IPTG were added, prior to the addition of 180  $\mu\text{l}$  standardised culture of strains *As-fabI* or *As-murA*.

### **2.7 Assessment of target specificity using PNA**

To investigate whether peptide conjugate PNA with antisense sequences could discriminate between gene homologues, and silence an essential gene target in different bacterial species, a mixed microbial assay was performed.

### **2.7.1 Design of Species-specific PNA**

The design of species specific PNA was performed using Artemis (Rutherford *et al.*, 2000), according to established rules (Good and Nielsen, 1997). Gene homologues from *Salmonella enterica* serovar *Typhimurium* LT2 (Knuth *et al.*, 2004) and *Escherichia coli* DH10B (Durfee *et al.*, 2008b) were identified by BLAST (Altschul *et al.*, 1990) from the Database of essential genes (Zhang and Lin, 2009b). Using Artemis, a 20 base pair sequence (-10 to +10) relative to the start codon of essential gene homologues were identified and aligned in Clustal X version 2 (Larkin *et al.*, 2007) to determine base pair mismatches. A 2 base pair cut-off was assigned to identify potential PNA binding sites. A custom perl script was used to identify -5 to +5 bases relative to the start codon for all against comparison (McGinnis and Madden, 2004b), using BLAST to identify essential genes with TIR's amenable to the design of PNA's. From the generated list, a 10 base pair sequence (aacataatct) targeting the TIR of *ftsZ* was identified (PNA Se002). To enable PNA's to transverse the bacterial cell wall, a short peptide (KFFKFFKFFK) was conjugated to the PNA. The peptide sequence is has previously been shown to increase uptake and target specificity (Good *et al.*, 2001b).

### **2.7.2 Transformation of *S. typhimurium* and *E.coli* with reporter gene plasmids**

To highlight phenotypic variation arising from the effects of PNA silencing of *ftsZ*, *Salmonella typhimurium* and *E.coli* were transformed with pGLO expressing Green Fluorescent Protein (Bio-Rad) and pDs-Red expressing red fluorescent protein (Clontech) respectively. *Salmonella typhimurium* LT2 strains JR501 was obtained from the Salmonella Genetic Stock Centre, (University of Calgary, U.S.A). *E.coli* TOP10 was obtained from Invitrogen. Transformation of *S. typhimurium* with pGlo was based on an adapted protocol for the transformation of enteric bacteria (Tsai *et al.*, 1989). An overnight culture of *S. typhimurium* LT2 JR501, was diluted 1/20 in SOB medium (see Appendix: A) and then incubated at 37°C with aeration until the OD<sub>550</sub> reached mid log phase (0.5-0.6). A 50 ml aliquot of the culture was chilled and cells were harvested by centrifugation at 10,000 x g for 10 minutes. Harvested cells were washed with 50 ml of cold (4°C) 0.1 M MgCl<sub>2</sub> and then resuspended in 25 ml 0.1 M CaCl<sub>2</sub> and incubated on ice for 20 minutes. Cells were centrifuged at 10,000 x g for 10

minutes and resuspended in 5 ml of cold 0.1 M CaCl<sub>2</sub>, which was dispersed as 0.2 ml aliquots into thin wall 15 ml tubes (Falcon). Upon addition of plasmid DNA, cells were incubated on ice for 20 minutes prior to heat shocking for 2 minutes at 42°C without shaking. A total volume of 250 µl of pre-warmed SOC medium was added to Transformants, which were then incubated at 37°C for 1 hour with aeration. A suitable volume of transformants (100 µl) was pipetted onto pre-warmed LB agar containing ampicillin and incubated at 37°C overnight. *E.coli* TOP10 was transformed with pDs-Red as per previous *E.coli* transformations (see methods section: 2.1.8)

### **2.7.3 Mixed microbial assay**

Overnight cultures of *E.coli* Top 10 harbouring pDs-Red and *Salmonella typhimurium* LT2 JR501, containing pGlo, were incubated overnight in low salt LB media containing ampicillin. Overnight cultures were diluted in fresh low salt LB containing 0.2% L-arabinose (Sigma) and standardized by OD<sub>550</sub> readings to approximately 2×10<sup>4</sup> cfu/ml. Equal volumes were then combined to produce a mixed culture. Prior to use, PNAs (Panagene, Korea) were heated at 55°C for 10 minutes as per manufacturer's instructions and added to a final concentration of 2.5 and 1.25 µM, in a 96 well tissue culture plate (Sarstedt). The well volume was made up to 100 µl with low salt LB containing 0.2% L-arabinose, prior to the addition of 100 µl of mixed bacterial culture. The 96 well plate was sealed with breathable self-adhesive film (Starlab) and incubated in a Powerwave HT plate reader (BioTek) at 37°C with medium intensity shaking. After 6 hours the plate was removed and 10µl samples were removed and harvested by centrifugation, prior to re-suspension in 1 x PBS (Sigma). Microscope slides were prepared by addition of 2 µl of sample to a 1% agarose pad (Levin, 2002a) and heat fixing for Fluorescent Microscopy. Images were captured using a Lecia DMRB microscope with Aqua EXI bioimaging camera (Qimiaging).and processed using Image pro plus software (MediaCybernetics).

### **2.8 Statistics**

All statistical analyses used in this study were performed using Minitab (Minitab Inc). Before the selection of statistical analysis methods, data was assessed for normality using a Kolomogorov-Smirnov test prior to the use of parametric tests

(T-test). Where data did not confirm to a normal distribution non-parametric statistical analyses (Mann-Whitney U test) were undertaken.

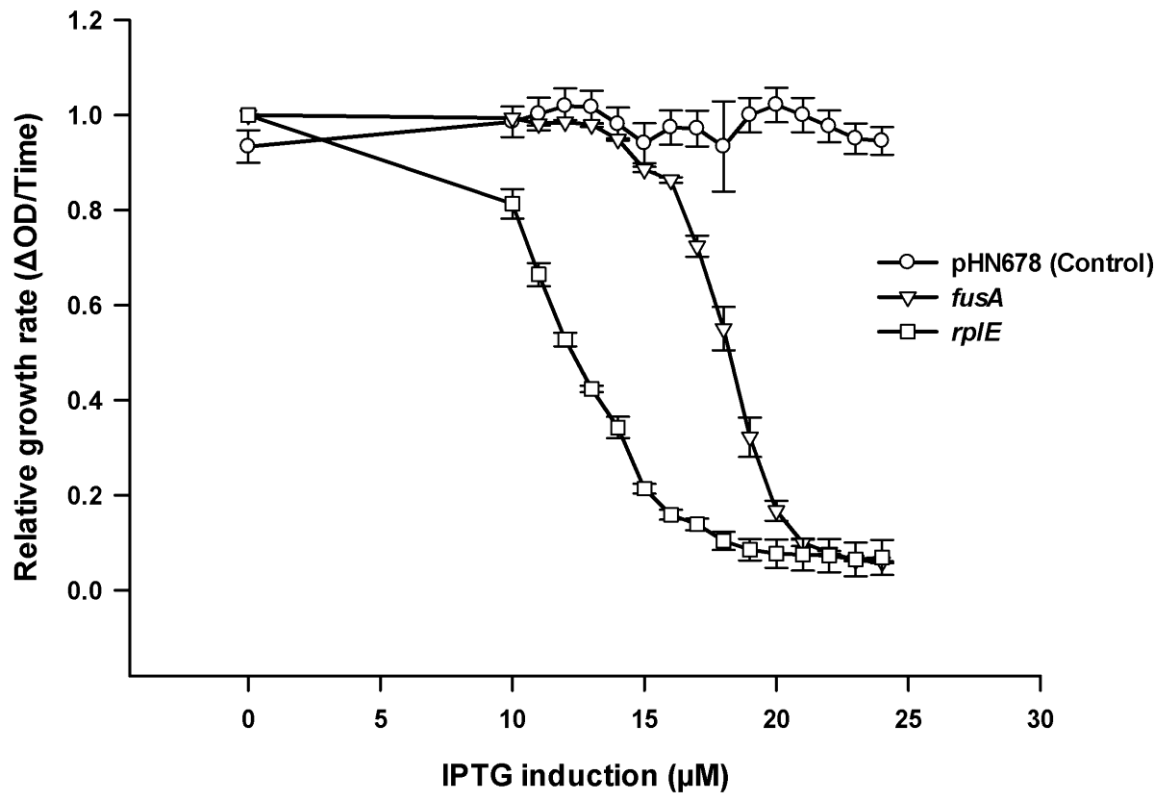
## Chapter 3 Results

### 3.1 Evaluation of gene silencing of *fusA* and *rpIE*

#### 3.1.1 Specificity of expressed antisense RNA constructs

The assessment of essential gene stringency requires exclusive silencing of a target gene, to generate a reduction in growth rate. Determining the specificity of expressed antisense RNA sequences is therefore necessary to ensure no additional genes are inadvertently targeted. Antisense sequences of approximately 101-152 nucleotides complementary to the ribosome binding region of mRNA for essential genes *fusA* and *rpIE*, were cloned into a paired termini antisense expression vector (pHN678) (Nakashima *et al.*, 2006) and transfected into *E.coli* (TOP10) to create strains Ash01, and Ash02 (see methods section 2.1.5-2.18). The growth rate of each strain was titrated down using a range of IPTG concentrations to induce increased expression of antisense RNA. The growth rates of silenced clones were measured relative to uninduced controls to obtain relative growth rate (see methods section: 2.2).

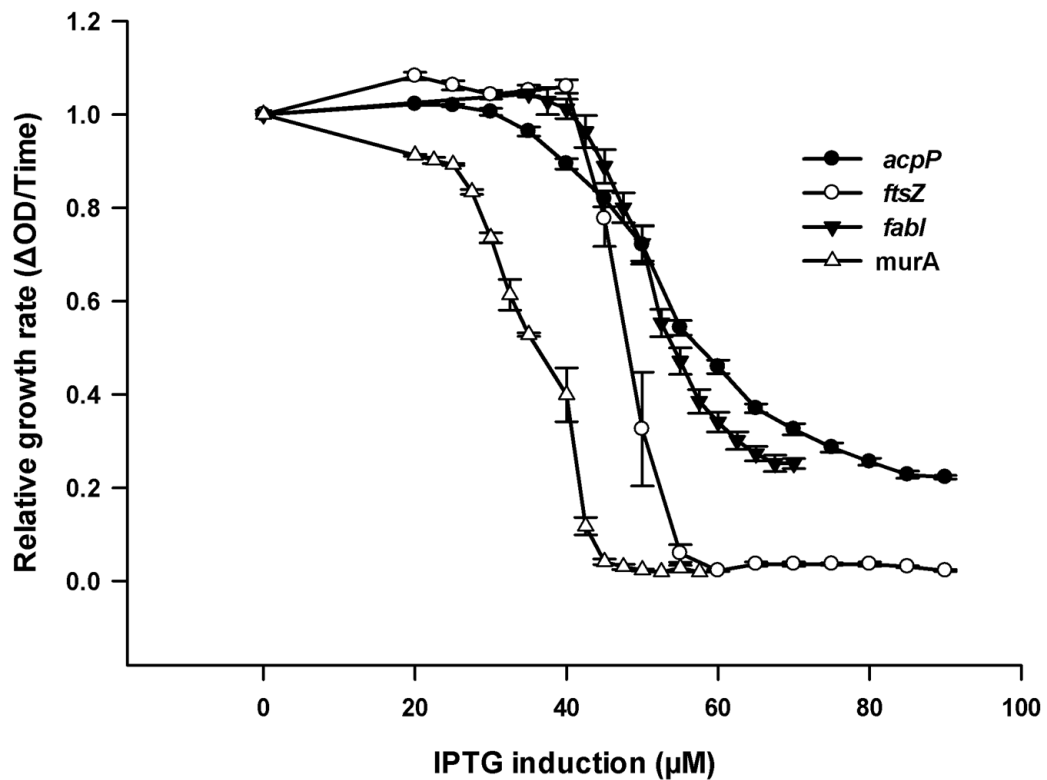
Both antisense RNA expression constructs demonstrated significant growth inhibition of *E.coli* (2 sample T-test  $n = 3$   $P < 0.005$ ), compared to controls containing empty constructs (Figure 3.1). Greater silencing efficiency was displayed for *rpIE*, as evident from the early decrease in the relative growth rate compared to *fusA*. The specificity of both *fusA* and *rpIE* constructs were compared to expressed antisense RNA constructs targeting essential genes *acpP*, *ftsZ*, *fabI* and *murA* (Goh *et al.*, 2009). The relative growth rates of *acpP*, *ftsZ*, *fabI* and *murA* (Figure 3.2) were comparable to those obtained previously. However a greater level of silencing efficiency was demonstrated for *fusA* and *rpIE* over a low IPTG induction range, with growth inhibition occurring at 20-25  $\mu\text{M}$  compared to *acpP* (80  $\mu\text{M}$ ), *ftsZ* (60  $\mu\text{M}$ ), *fabI* (70  $\mu\text{M}$ ), and *murA* (50  $\mu\text{M}$ ). Antisense RNA expression constructs for *fusA* and *rpIE* demonstrate the capacity to reduce bacterial growth, which suggests specificity for their respective essential gene targets in *E.coli*.



**Figure 3.1** Relative growth rate of *E.coli* following expressed antisense RNA silencing of essential gene targets *fusA* and *rplE*.

Growth rate titration was achieved by induction with increasing concentrations of IPTG to increase the expression of antisense RNA and measuring OD<sub>550</sub>. Raw OD<sub>550</sub> data corresponding to the exponential phase of growth was plotted and a trendline applied to obtain the rate of growth from the straight line equation  $y = mx + c$ . Relative growth rate was determined by normalising the growth rate of induced samples to uninduced controls. Graph shows average relative growth rate with standard error bars (n=3).





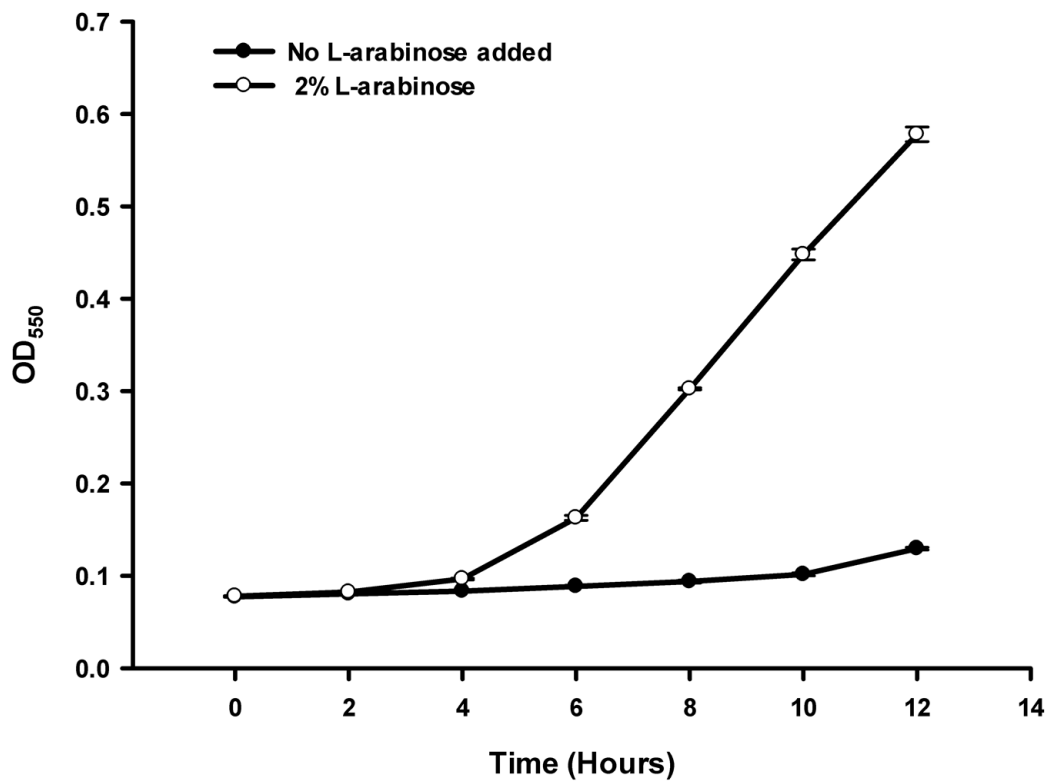
**Figure 3.2** Relative growth rates of *E.coli* following expressed antisense RNA silencing of essential gene targets *acpP*, *ftsZ*, *fabI*, and *murA*.

Growth rate titration was achieved by induction with increasing concentrations of IPTG to increase the expression of antisense RNA and measuring OD<sub>550</sub>. Raw OD<sub>550</sub> data corresponding to the exponential phase of growth was plotted and a trendline applied to obtain the rate of growth from the straight line equation  $y = mx + c$ . Relative growth rate was determined by normalising the growth rate of induced samples to uninduced controls. Graph shows average relative growth rate with standard error bars (n=3).

### **3.1.2 Validation of antisense construct specificity by transcomplementation**

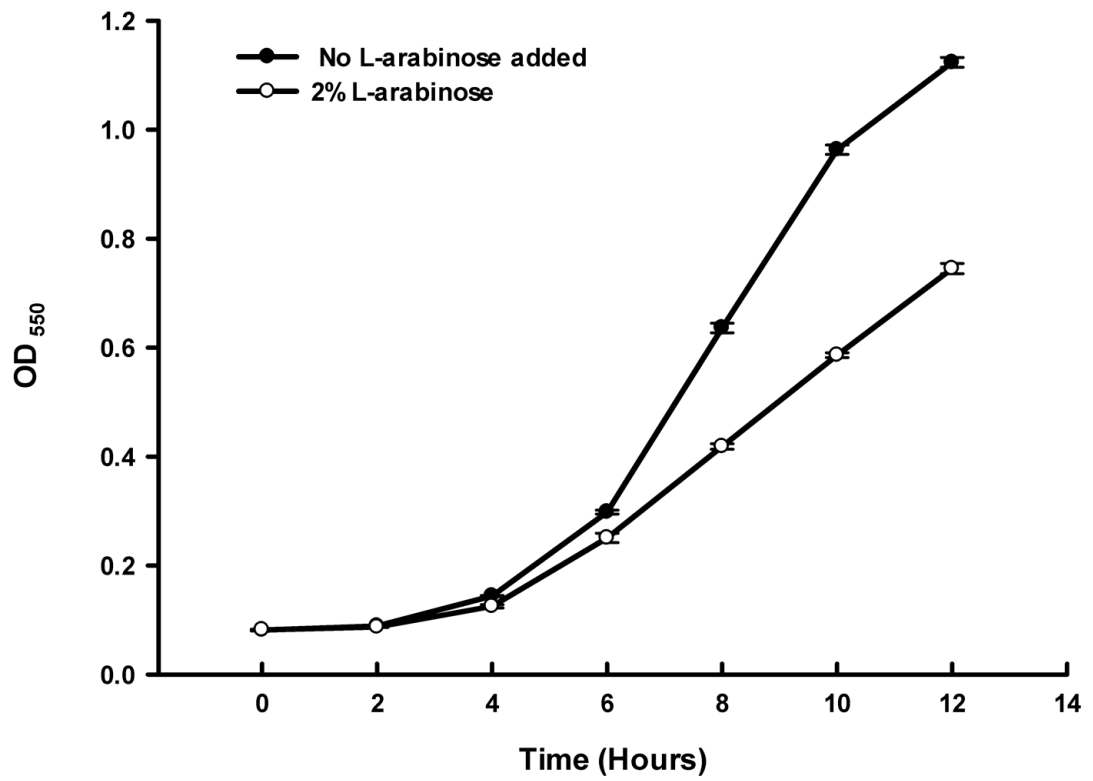
The validation of target specificity of expressed antisense RNA constructs was attempted using a transcomplementation strategy. This approach aims to rescue cells from growth inhibition induced by antisense RNA silencing, through over-expression of the complete open reading frame (ORF) for each target gene from a separate plasmid. Target specificity can then be confirmed following the rescue of clones from growth inhibition and restoration of normal growth. The entire ORF's for *fusA* and *rpIE* were cloned into pBAD-HisA containing an L-arabinose inducible promoter to generate strains *E.coli* Ash03 and Ash04 (see methods section 2.1.1-2.1.9). The rescue constructs were extracted and transfected with their corresponding expressed antisense RNA constructs to create *E.coli* strains, Ash05 and Ash06 respectively. Strains was grown in the presence of IPTG and L-arabinose to induce expression of both antisense RNA and corresponding episomal copy of the target genes, to determine appropriate levels of induction required to demonstrate rescue of growth rate (see methods section 2.3).

Growth inhibition of *E.coli*, following expressed antisense RNA silencing of *rpIE* at 25  $\mu$ M IPTG, was prevented following gene complementation using 2% L-arabinose (Figure 3.3). However this was deemed statistically insignificant (Mann Whitney,  $P=0.104$ ,  $n=7$ ). Difficulties were encountered with the transcomplementation of *fusA*, for which suitable levels of induction for expressed antisense RNA and gene overexpression could not be determined. Subsequently no significant rescue of growth rate following silencing of *fusA* (Mann Whitney,  $P=0.734$ ,  $n=7$ ) was observed with L-arabinose induction (Figure 3.5). To investigate the potential influence of metabolic load associated with expression of two plasmids, strain Ash07 containing empty expression vectors, was examined using identical rescue conditions for both *rpIE* (Figure 3.4) and *fusA* (Figure 3.6). Induction with IPTG and L-Arabinose concentrations did not significantly affect the growth rate of *E.coli* using the same rescue conditions for *rpIE* (Mann Whitney,  $P=0.610$ ,  $n=7$ ) or *fusA* (Mann Whitney,  $P=0.710$ ,  $n=7$ ). Higher OD<sub>550</sub> readings were observed in the control strains. In summary using transcomplementation validated target specificity of *rpIE* but not for *fusA*.



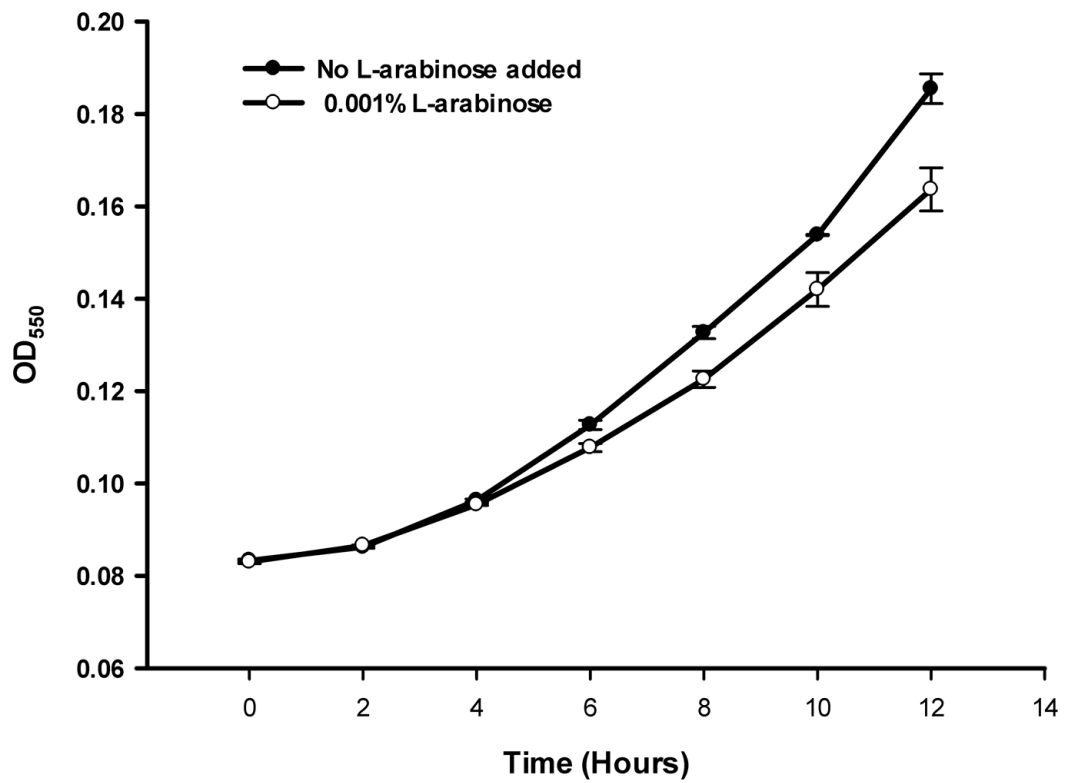
**Figure 3.3 Transcomplementation of expressed antisense RNA silencing effects of the essential gene *rpIE* in *E.coli*.**

Growth rate of *E.coli* strain Ash05 following *rpIE* over-expression, induced with 2% L-arabinose. Antisense RNA expression was induced with 25  $\mu$ M IPTG. Graph shows bacterial growth curves derived from average OD<sub>550</sub> readings with standard error bars (n=3).



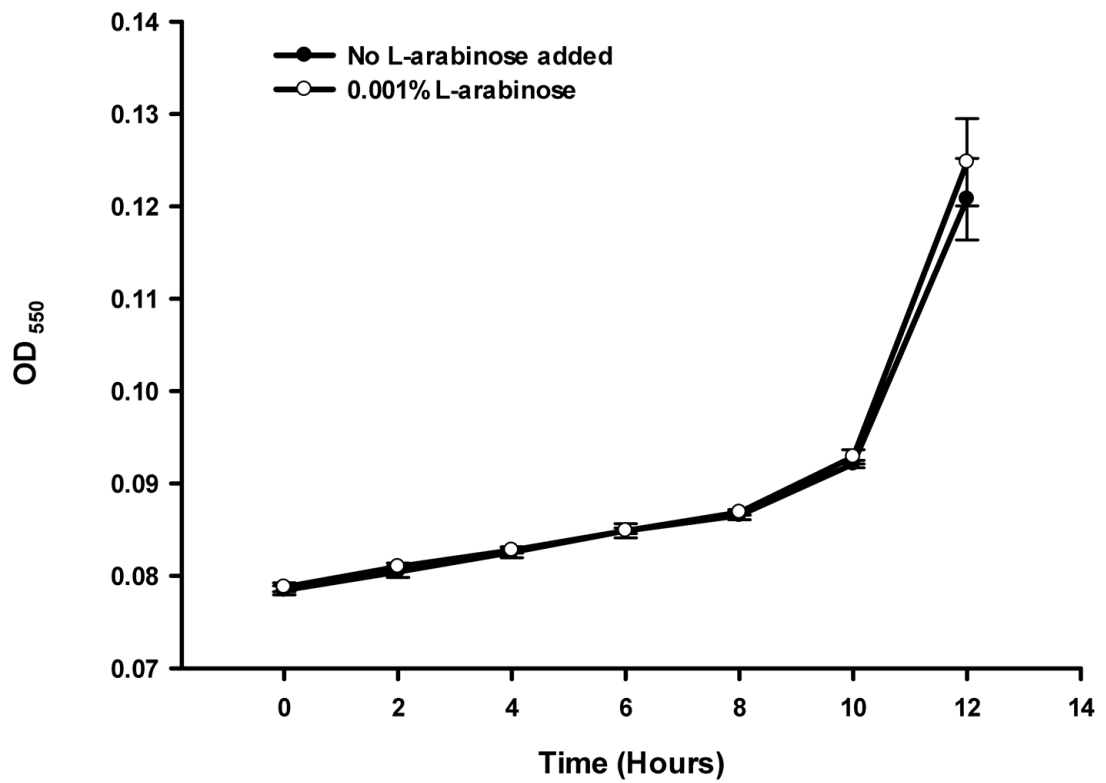
**Figure 3.4 Transcomplementation control mimicking the expression conditions used for the rescue of *rplE*.**

Expression of *E.coli* strain Ash07 was induced with 25  $\mu$ M IPTG and 2% L-arabinose as used in the rescue of *E.coli* strain Ash05. Graph shows bacterial growth curves derived from average OD<sub>550</sub> readings with standard error bars (n=3).



**Figure 3.5 Transcomplementation of expressed antisense RNA silencing effects of the essential gene *fusA* in *E.coli*.**

Growth rate of *E.coli* strain Ash06 following *fusA* over-expression, induced with 0.001% L-arabinose. Antisense RNA expression was induced with 25  $\mu$ M IPTG. Graph shows bacterial growth curves derived from average OD<sub>550</sub> readings with standard error bars (n=3).



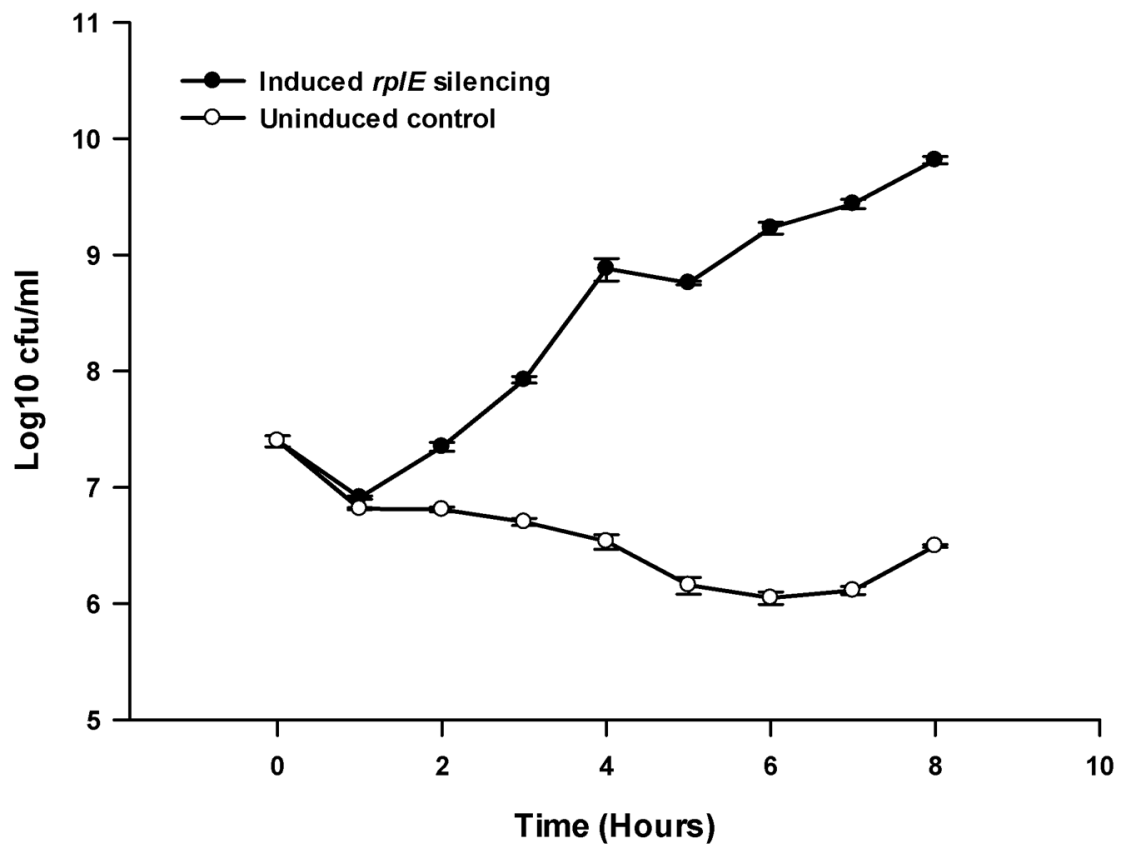
**Figure 3.6** Transcomplementation control mimicking the expression conditions used for the rescue of *fusA*.

Expression of *E.coli* strain Ash07 was induced with 25  $\mu$ M IPTG and 0.001% L-arabinose as used in the rescue of *E.coli* strain Ash06. Graph shows bacterial growth curves derived from average OD<sub>550</sub> readings with standard error bars (n=3).

### **3.1.3 Delineating inhibitory effects caused by gene silencing of *fusA* and *rpIE***

To ascertain whether the effects of expressed antisense RNA silencing of *fusA* and *rpIE* promoted a bactericidal or bacteriostatic effect on cell growth, a survival assay was performed (see methods section 2.4). Silencing of *fusA* and *rpIE* was induced using 50  $\mu$ M IPTG with viable cell counts performed over 8 hours. Prior to the assay, It was postulated that silencing of *fusA* would cause a bacteriostatic effect on cell growth, in accordance with inhibitory effects observed in *S.aureus* by the target specific antibiotic fusidic acid (Howden and Grayson, 2006). Similarly silencing of *rpIE* was also expected to cause a bacteriostatic effect, based upon the inhibitory action exerted by the majority of antibiotic classes that target the ribosome, with the exception of aminoglycosides (Kohanski *et al.*, 2010).

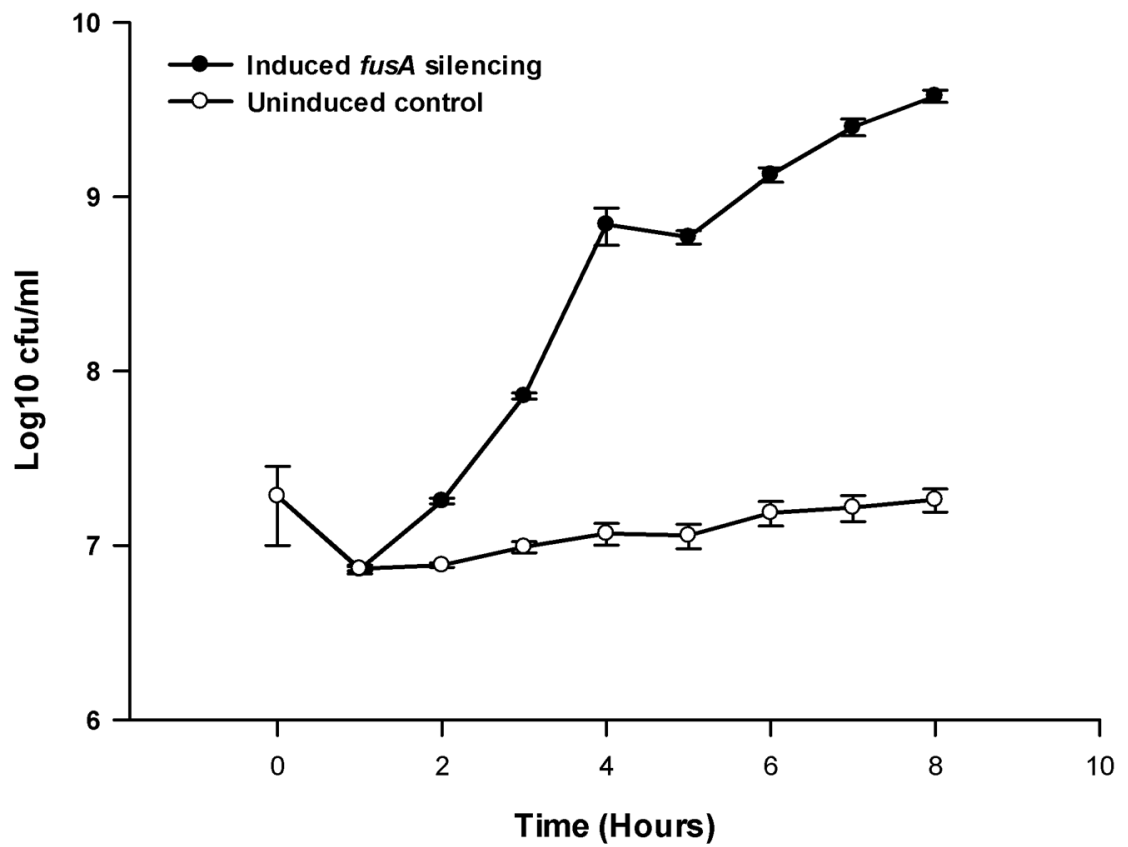
Both *fusA* and *rpIE* silenced strains exhibited significant growth inhibition rather than cell death following gene silencing. A significant reduction in viable cell numbers was observed upon silencing for both *fusA* (Mann Whitney U Test,  $P=0.0118$ ,  $n= 9$ ) and *rpIE* (Mann Whitney U Test,  $P = 0.0009$   $n=9$ ). In the case of *rpIE* (Figure 3.7) the viable number of cells initially decreased prior to increasing after 8 hours. For *fusA* (Figure 3.8) the number of viable cells remained relatively static throughout the 8 hour growth period. The growth effects of gene silencing of both *rpIE* and *fusA* were determined to be bacteriostatic, since cell number decline was below the  $>3$  log reduction of the final inoculum required for the demonstration of bactericidal activity. One particular observation was that growth curves generated for uninduced antisense RNA expression of *fusA* and *rpIE* displayed an exponential increase with a plateau at approximately 4 hours. This anomaly was not observed with both the uninduced and induced control plasmid, where no difference in viable cell count was observed (Figure 3.9), following induction (Mann Whitney U Test,  $P=0.8946$ ,  $n= 9$ )



**Figure 3.7 Delineation of growth effects following expressed RNA silencing of the essential gene *rpIE* in *E.coli*.**

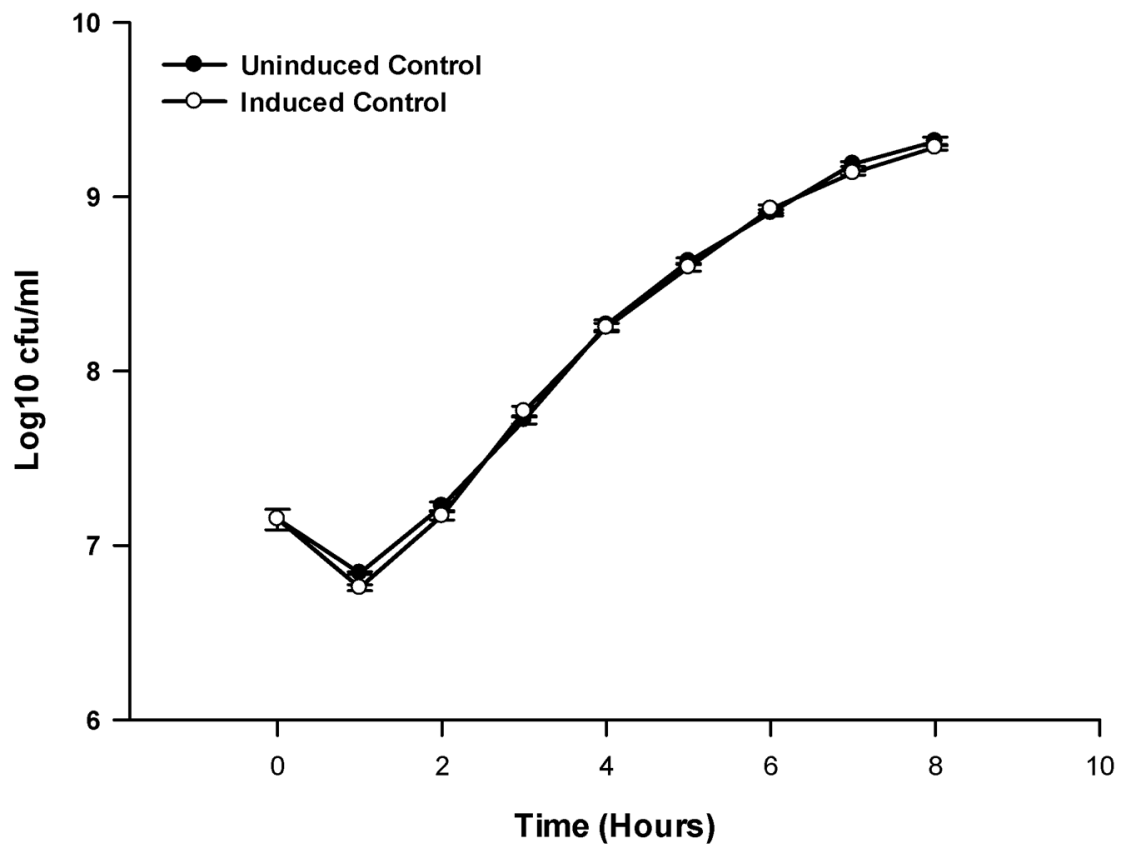
Antisense RNA expression was induced using 50  $\mu$ M IPTG *E.coli* strain Ash01 and viable cell counts were performed over 8 hours. Graph shows average viable cell count with standard error bars (n=3).





**Figure 3.8 Delineation of growth effects following silencing of the essential gene *fusA* in *E.coli*.**

Antisense RNA expression was induced in *E.coli* strain Ash02 using 50  $\mu$ M IPTG and viable cell counts were performed over 8 hours. Graph shows average viable cell count with standard error bars (n=3).



**Figure 3.9** Viable cell counts of *E. coli* expressing empty pHN678 construct.

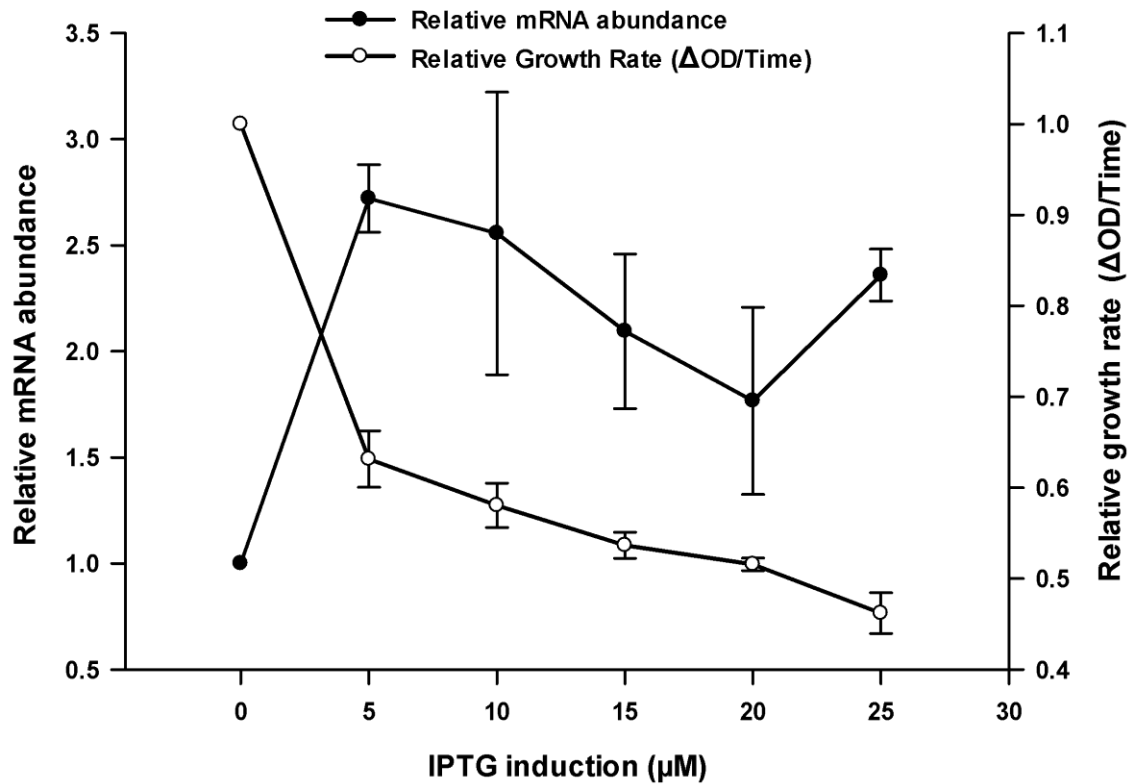
As per previous experimental conditions 50  $\mu$ M IPTG was used to induce expression of empty antisense expression plasmid in *E. coli* strain Ash03 and viable cell counts were performed over 8 hours. Graph shows average viable cell count with standard error bars (n=3).

### **3.1.4 Evaluation of gene stringency of *fusA* and *rpIE* using Mean Transcript Level 50 (MTL<sub>50</sub>)**

To enable comparisons of stringency among essential genes, a Mean Transcript Level (MTL<sub>50</sub>) value, representing the amount of mRNA required to maintain 50% of relative growth has been proposed, which reflects the relationship between transcript abundance and growth rate decline. (Goh *et al.*, 2009). The MTL<sub>50</sub> of *rpIE* and *fusA* was initially evaluated by performing a titration of growth rates of *E.coli* strains Ash01 and Ash02, using increasing concentrations of IPTG, to promote expressed antisense RNA expression and measuring the corresponding mRNA abundance by performing relative quantification with QPCR, (see methods section 2.5). Examination of the relative growth rate and corresponding mRNA abundance for *rpIE* and *fusA* (Figure 3.10 and Figure 3.11 respectively), reveals an increase in mRNA abundance with relative growth rate decline. This is contrary to the expectation that mRNA abundance would decline with relative growth rate, due to antisense mediated degradation of mRNA transcripts.

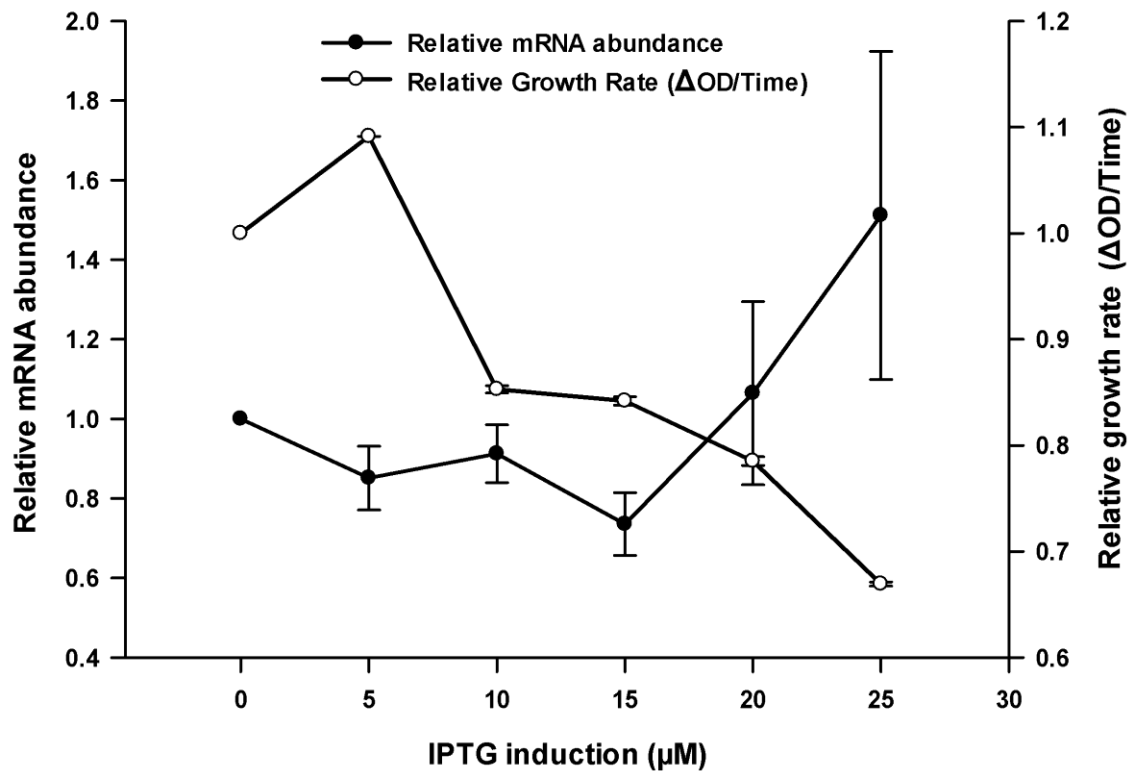
A reduction in growth rate was observed following silencing of *rpIE* yet mRNA abundance initially decreased then remained stable, prior to an increase in abundance at the lowest relative growth rate. Silencing of *fusA*, caused the relative growth rate and mRNA abundance to initially decline, followed by a large increase in mRNA abundance at the lowest relative growth rate. No significant correlation between relative growth rate decline and mRNA abundance, following silencing for either *rpIE* ( $r_s = -0.02$ ) or *fusA* ( $r_s = -0.6$ ) was observed.

To compare the stringency of *rpIE* and *fusA* against previously validated targets, the MTL<sub>50</sub> value was determined by plotting relative growth rate against relative mRNA abundance (Figure 3.12 and Figure 3.13 respectively). The MTL<sub>50</sub> reveals an inverse trend to previously investigated gene targets, whereby mRNA abundance increases as relative growth rate declines. The number of *rpIE* transcripts required to maintain 50% cell viability increases to approximately 2.4 relative value (or 240% of a normal cell growth). For *fusA* the 50% decrease in growth rate was not achieved, but indicates that a large number of transcripts are present, approximately 1.5 relative value (or 150% of a normal cell growth).



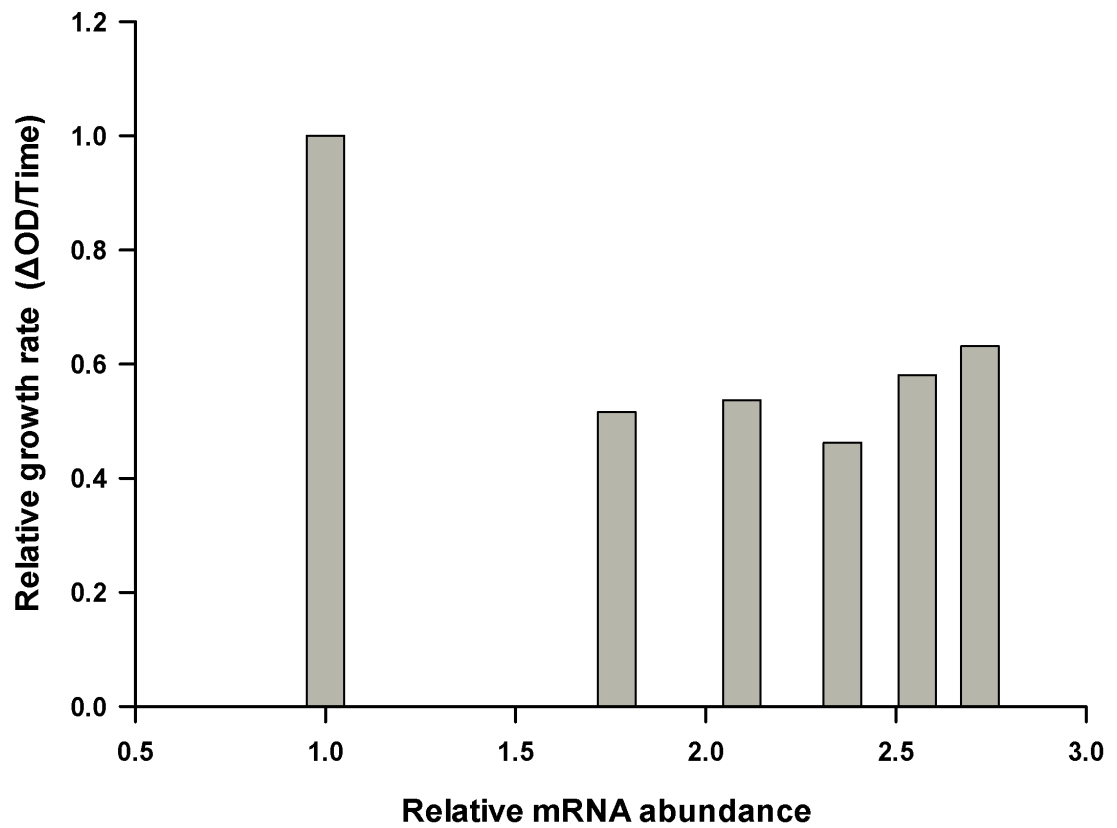
**Figure 3.10** Relative growth rate and mRNA abundance profiles following expressed antisense RNA silencing of the essential gene *rpIE* in *E.coli*.

Transcript and relative growth rate profile for *E.coli* strain Ash01. Total RNA was isolated from cultures, which were harvested once the OD<sub>550</sub> of the uninduced control had reached 0.1. mRNA abundance was determined by QPCR using *E.coli* genes *16S* and *ZipA* as endogenous references for relative quantification by  $\Delta\Delta C_t$  method. Graph shows average relative growth rate and average relative mRNA abundance with standard error bars (n=3).



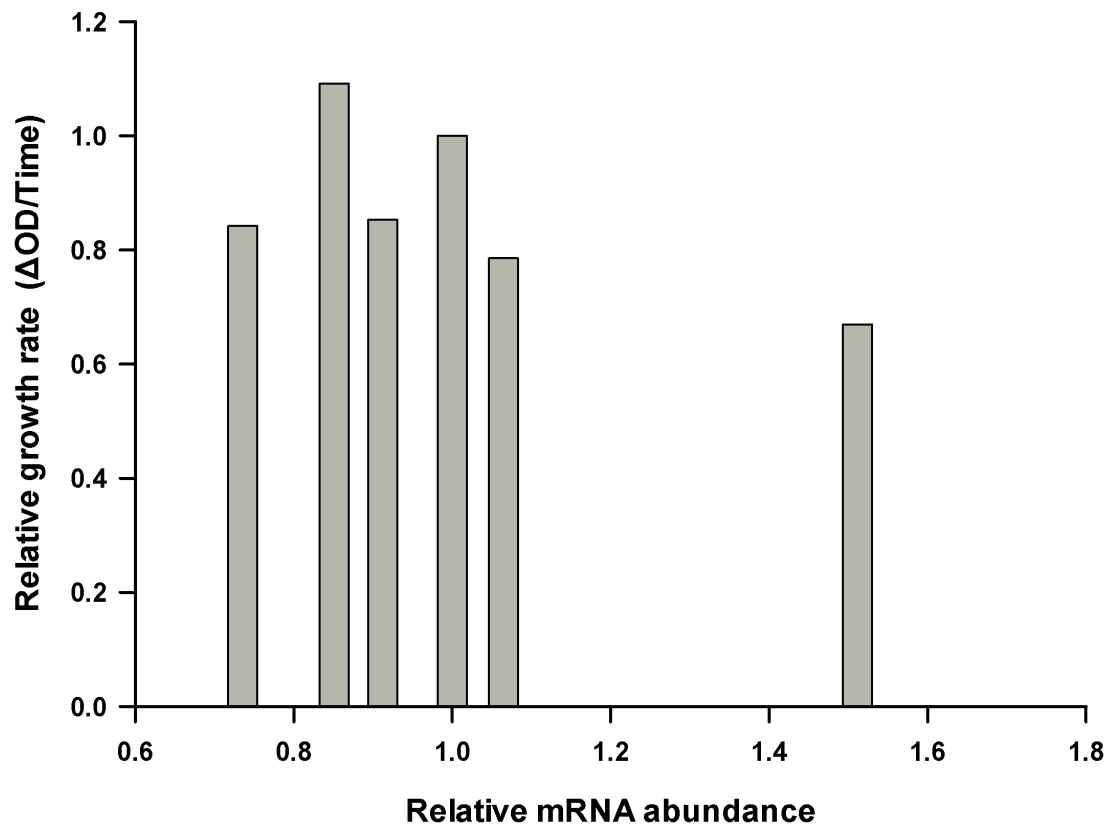
**Figure 3.11 Relative growth rate and mRNA abundance profiles following expressed antisense RNA silencing of the essential gene *fusA* in *E.coli*.**

Transcript and relative growth rate profile for *E.coli* strain Ash02. Total RNA was isolated from cultures, which were harvested once the OD<sub>550</sub> of the uninduced control had reached 0.1. mRNA abundance was determined by QPCR using *E.coli* genes *16S* and *ZipA* as endogenous references for relative quantification by  $\Delta\Delta C_t$  method Graph shows average relative growth rate and average relative mRNA abundance with standard error bars (n=3).



**Figure 3.12 MTL<sub>50</sub> for essential gene *rplE*.**

The MTL<sub>50</sub> was calculated by plotting the mean relative mRNA abundance against its corresponding mean relative growth rate. This enables stringency of essential genes to be determined by examining the number of transcripts required to maintain 50% relative growth rate.



**Figure 3.13 MTL<sub>50</sub> for essential gene *fusA*.**

The MTL<sub>50</sub> was calculated by plotting the mean relative mRNA abundance against its corresponding mean relative growth rate. This enables stringency of essential genes to be determined by examining the number of transcripts required to maintain 50% relative growth rate.

## 3.2 Development of LC-MS/MS protein quantification method

### 3.2.1 <sup>15</sup>N labelled protein internal standard preparation for LC-MS/MS

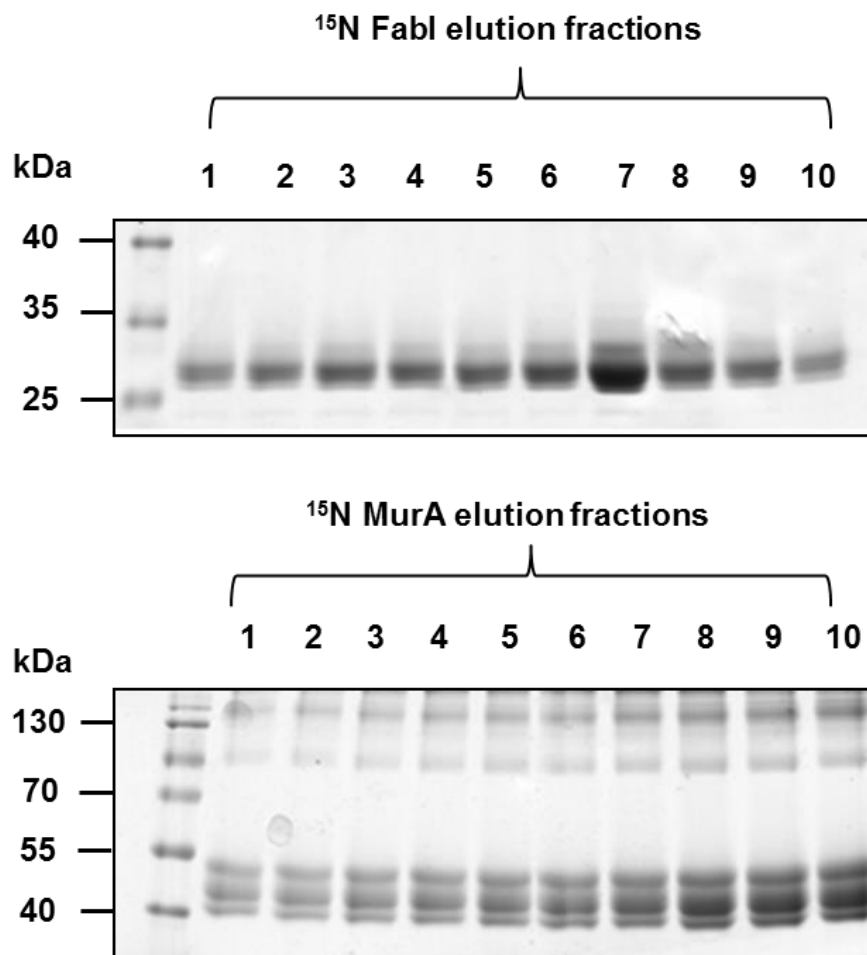
The evaluation of stringency in essential gene targets *fusA* and *rpIE* in this study, suggests that mRNA may be limited as a surrogate for expressed protein, and therefore inappropriate as a measure of gene essentiality. Indeed correlations between mRNA and protein abundance are known to vary widely, due to numerous post-transcriptional and post-translational mechanisms that operate within prokaryotes (Maier *et al.*, 2009).

To circumvent the use of mRNA for determining essential gene stringency, an LC-MS/MS assay was developed to provide absolute quantification of target protein, following expressed antisense RNA silencing. Development of the assay required the synthesis of a heavy labelled (<sup>15</sup>N) target protein for use as an internal standard. The use of a fully labelled protein enables an initial LC-MS/MS analysis to identify the most suitable tryptic peptides, possessing the same physiochemical properties as their native equivalent, but distinguishable by mass difference for the purpose of quantifying protein in experimental samples. The essential genes *fabI* and *murA* were selected as candidate targets for use as internal standards, due to their extensive characterization and previous evaluation as stringently required genes (Goh *et al.*, 2009).

As an alternative to commercially synthesized heavy labelled peptides, fully <sup>15</sup>N labelled proteins were generated from *E.coli* MG1 AE5305 obtained from the ASKA collection (Kitagawa *et al.*, 2005a). Using a complete stable isotope labelled form enabled correction for variability in the analytical workflow, and has been shown to be a preferable choice compared to other forms of internal standard (Bronsema, 2013). Candidate proteins were overexpressed in minimal media containing <sup>15</sup>N labelled NH<sub>4</sub> as the sole nitrogen source (see methods section 2.6.1), extracted from whole cell *E.coli* lysate by Immobilised metal affinity chromatography (see methods section 2.6.2) and subject to acetone precipitation to remove contaminants (see methods section 2.6.4). Purified <sup>15</sup>N labelled FabI and MurA (Figure 3.14) revealed both target proteins (27.9 and 44.8 kDa respectively), in addition to high molecular weight contaminants. Contamination was more prevalent for MurA, which revealed numerous bands in the range of 100 – 130 kDa, including two low molecular weight bands in the



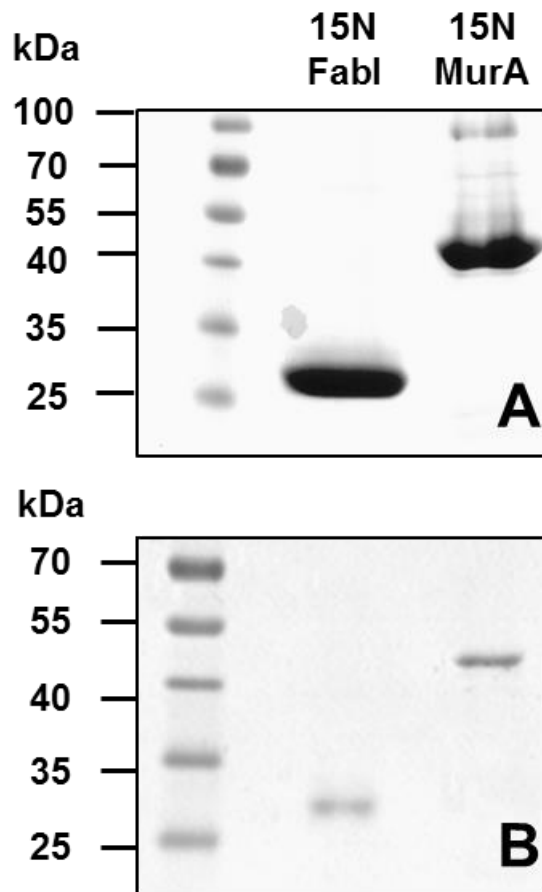
predicted range of MurA. Unlike FabI which resides in the cytosol, MurA is a membrane associated protein; therefore contaminating bands were likely to have been accessory proteins that co-eluted. The identity of each protein was confirmed by LC-MS/MS (see results section 3.2.2).



**Figure 3.14 Purification of <sup>15</sup>N labelled FabI and MurA protein from *E.coli* whole cell lysate.**

*E.coli* MG1 ME5305 overexpressing His-tagged FabI and MurA was grown in minimal media until mid log phase and then induced with 1 mM IPTG and grown over 24 hours prior to harvesting. Extracted protein was eluted from a nickel column over an imidazole gradient generating 10 eluted fractions. A protein ladder (first lane) was used to confirm approximate protein size.

Both FabI and MurA protein elutes were subsequently concentrated and purified further using Amicon filters with appropriate molecular weight ranges. However SDS-PAGE of filtered protein revealed the presence of warped bands indicative of high salt concentration (Figure 3.15 A), that could detrimentally effect electrospray ionization efficiency. Both proteins were therefore subjected to an additional purification step using acetone precipitation (Figure 3.15 B), which subsequently removed contaminants. Following purification, the yield of FabI and MurA appeared to be reduced. This however was not deemed to affect the quantification strategy as both protein batches would be quantified independently using a standard curve, prior to use as internal standards (see methods section: 2.6.8). This would ensure known quantities of internal standard would be spiked into experimental samples.



**Figure 3.15 Purification and concentration of  $^{15}\text{N}$  labelled FabI and MurA.**

(A) Contamination in  $^{15}\text{N}$  FabI and MurA, following initial clean-up after elution from IMAC. High molecular weight bands corresponding to co-eluted contaminants were observed for MurA (B) Acetone precipitation of  $^{15}\text{N}$  FabI and MurA, revealing the removal of contaminants. Purified protein was pooled and 10  $\mu\text{l}$  aliquots were ran on a 15% polyacrylamide gel. A protein ladder (first lane) was used to confirm protein size.

### **3.2.2 Confirmation of complete $^{15}\text{N}$ labelling of protein internal standards**

For reliable quantification, the heavy labelling of an internal protein standard requires complete and stable  $^{15}\text{N}$  incorporation to a level approaching 95 %, as a minimum for quantitative analysis (Huttlin *et al.*, 2008). The use of  $^{15}\text{N}$  is particularly suited as a heavy label, since peptides contain fewer types of nitrogen compared to carbons. This has the effect of reducing the complexity of isotopic envelopes in  $^{15}\text{N}$  labelled peptides, which subsequently reduces the distribution of the  $m/z$  signal thereby increasing the signal to noise ratio. This subsequently increases confidence in identification and quantification (Huttlin *et al.*, 2008). Purified FabI and MurA samples were digested with trypsin and analysed by LC-MS/MS to determine the relative degree of  $^{15}\text{N}$  incorporation. The degree of  $^{15}\text{N}$  incorporation in FabI was assessed by submitting the raw LC-MS/MS data to the gpm (Global Protein Machine), a publically available system, comprising a data analysis server, user interface and a relational database, containing experimentally generated peptide mass data (Craig *et al.*, 2004). Within this system, raw tandem mass spectra is compared to spectra derived from the same peptide sequences using X! Tandem, a class B open source search engine. An algorithm performs a two step calculation, initially a 'survey' is performed whereby experimentally derived spectra are compared to the protein sequences, under the assumption that complete peptide cleavage occurs. Peptides identified with statistically significant matches are then re-matched taking into consideration potential chemical modifications (e.g.: oxidation). To ascertain the level of incorporation the gpm search was initially performed with the assumption of no  $^{15}\text{N}$  incorporation (i.e. the search engine would only identify peptide sequences with spectra relating to native unlabelled protein sequences specific for FabI or MurA). The gpm search (Figure 3.16) did not match any spectra corresponding to native FabI or MurA, with spectra generated from  $^{15}\text{N}$  labelled internal standards, or any other *E.coli* proteins. The only positive identifications made were keratin and bovine trypsin, which represent typical contaminants arising from sample preparation. Due to financial constraints, a decision was made to proceed with developing an LC-MS/MS assay for *fabI* only.



Models from "W-MS\_Orbitrap-1104-110404\_08\_PR11\_0010\_murA\_fabl.mgf": Main model display

| [model](#) | [context](#) | [group](#) | [gel](#) | [chip](#) | [peptide](#) | [table](#) | [details](#) | [mh](#) | [ζ](#) | [XML](#) | [ms/ms](#) | [wiki](#) |

GPM28700000139 | [metadata](#) | [FASTA](#) | [asl](#) | [get annotation](#)

Contributor: anonymous

log(e) <  & # >  Display as: [html](#) Show Ω:  [go](#) valid log(e) < -1.3, p = 75, FDR = 0.62%

rank	log(e) <sup>▲</sup>	log(l)	%/%	#	total	Mr	Accession
1	-76.1	4.98	12/20	8	10	65.8	sp K2C1_HUMAN  <a href="#">gpmDB [19959/39662]</a> <a href="#">homo (1/1)</a> <a href="#">protein</a> Keratin, type II cytoskeletal 1; 67 kDa cytokeratin; Cytokeratin-1; CK-1; Hair alpha protein; Keratin-1; K1; Type-II keratin Kb1;
2	-72.1	4.80	14/18	7	7	65.8	(H) sp K2E_HUMAN  <a href="#">gpmDB [16417/32211]</a> <a href="#">homo (1/1)</a> <a href="#">protein</a> Keratin, type II cytoskeletal 2 epidermal; Cytokeratin-2e; CK-2e; Epithelial keratin-2e; Keratin-2 epidermis; Keratin-2e; K2e; Type-II keratin Kb2;
3	-71.7	5.04	14/28	9	10	59.5	sp K1C10_HUMAN  <a href="#">gpmDB [15916/30982]</a> <a href="#">homo (0/9)</a> <a href="#">protein</a> Keratin, type I cytoskeletal 10; Cytokeratin-10; CK-10; Keratin-10; K10;
4	-58.6	4.73	14/22	7	8	62.1	sp K1C9_HUMAN  <a href="#">gpmDB [14240/28247]</a> <a href="#">protein</a> Keratin, type I cytoskeletal 9; Cytokeratin-9; CK-9; Keratin-9; K9;
5	-46.0	5.84	20/26	5	12	24.4	sp TRYP_PIG  <a href="#">gpmDB [25919/50475]</a> <a href="#">protein</a> Trypsin; EC 3.4.21.4; Flags: Precursor;
6	-17.2	4.57	7.2/9	3	3	69.2	sp ALBU_BOVIN  <a href="#">gpmDB [12551/24396]</a> <a href="#">homo (0/1)</a> <a href="#">protein</a> Serum albumin; BSA; Bos d 6; Flags: Precursor;
7	-1.8	4.05	1.9/2	1	1	60.3	gi 170083053  <a href="#">gpmDB [39/56]</a> <a href="#">homo (1/1)</a> <a href="#">protein</a> no protein information available
8	-1.8	3.58	1.6/2	1	1	55.8	gi 170081181  <a href="#">gpmDB [0/1]</a> <a href="#">homo (1/1)</a> <a href="#">protein</a> no protein information available
9	-1.6	3.48	5.3/6	1	1	14.2	gi 170080930 :reversed <a href="#">homo (1/1)</a> <a href="#">protein</a> no protein information available
10	-1.5	3.81	4.1/6	1	1	25.4	sp TRY1_BOVIN  <a href="#">gpmDB [4878/9587]</a> <a href="#">protein</a> Cationic trypsin; EC 3.4.21.4; Beta-trypsin; Contains: Alpha-trypsin chain 1; Contains: Alpha-trypsin chain 2; Flags: Precursor;

Figure 3.16 GPM Results for unlabelled FabI and MurA.

No hits for native (unlabelled) FabI and MurA were obtained when gpm search parameters were modified to assume the absence of <sup>15</sup>N incorporation. This supports complete incorporation of <sup>15</sup>N in both FabI and MurA.

The gpm search was repeated by altering the search parameters to accommodate for  $^{15}\text{N}$  incorporation (Figure 3.17) in peptides specific to FabI and MurA proteins. The probability of peptide identification was ranked according to a logE score, which represent the base -10 logarithm of the probability that a peptide identification is a random match. Log E scores of -251.6 and -220.4 were obtained for MurA and FabI respectively, indicating that the chance of false positive identification is low (for reference a logE score of -3 corresponds to 1 in 1000 chance). The logE scores for  $^{15}\text{N}$  labelled FabI and MurA correspond to approximately 1 in  $1 \times 10^{2000}$  chances that identification has been made at random, which represents a statistically significant probability of identification.

To provide direct evidence of complete  $^{15}\text{N}$  labelling, characteristic peptide sequences for FabI (ILVTGVASK and SMLNPGSALLTSLYLGAER) and MurA (MGAHAEIESNTVICHGVEK and LQGEVTISGAK), were selected to confirm the degree of  $^{15}\text{N}$  incorporation. Incomplete labelling of each protein would be confirmed by the presence of mass spectra corresponding to unlabelled peptides. The mass spectrum for peptide ILVTGVASK (Figure 3.18) revealed an isotopic envelope corresponding to  $^{15}\text{N}$  labelled pseudo-molecular ion for the doubly charged peptide, with a  $m/z$  of 449.27 and no trace of the unlabelled native protein with a  $m/z$  of 444.27. The mass spectrum for SMLNPGSALLTSLYLGAER (Figure 3.19) also revealed a characteristic isotopic envelope corresponding to a  $^{15}\text{N}$  labelled pseudo-molecular 3+ ion for the doubly charged peptide, with a  $m/z$  of 677.9 and the absence of corresponding unlabelled peptide. For MurA the mass spectrum of MGAHAEIESNTVICHGVEK revealed the presence of a  $^{15}\text{N}$  labelled pseudomolecular 3+ ion with a  $m/z$  of 702.9 (Figure 3.20). The mass spectrum of LQGEVTISGAK (Figure 3.21) revealed the presence of a  $^{15}\text{N}$  labelled pseudomolecular 2+ ion with  $m/z$  of 558.2. No unlabelled species were observed for either peptide. The absence of unlabelled species ( $^{14}\text{N}$ ) indicates that complete labelling (i.e. every  $^{14}\text{N}$  atom is replaced by  $^{15}\text{N}$ ) was achieved, as expected in unicellular systems grown over eight generations (Snijders *et al.*, 2005). The complete  $^{15}\text{N}$  incorporation of FabI and MurA validates their use as internal standards for absolute quantification of cellular protein, since the high level incorporation permits an optimal signal to noise ratio, which aids identification and permits accurate quantification.

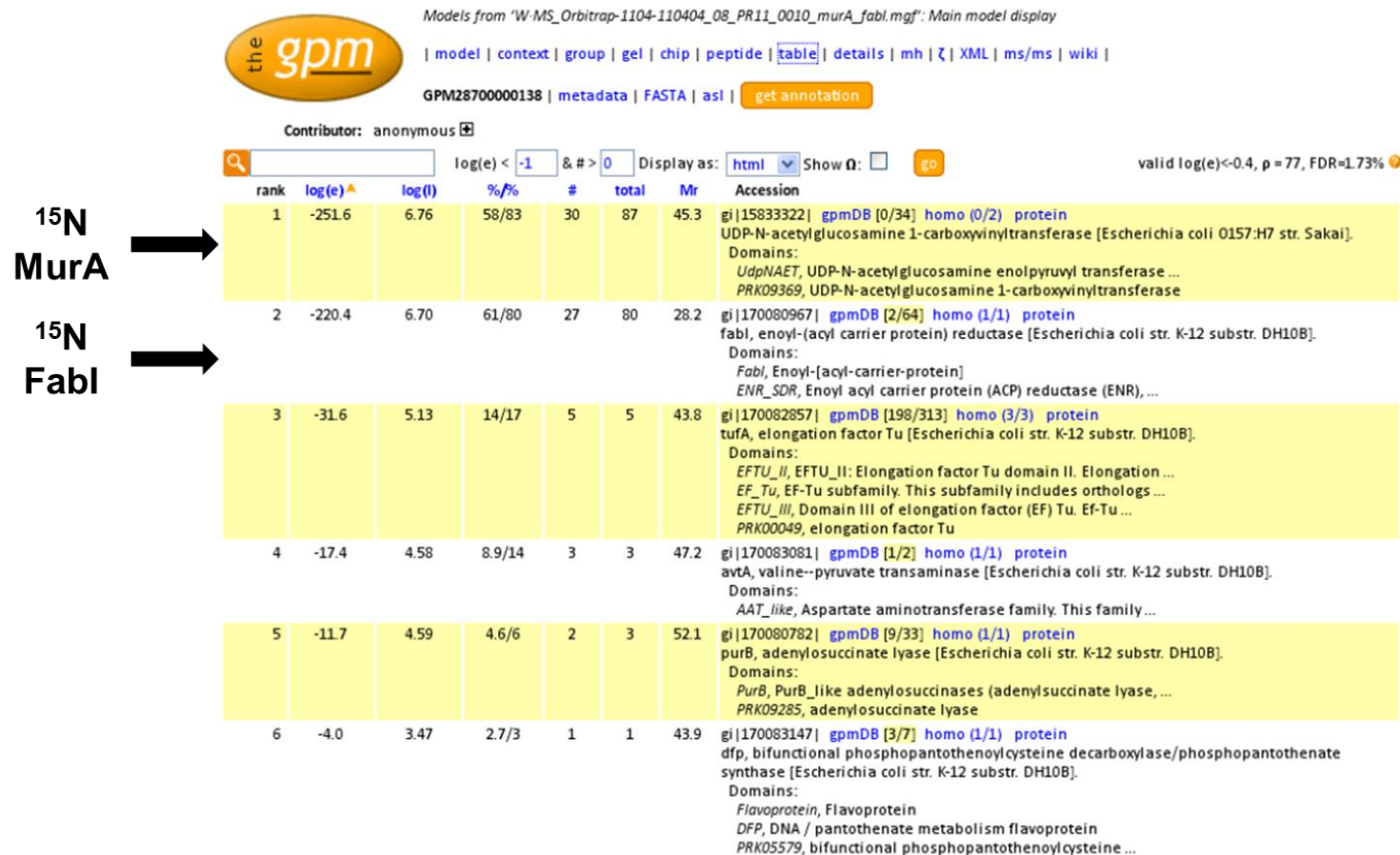


Figure 3.17 GPM Results examining <sup>15</sup>N Incorporation in FabI and MurA.

The gpm search parameters were adjusted to assume complete <sup>15</sup>N labelling of FabI and MurA, which revealed significant log (e) scores, indicating a significant probability that labelled protein internal standards were fully labelled.

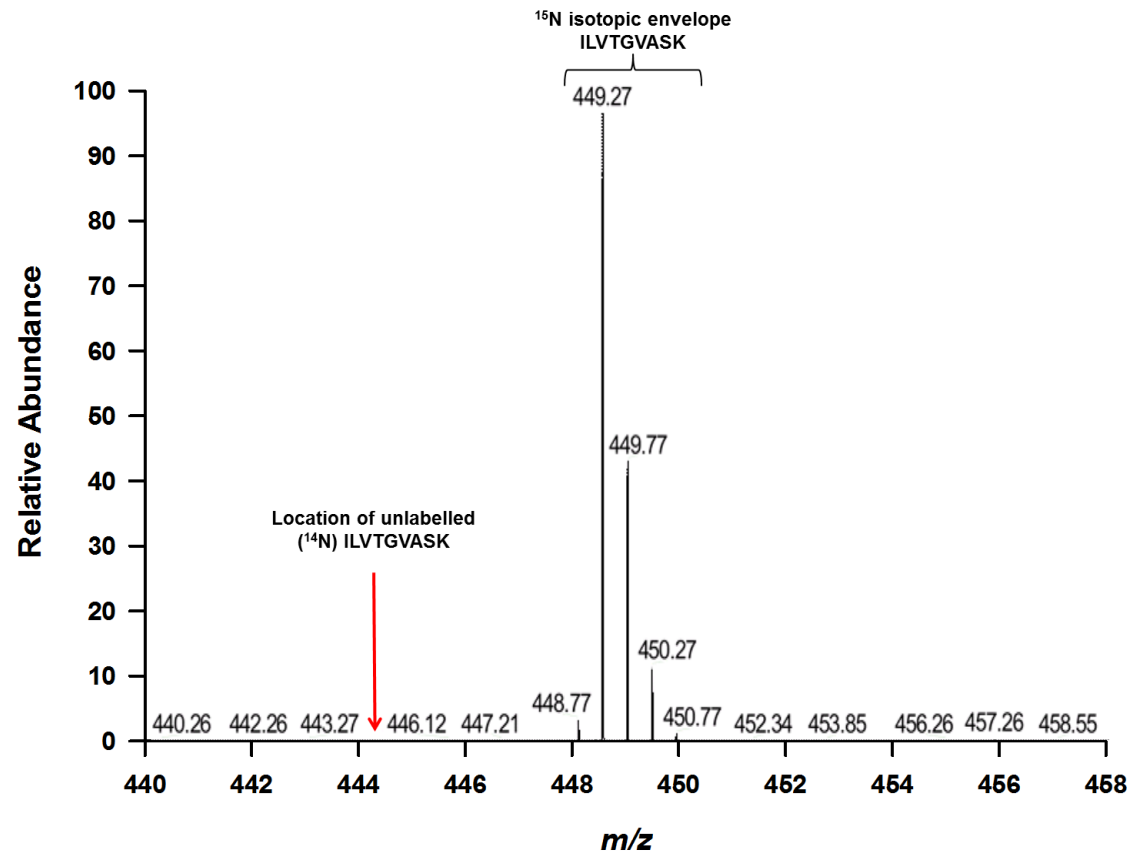


Figure 3.18 Mass spectrum of tryptic peptide ILVTGVASK used for establishing the level of <sup>15</sup>N incorporation in FabI.

The red arrow indicates where a completely unlabelled (<sup>14</sup>N labelled) peptide would be observed for peptide ILVTGVASK.



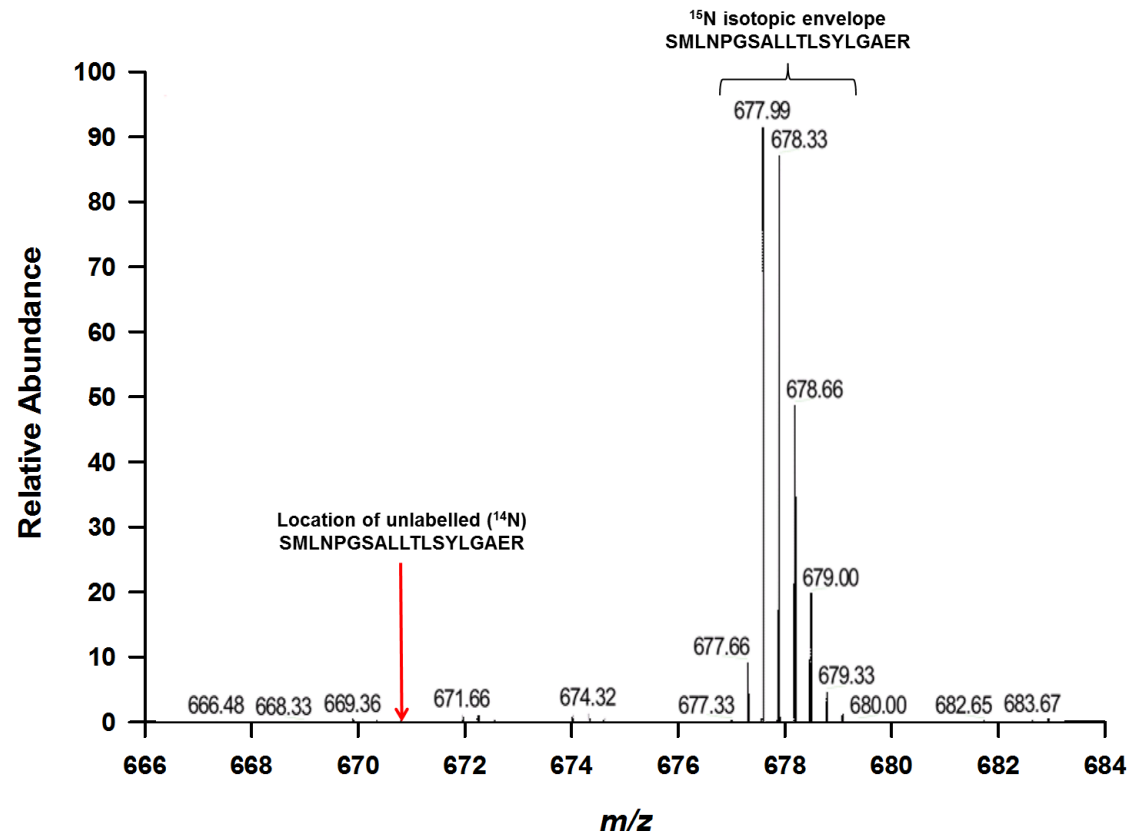
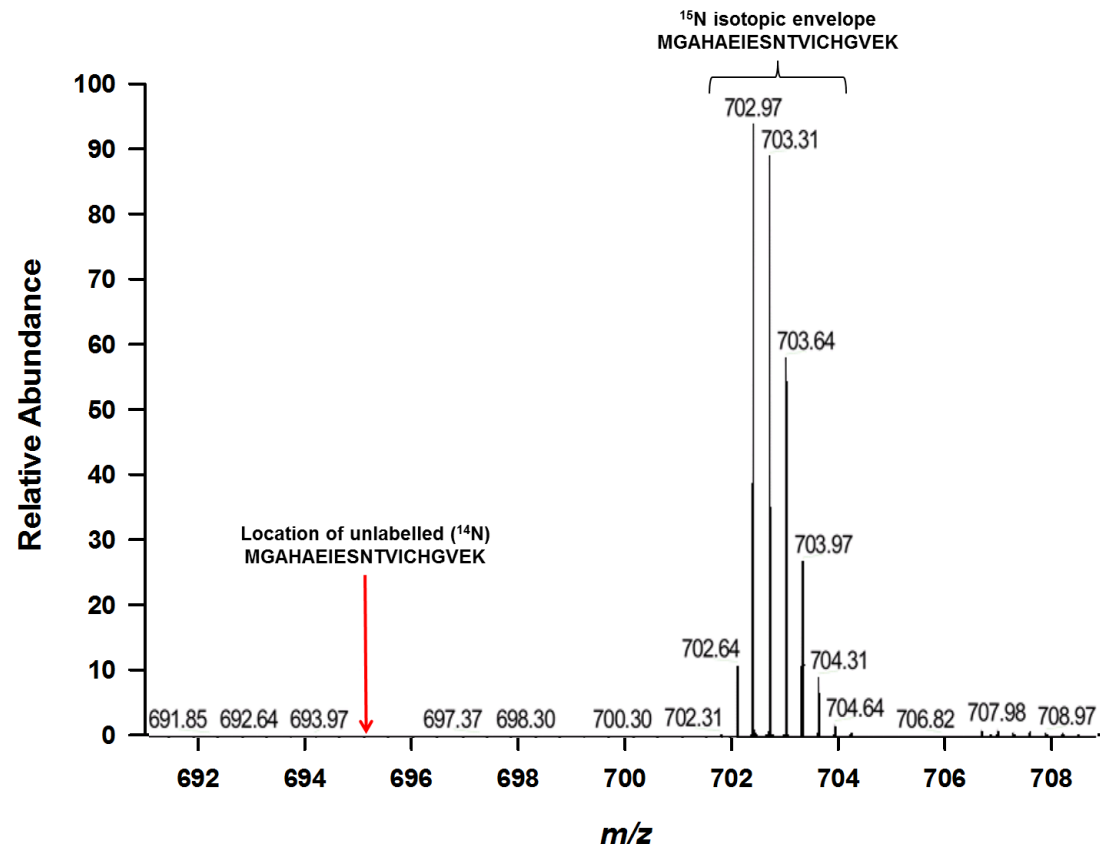


Figure 3.19 Mass spectrum of tryptic peptide SMLNPGSALLTSLYLGAER used for establishing the level of  $^{15}\text{N}$  incorporation in FabI.

The red arrow indicates where a completely unlabelled ( $^{14}\text{N}$  labelled) peptide would be observed for peptide SSMLNPGSALLTSLYLGAER.



**Figure 3.20** Mass spectrum of tryptic peptide MGAHAEIESNTVICHGVEK used for establishing the level of  $^{15}\text{N}$  incorporation in MurA.

The red arrow indicates where a completely unlabelled ( $^{14}\text{N}$  labelled) peptide would be observed for peptide MGAHAEIESNTVICHGVEK.

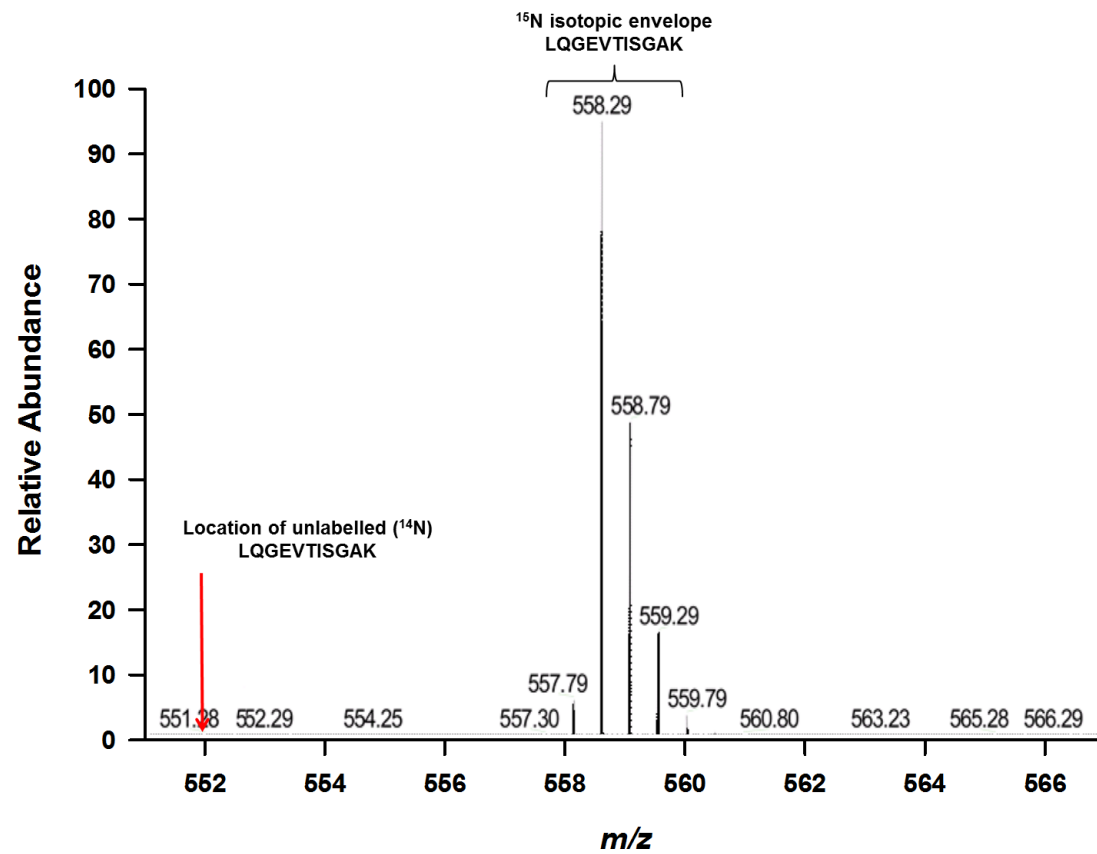


Figure 3.21 Mass spectrum of tryptic peptide LQGEVTISGAK used for establishing the level of <sup>15</sup>N incorporation in MurA.

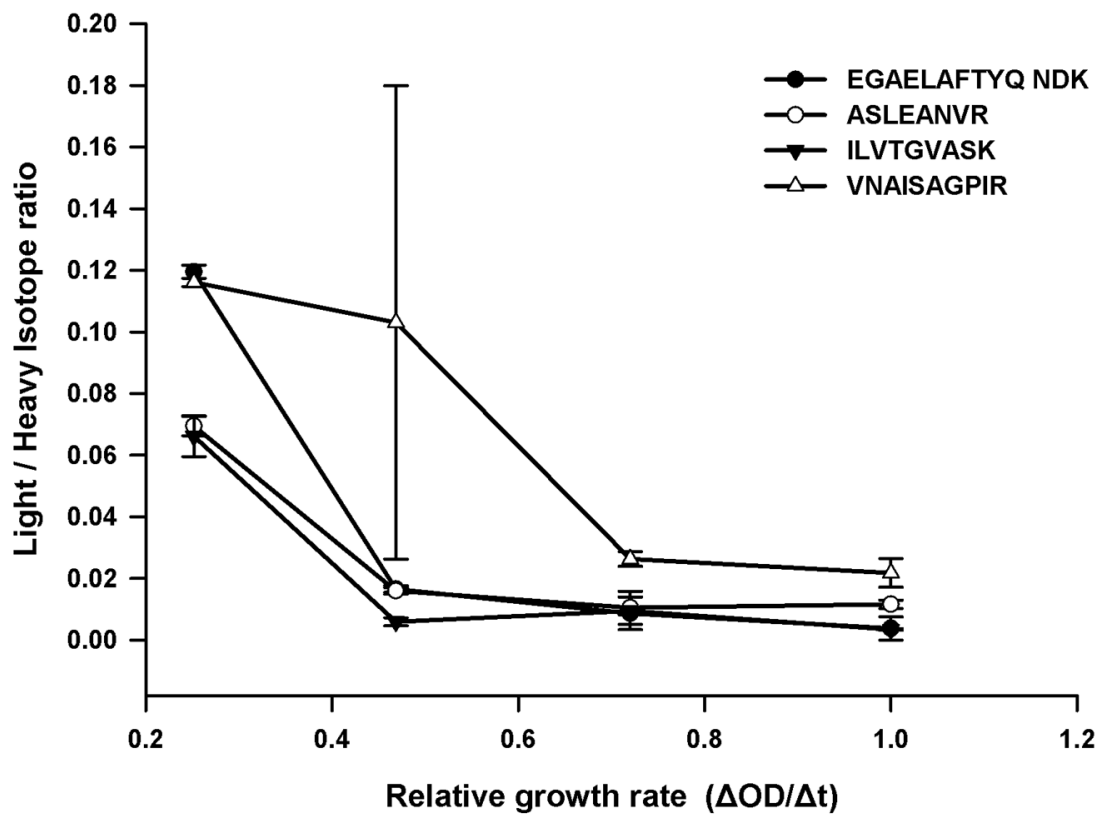
The red arrow indicates where a completely unlabelled (<sup>14</sup>N labelled) peptide would be observed for peptide LQGEVTISGAK.

### **3.2.3 Selection of peptides for quantification of protein internal standard by LC-MS/MS**

The strategy for evaluating essential gene stringency in this study incorporates the use of absolute quantification of cellular protein using LC-MS/MS. Quantification is achieved by comparing the ratio of peak areas of extracted ion chromatograms for native peptide to an internal standard of known amount. It is critical that tryptic peptides representative of both labelled and unlabelled protein generate an equivalent response during electrospray ionization. Ideally heavy labelled and native peptides should be equivalent in elution times, generate characteristic spectra and be consistently detected in both  $^{15}\text{N}$  labelled and unlabelled forms over orders of magnitude. Furthermore, the detection of peptides should be resilient to potential effects arising from the sample matrix. A key requirement of LC-MS/MS method validation is therefore to assess for matrix effects that can alter the instrument response through the suppression or enhancement of electrospray ionization. Such effects are associated with endogenous components of sample (biological) matrices that co-elute and compete with peptides or interfere with ionization, specifically the efficiency of droplet formation (Van Eeckhaut *et al.*, 2009).

To evaluate any potential matrix effects associated with whole cell *E.coli* lysate, a post-extraction spike method was employed (see methods section 2.6.7). A known volume of internal standard was spiked prior to in-gel digestion with trypsin into replicate samples of cell lysate. The cell lysate originated from *E.coli* silenced for *fabI* expression to achieve a titration of relative growth rate. Induction concentrations of IPTG were examined and chosen to achieve an approximate 25%, 50% and 75% reduction in the relative growth rate. LC-MS/MS analysis identified 30 tryptic peptide sequences indicative of FabI, which were evaluated according to established criteria (Han and Higgs, 2008). Suitable peptides were selected based on optimal length, (7-30 residues), lack of extreme hydrophilic or hydrophobic regions and absence of residues susceptible to chemical modification, that could alter  $m/z$  values thereby complicating identification and quantification. Peptide sequences containing specific residues were avoided particularly those containing methionine (subject to oxidation), asparagine (can be glycosylated or deaminated), glycine or glutamic acid (undergo cyclization to pyroglutamic acid), or peptides with

internal trypsin cleavage sites. The absolute areas of extracted ion chromatogram peaks for selected peptides, identified in both  $^{15}\text{N}$  labelled and unlabelled FabI, were determined and converted to a Light/Heavy ratio. An arbitrary cut-off value of 0.15 was used to identify peptides displaying the lowest L/H ratio (approximately 1:1). Four candidate peptides were subsequently determined as suitable for quantification standards that could be used to quantitate the FabI Internal standard (Figure 3.22).



**Figure 3.22 Tryptic peptides identified for the quantification of FabI internal standard.**

The Light/Heavy ratio for four tryptic peptides specific to FabI were generated from extracted ion chromatograms and plotted against the relative growth rate of *E.coli*, which was titrated using different concentrations of IPTG to achieve increased antisense RNA silencing of *fabI*. Graphs show average peptide L/H ratio with standard error bars (n=3). Peptide VNAISAGPIR was discounted from analysis due to substantial variability in the L/H peptide ratio obtained for 50 % relative growth rate

For quantitation of the  $^{15}\text{N}$  labelled internal standard, two tryptic peptides EGAELAFTYQNDK and ILVTGVASK were selected due to their similarity in L/H ratio's across all relative growth conditions. Significant variation was displayed for VNAISAGPIR at 50% relative growth, hence it was discarded from selection with ASLEANVR.

In order to provide accurate quantitation of the FabI internal standard, the mass spectra and elution characteristics for respective peptide ions was determined. Since these characteristics are specific to each peptide ion, they can be used to confirm that extracted ion chromatogram peaks are assigned to the correct peptide ion, which serves a proxy for both the internal standard and the protein under investigation.

Evaluation of EGAELAFTYQNDK revealed spectra corresponding to both unlabelled and  $^{15}\text{N}$  labelled tryptic peptides (Figure 3.23 and Figure 3.24 respectively). A pseudomolecular 2+ ion was identified with a  $m/z$  of 743.35 for the unlabelled peptide and 751.32 for the  $^{15}\text{N}$  labelled counterpart (Figure 3.25). Extracted chromatograms revealed an elution time of 16.85 minutes (Figure 3.26 A and B). Similarly ILVTGVASK also revealed characteristic spectra for unlabelled and  $^{15}\text{N}$  labelled forms of the peptide (Figure 3.27 and Figure 3.28). The pseudomolecular 2+ ion was identified with a  $m/z$  of 444.28 and 449.27 for the unlabelled and  $^{15}\text{N}$  labelled counterpart respectively (Figure 3.29). Extracted ion chromatograms revealed a peptide elution time of approximately 15 minutes (Figure 3.30 A and B). Due to the consistent detection of tryptic peptides EGAELAFTYQNDK and ILVTGVASK in all relative growth conditions, both peptides were selected for incorporation as template profiles into LC-MS/MS data analysis software, for subsequent identification and quantitation of the  $^{15}\text{N}$  labelled FabI internal standard.

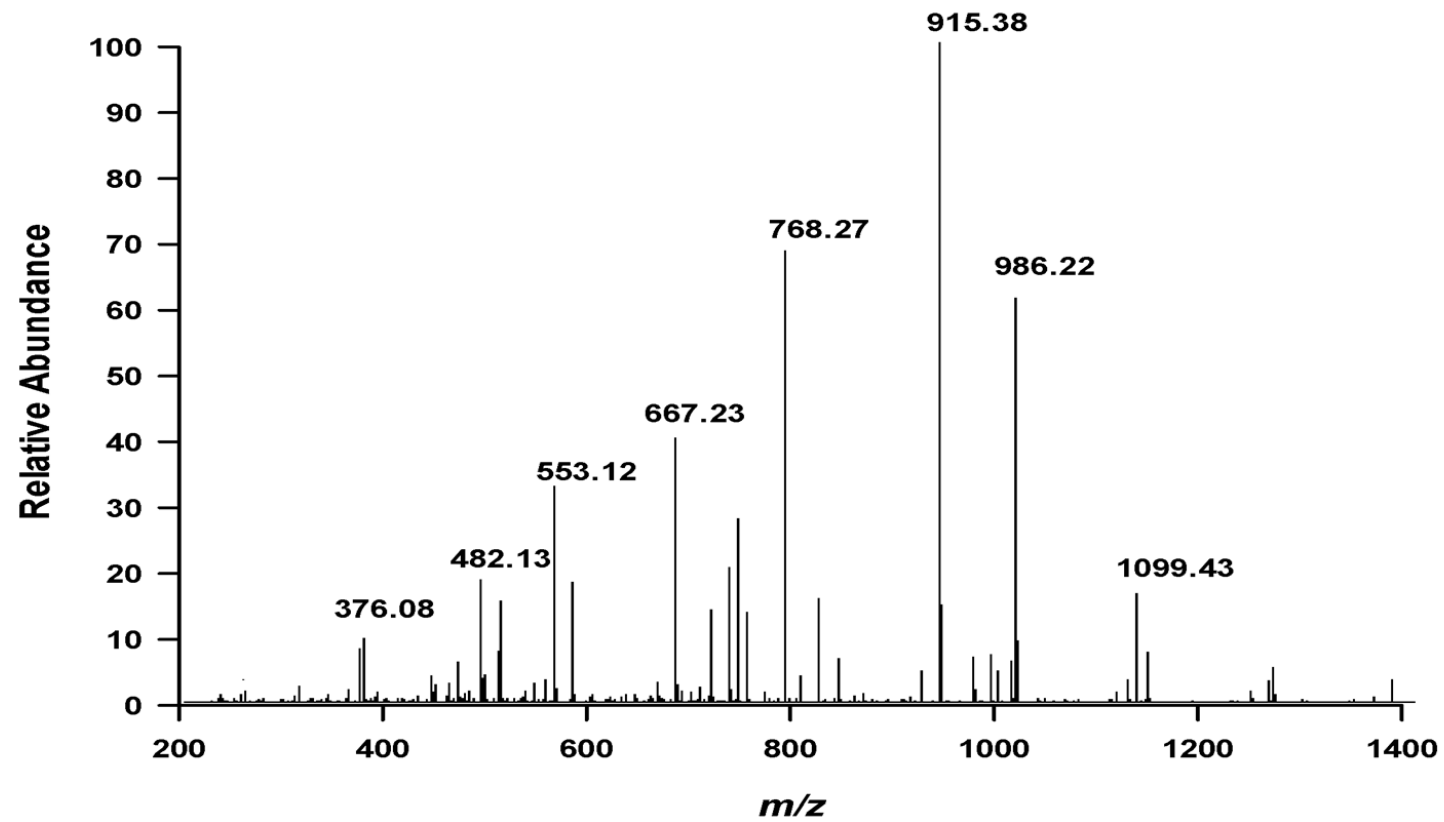


Figure 3.23 LC-MS/MS spectra for unlabelled EGAELAFYQNDK.

The spectra for peptide EGAELAFYQNDK revealed eight individual  $m/z$  peaks that could be used to confirm identity of the native ( $^{14}\text{N}$ ) peptide.



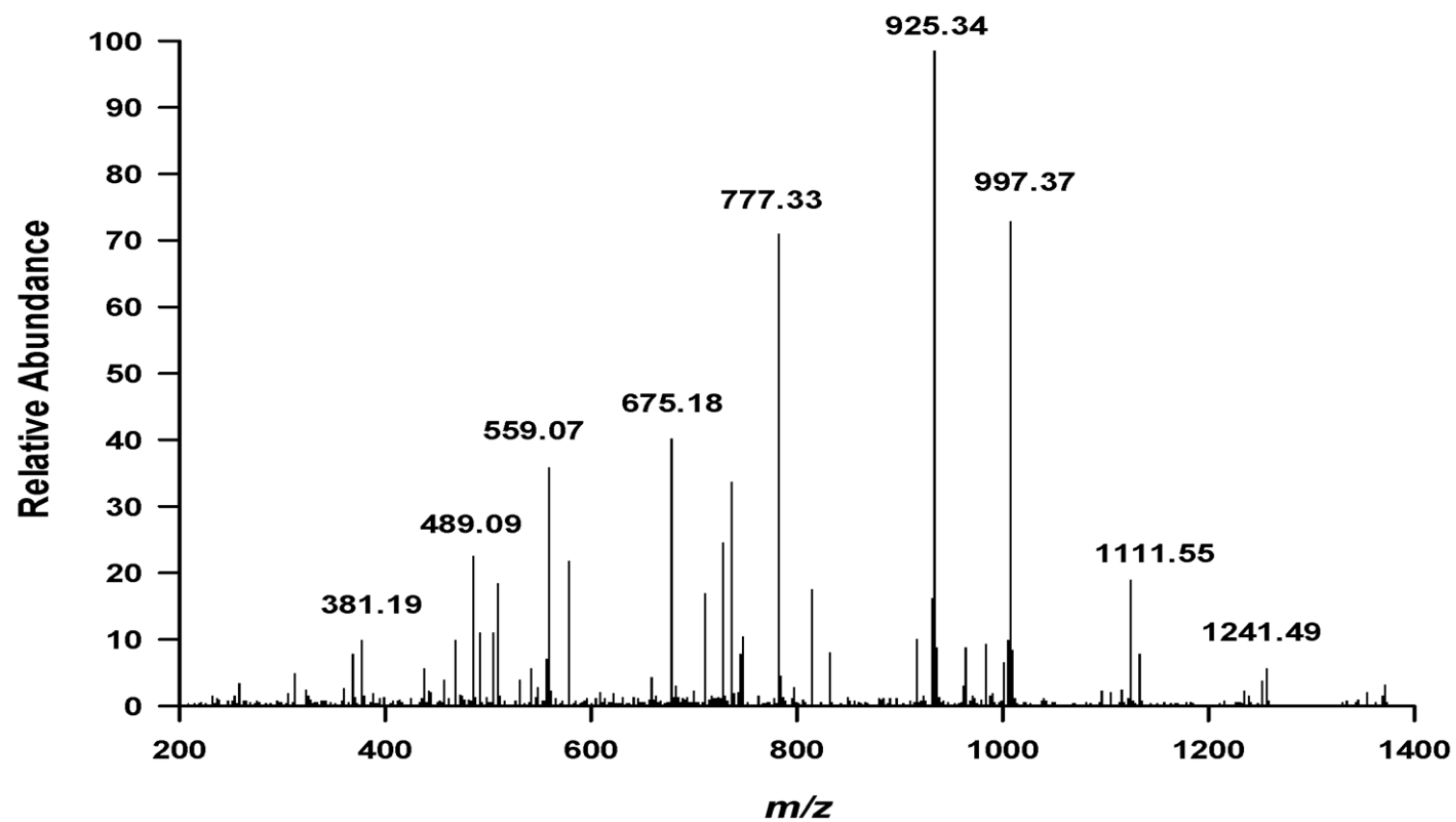


Figure 3.24 LC-MS/MS spectra for  $^{15}\text{N}$  labelled peptide EGAELAFTYQNDK.

The spectra for  $^{15}\text{N}$  labelled EGAELAFTYQNDK revealed eight individual  $m/z$  peaks that could be used to confirm identity. A noticeable shift in  $m/z$  values was observed compared to the spectra for the native peptides.

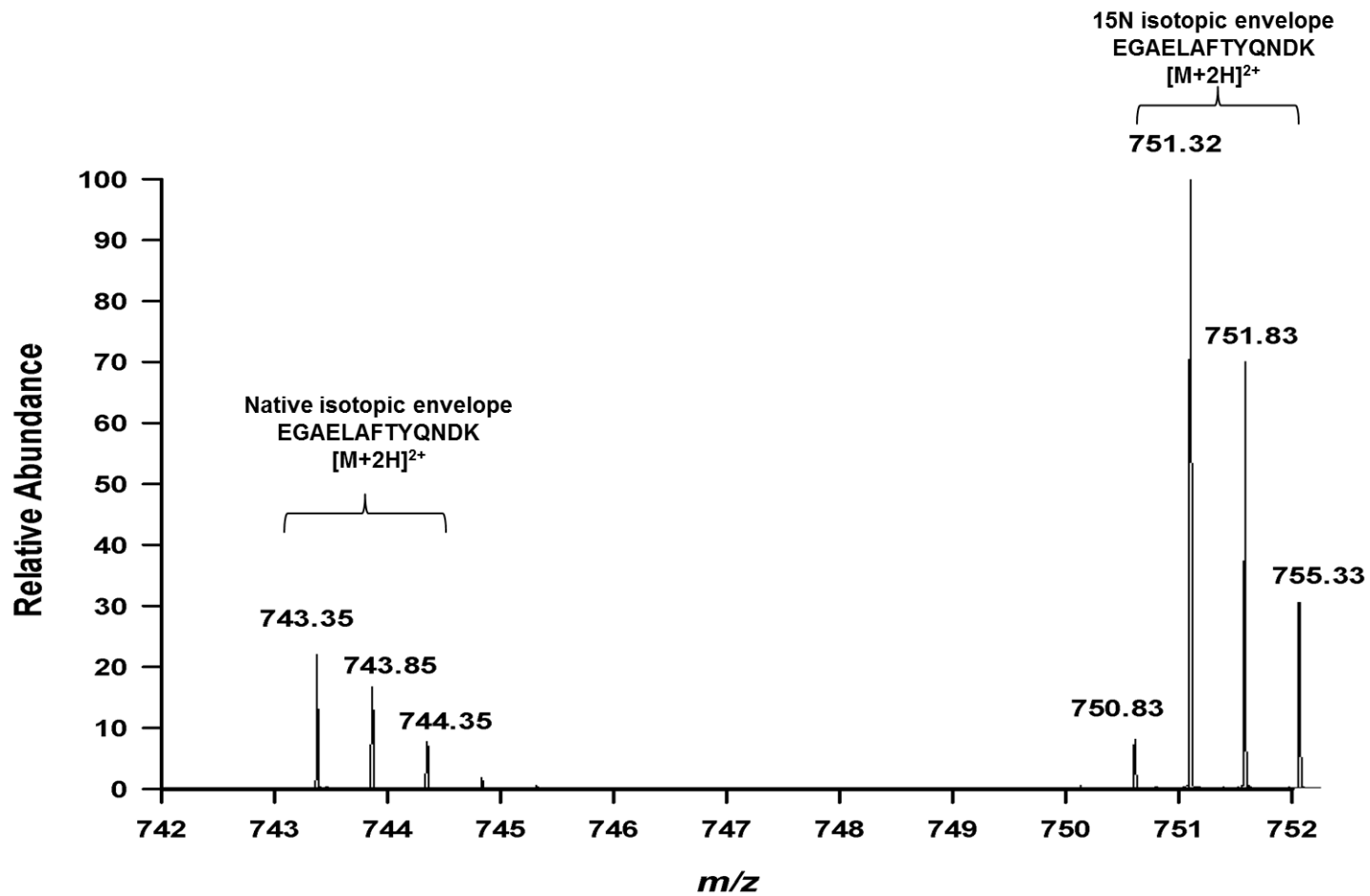
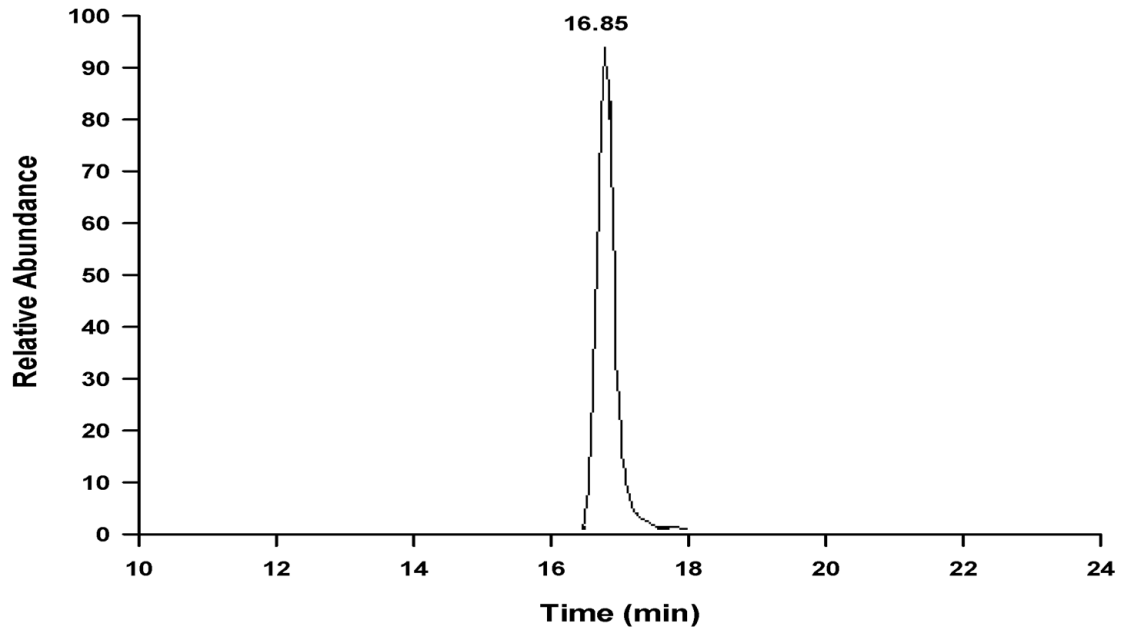
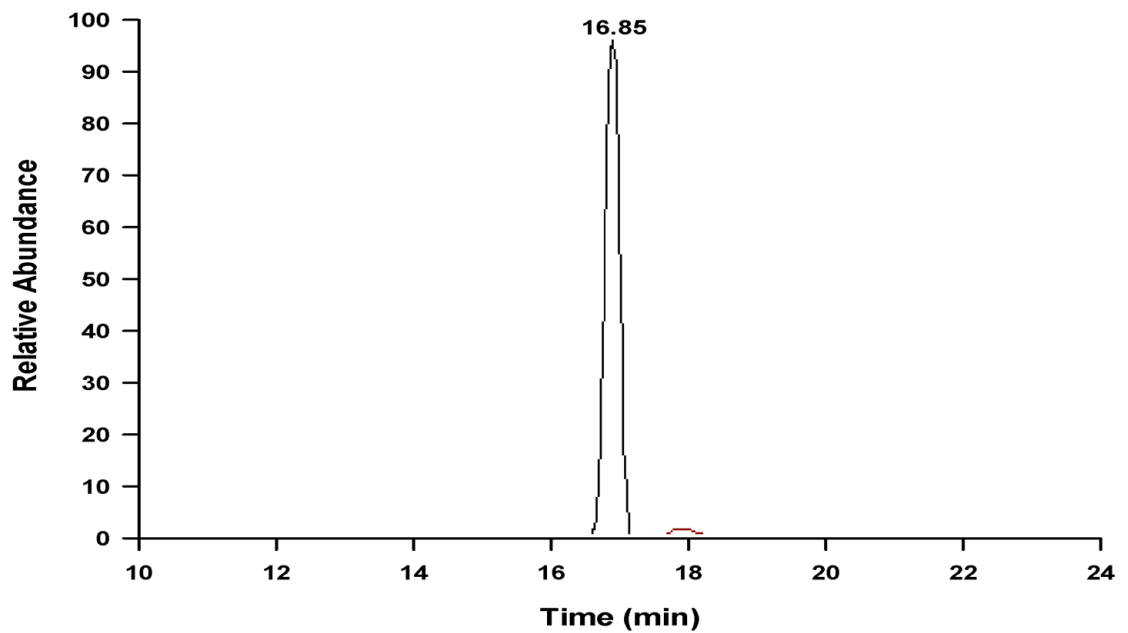


Figure 3.25 LC-MS/MS characteristics of peptide EGAELAFTYQNDK.

Zoomed MS spectra showing characteristic isotopic envelope of native and <sup>15</sup>N labelled EGAELAFTYQNDK with m/z of 743.35 and 751.32 respectively.

**A****B**

**Figure 3.26 LC-MS/MS characteristics of peptide EGAELAFYQNDK.**

Extracted ion chromatogram of native (A) and <sup>15</sup>N labelled (B) **EGAELAFYQNDK** peptide with a co-elution time of 16.85 minutes. **Relative abundance** represents the ion intensity

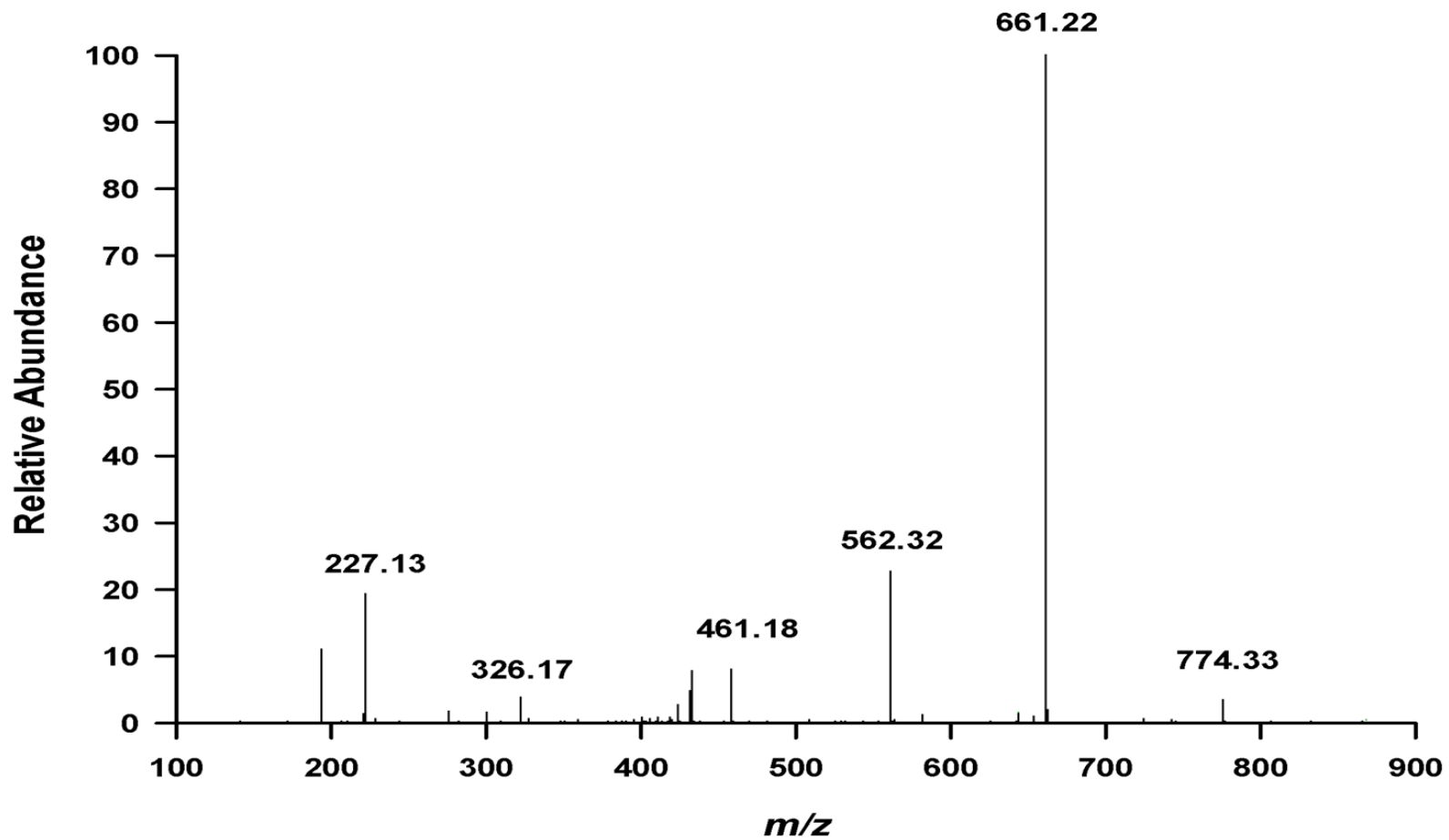


Figure 3.27 LC-MS/MS spectra for unlabelled and  $^{15}\text{N}$  labelled peptide ILVTGVASK.

The spectra for peptide ILVTGVASK revealed six individual  $m/z$  peaks that could be used to confirm identity of the heavy labelled peptide.

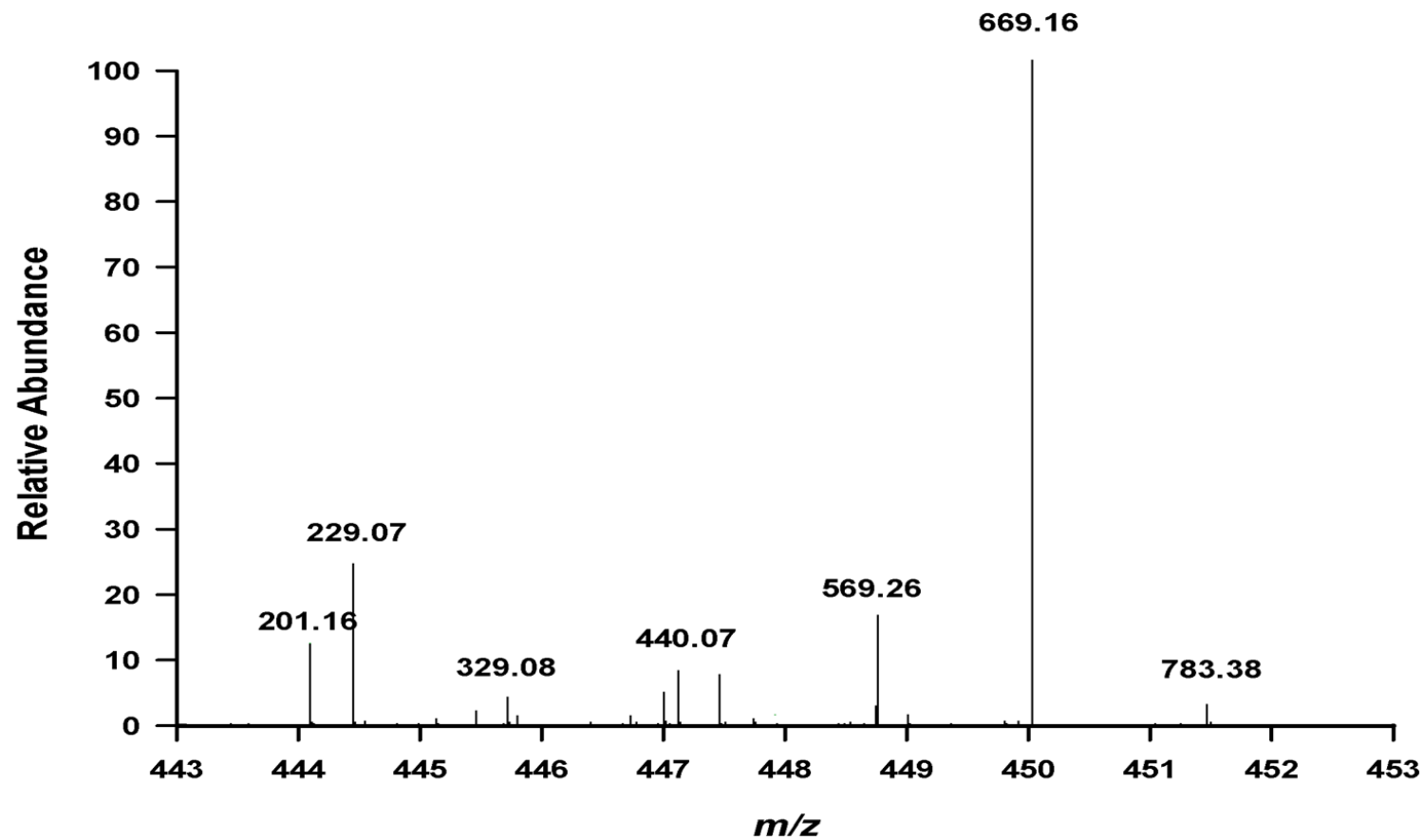


Figure 3.28 LC-MS/MS spectra for <sup>15</sup>N labelled peptide ILVTGVASK.

The spectra for peptide ILVTGVASK revealed seven individual *m/z* peaks that could be used to confirm identity of the heavy labelled peptide. A noticeable shift in *m/z* values is observed compared to spectra for corresponding unlabelled peptides.

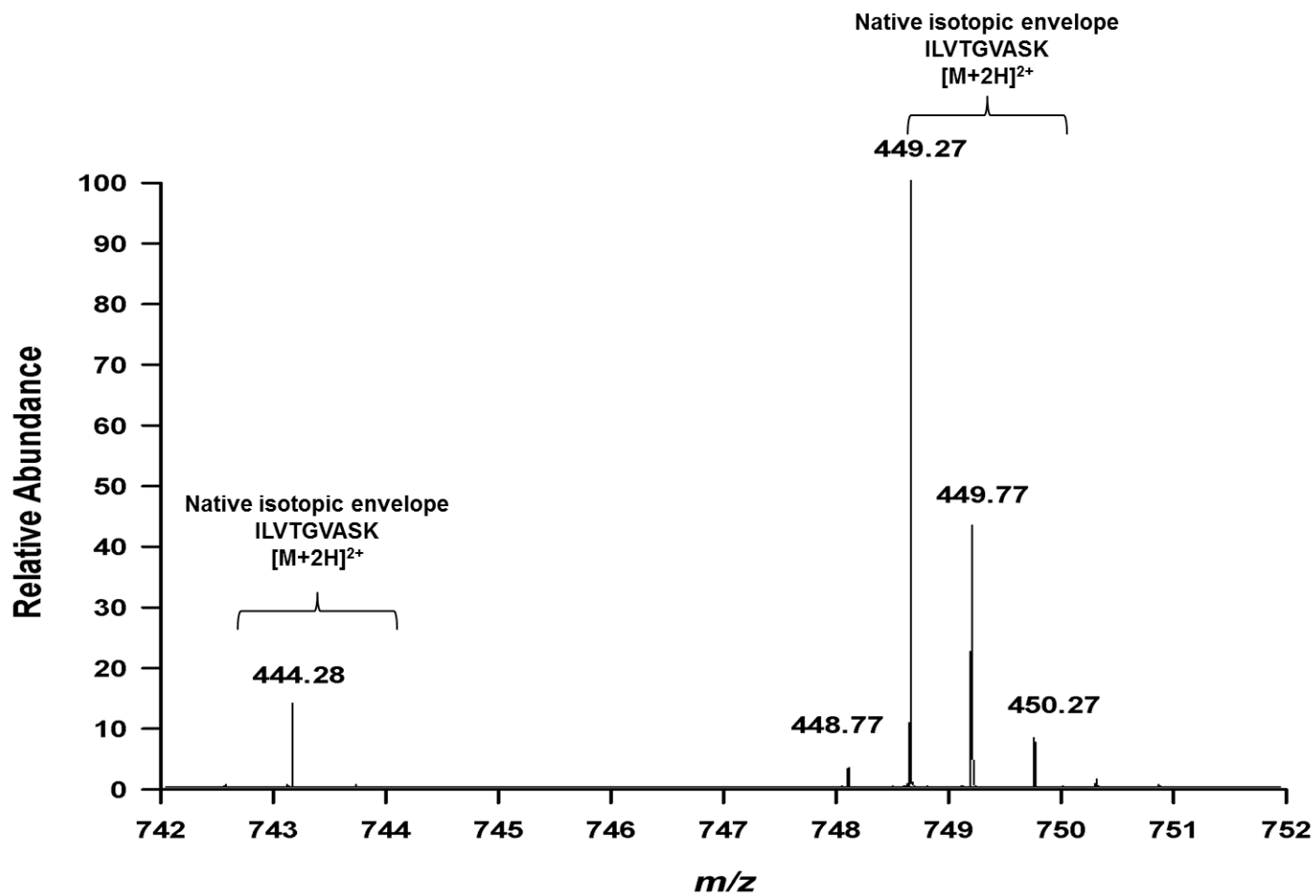
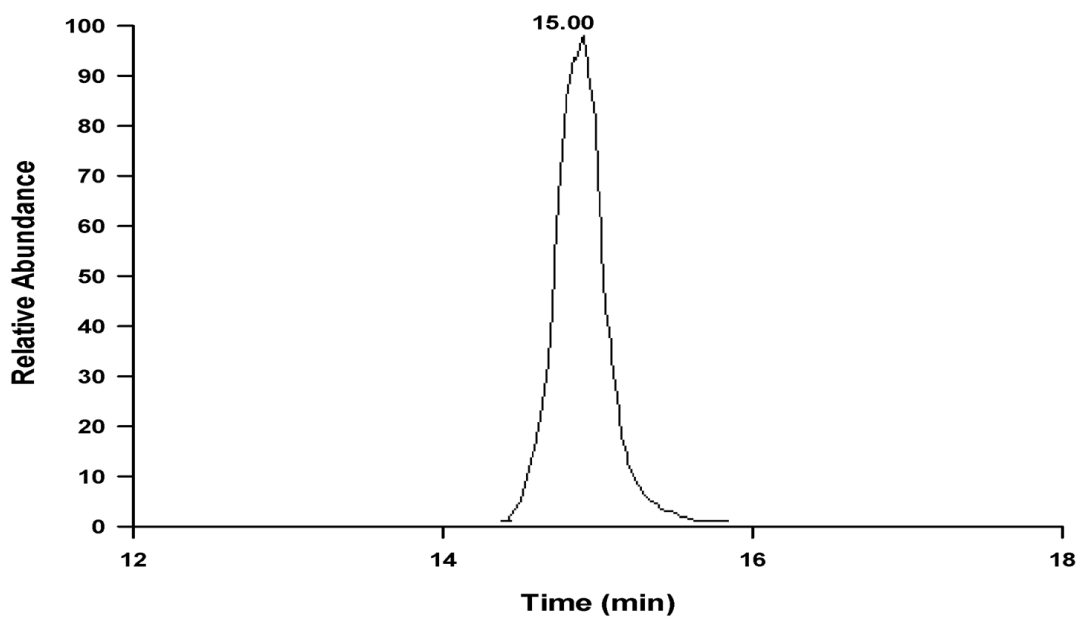
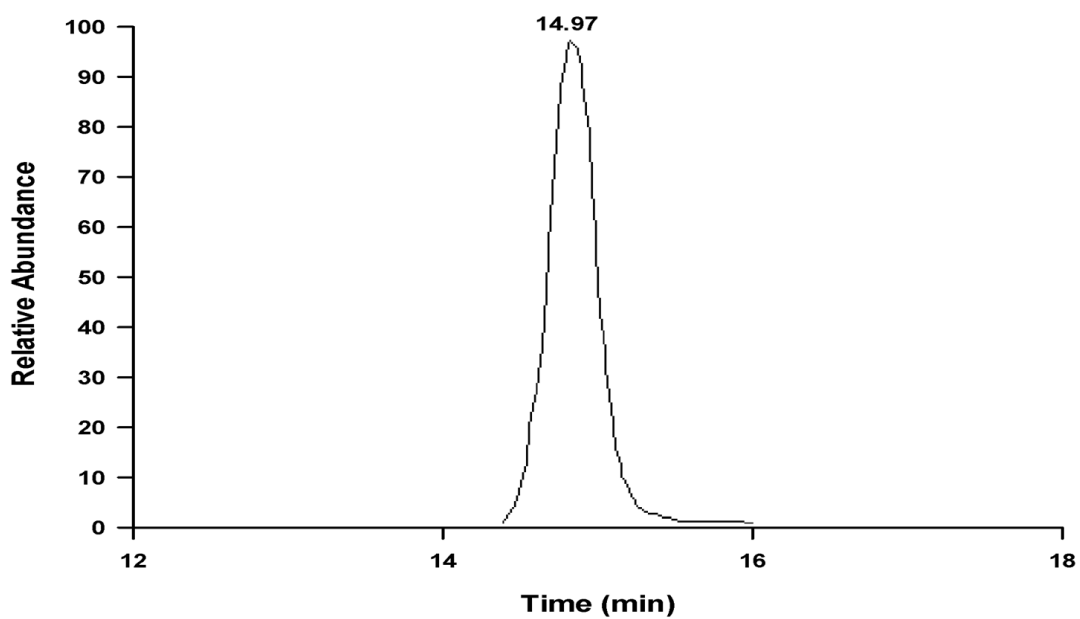


Figure 3.29 LC-MS/MS characteristics of peptide ILVTGVASK.

Zoomed MS spectra showing characteristic isotopic envelope of native and  $^{15}\text{N}$  labelled EGAELAFTYQNDK with  $m/z$  of 444.28 and 449.27 respectively.

**A****B**

**Figure 3.30 LC-MS/MS characteristics of peptide ILVTGVASK.**

Extracted ion chromatogram of native (A) and  $^{15}\text{N}$  labelled (B) peptide ILVTGVASK with a co-elution time of approximately 15 minutes.

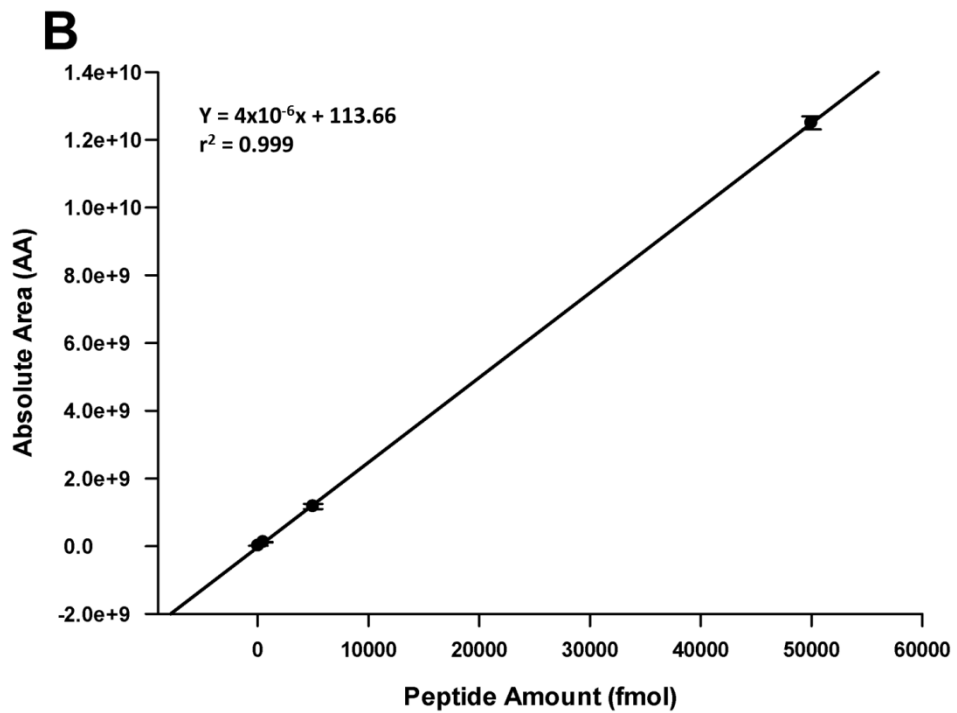
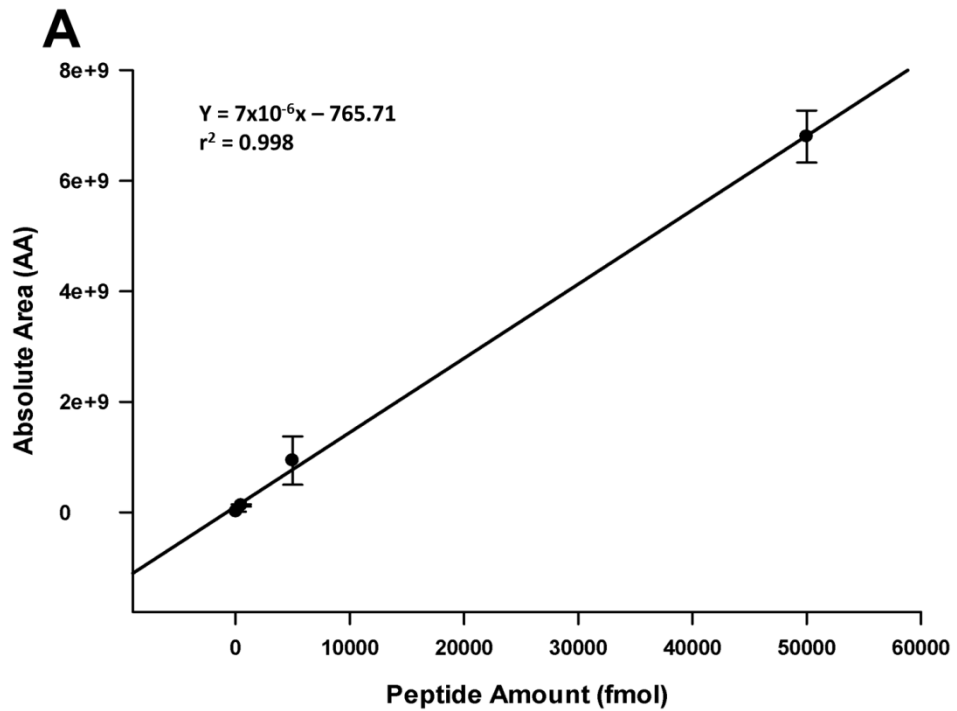
### 3.3 Quantification and assessment of gene stringency using protein abundance

#### 3.3.1 Quantification of <sup>15</sup>N labelled FabI protein internal standard

Prior to utilizing the <sup>15</sup>N labelled fabI as an internal standard, quantification of the purified protein was performed using commercially synthesized (pre-quantified) peptide analogues of EGAELAFTYQNDK and ILVTGVASK (see methods section 2.6.8). Both peptides were serially diluted to generate individual standard curves spanning 6 orders of magnitude, to enable extrapolation of the amount of internal standard. Serial dilutions of each peptide were spiked into trypsin digests containing a known volume of FabI internal standard and analysed by LC-MS/MS.

Insufficient data was obtained for both peptides corresponding to spiked amounts in the lower range of the standard curve (0.5 and 5 fmol). Consequently standard curves were generated over 4 orders of magnitude (50 – 50,000 fmol). Standard curves for EGAELAFTYQNDK and ILVTGVASK (Figure 3.31 A and B respectively), generated by linear regression displayed good correlation ( $r^2 = 0.99$ ). Using the linear regression equations  $y = 7 \times 10^{-6}x - 765.71$  and  $y = 4 \times 10^{-6}x + 113.66$ , the amount of internal standard was calculated as 675 pmol for EGAELAFTYQNDK and 4547 pmol for ILVTGVASK. The average amount of <sup>15</sup>N FabI internal standard was subsequently calculated as 2.61 nmol





**Figure 3.31** Tryptic peptide standard curves for quantification of  $^{15}\text{N}$  FabI internal standard.

(A) Standard curve for tryptic peptide EGAELAFTYQNDK. (B) Standard curve for tryptic peptide ILVTGVASK. Standard curves were constructed from 10-fold serial dilution of peptides ranging from 50-50,000 fmol. The average amount of  $^{15}\text{N}$  FabI was calculated using linear regression. Standard curves show average amount of peptide with standard error bars (n=3).

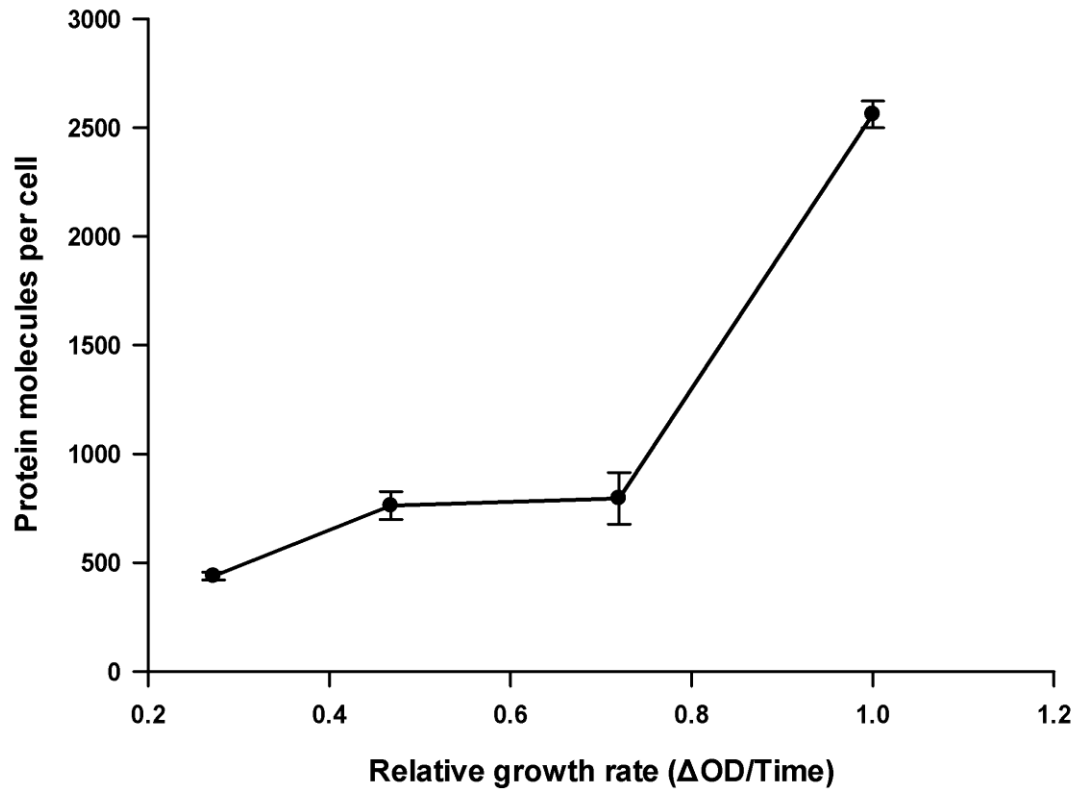
### **3.3.2 Quantification of FabI protein per cell following gene silencing of *fabI* in *E.coli***

To evaluate the differential growth requirement of the essential gene *fabI*, the growth rate of *E.coli* strain *As-fabI* was titrated down using expressed antisense RNA silencing. Concentrations of IPTG (50, 55 and 65  $\mu\text{M}$ ) were used to achieve approximate growth reductions of 25%, 50% and 75% (see methods section: 2.6.5). For comparative purposes the amount of FabI synthesised following overexpression in *E.coli* MG1 ME5305 was investigated using IPTG induction at 0.5 and 1 mM.

Transcript abundance was measured by QPCR and protein was quantified by LC-MS/MS. The peak area derived from extracted ion chromatograms for peptides EGAELAFTYQNDK and ILVTGVASK was used to generate ratios of unlabelled:  $^{15}\text{N}$  labelled peptide. The amount of protein was obtained by multiplying the calculated ratios for each peptide by the amount of internal standard spiked into tryptic digests. The calculated amount was converted to protein molecules by multiplying by Avogadro's number and corrected for viable cell counts to provide an estimation of FabI molecules per cell (Figure 3.32).

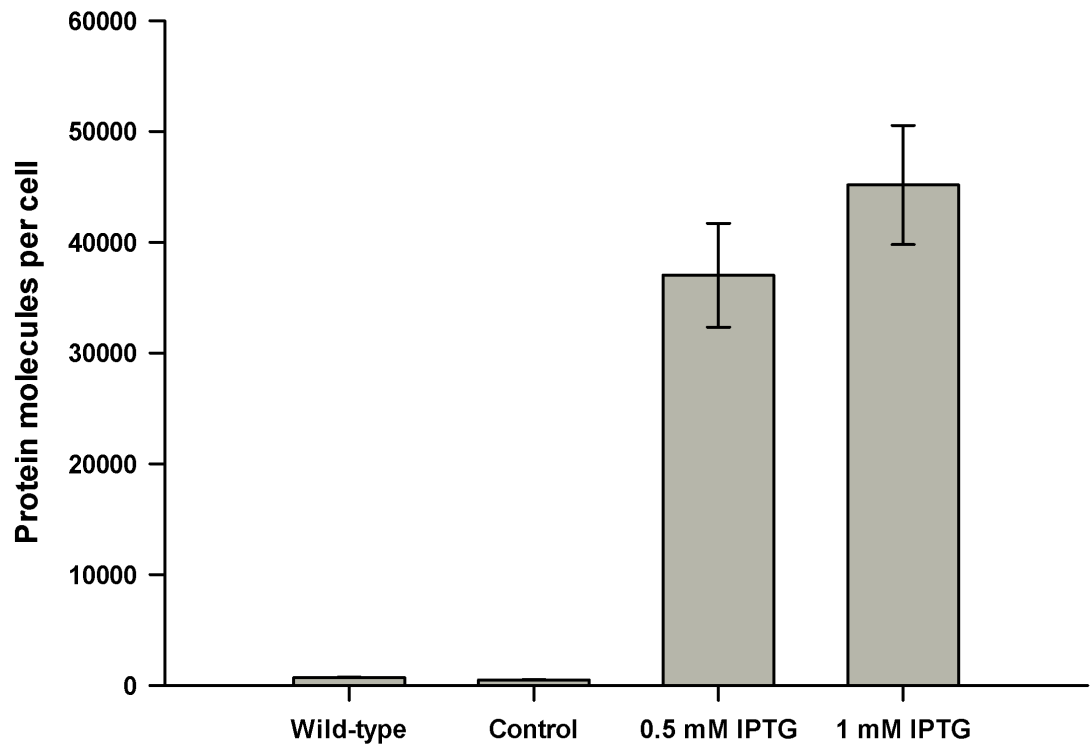
The number of FabI molecules per cell decreased with declining growth rate, following silencing of *fabI*. A 3-fold decrease in FabI molecules per cell was observed as the relative growth rate decreased to approximately 75% of normal growth. A smaller decrease in FabI molecules per cell (1.2 fold) was observed as the growth rate declined to approximately 50% of normal growth. A 2-fold decrease in FabI molecules per cell occurred as the relative growth rates dropped to approximately 25% of the normal growth rate.

When overexpressed the amount of FabI per cell increased by approximately 100-fold in comparison to both wild-type *E.coli* and uninduced controls (Figure 3.33). The number of FabI molecules per cell increased slightly (1.2-fold) when the concentration of IPTG was doubled from 0.5 to 1 mM. Interestingly the number of FabI molecules per cell in the antisense control (uninduced) was 3.5-fold higher than wild-type *E.coli* and 5-fold higher when compared to the ASKA control (uninduced). Both wild-type *E.coli* and the ASKA control revealed a similar basal expression of FabI.



**Figure 3.32 FabI protein molecules per cell following expressed antisense silencing of the cognate essential gene.**

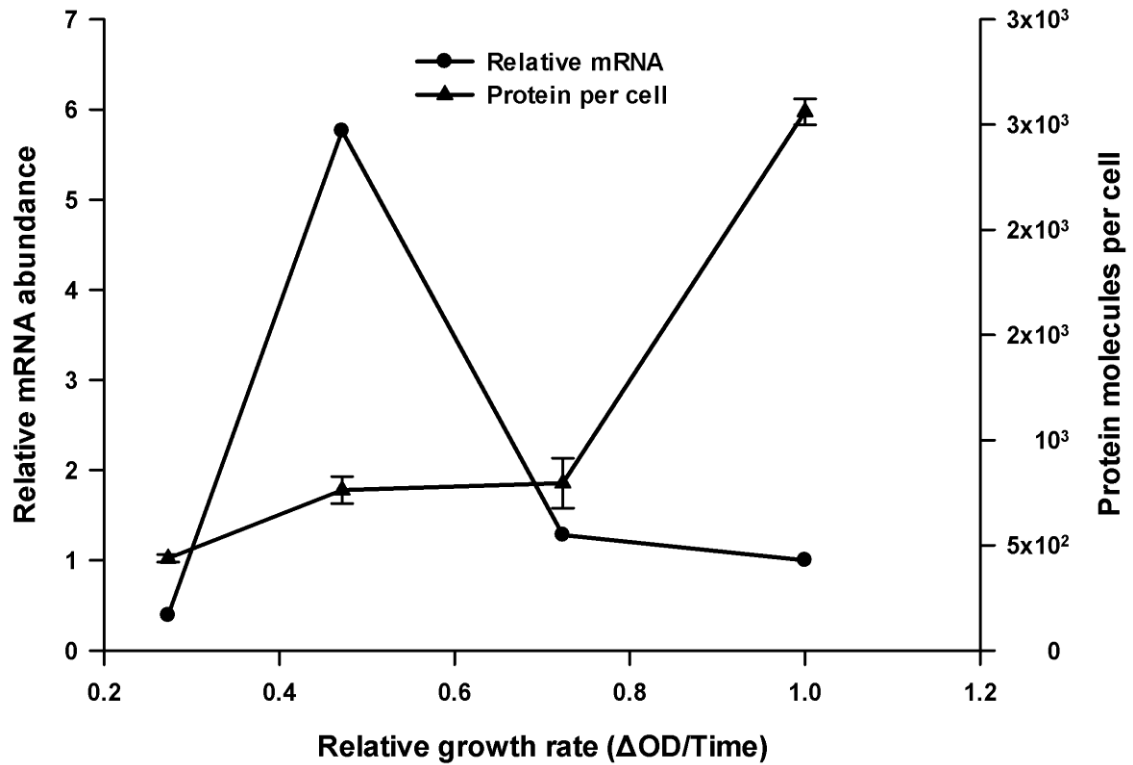
IPTG induction was used to titrate down the relative growth rate of *E.coli As-fabI* to approximately 75% 50% and 25% of normal growth. Protein was measured by comparing the extracted Ion chromatogram peak areas of light and  $^{15}N$  labelled forms of tryptic peptides EGAELAFYQNDK and ILVTGVASK. Protein amounts were corrected by viable cell counts. Graph shows average protein molecules per cell with standard error bars (n=3).



**Figure 3.33 FabI protein molecules per cell in *E.coli* MG1 ME5305 and Wild-type *E.coli*.**

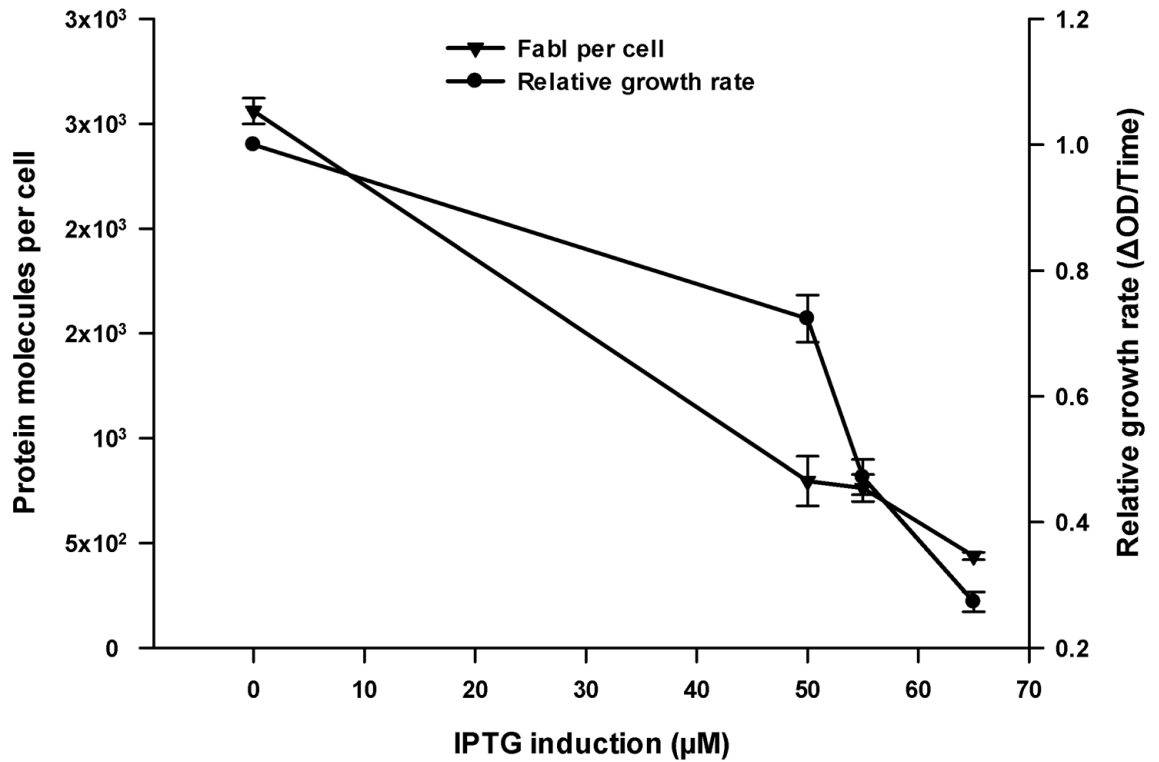
FabI overexpression was induced with 0.5 and 1 mM IPTG. Protein abundance was measured by comparing the extracted Ion chromatogram peak areas of light and <sup>15</sup>N labelled forms of tryptic peptides EGAELAFTYQNDK and ILVTGVASK. Protein amounts were corrected by viable cell counts. Graph shows average protein molecules per cell with standard error bars (n=3).

Both mRNA and protein abundance following gene silencing of *fabI* was evaluated to identify any correlation between the decrease in either mRNA or protein, with the observed decline in relative growth rate (Figure 3.34). No significant correlation (Pearson product correlation,  $n = 4$ ,  $P > 0.05$ ) was observed. The abundance of *fabI* transcripts appeared to initially increase prior to a large spike at approximately 50% of normal relative growth rate, after which mRNA abundance declined. When evaluated over the induction range of IPTG, the decline in protein abundance followed the decrease in growth rate (Figure 3.35), although again, no significant correlation was observed (Pearson product correlation,  $n = 4$ ,  $P > 0.05$ ). The decline in relative growth rate and mRNA abundance also revealed no significant correlation (Pearson product correlation,  $n = 4$ ,  $P > 0.05$ ) (Figure 3.36).



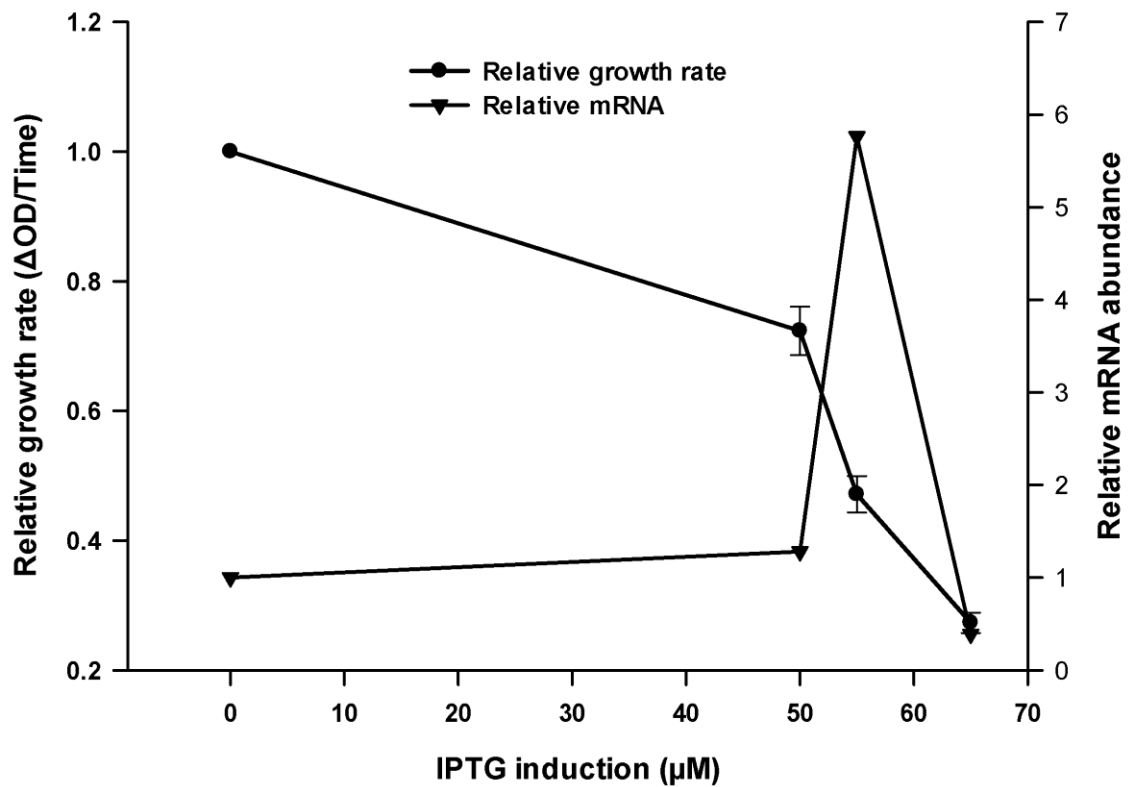
**Figure 3.34 mRNA transcript and protein abundance in *E.coli* following expressed antisense RNA silencing of the essential gene *fabI*.**

Relative mRNA abundance and protein molecules per cell were plotted against corresponding relative growth rate. Pearson product correlation revealed no significant correlation ( $n = 4$ ,  $P > 0.05$ ) between mRNA and protein abundance. Graph shows average protein molecules per cell and mRNA abundance with standard error bars ( $n=3$ ).



**Figure 3.35** Relative growth rate correlation with FabI molecules per cell following expressed antisense RNA silencing of the essential gene *fabI* in *E.coli*.

Growth rate titration of *E.coli* strain *As-fabI* was achieved with increasing concentrations of IPTG (50, 55 and 65 μM). Pearson product correlation revealed no significant correlation ( $n = 4$ ,  $P > 0.05$ ) revealed between FabI molecules per cell or relative growth rate decline. Graph shows average protein molecules per cell plotted with average relative growth rate against IPTG induction with standard error bars ( $n=3$ ).



**Figure 3.36 Relative mRNA abundance correlation with FabI per cell following expressed antisense RNA silencing of the essential gene *fabI* in *E.coli*.**

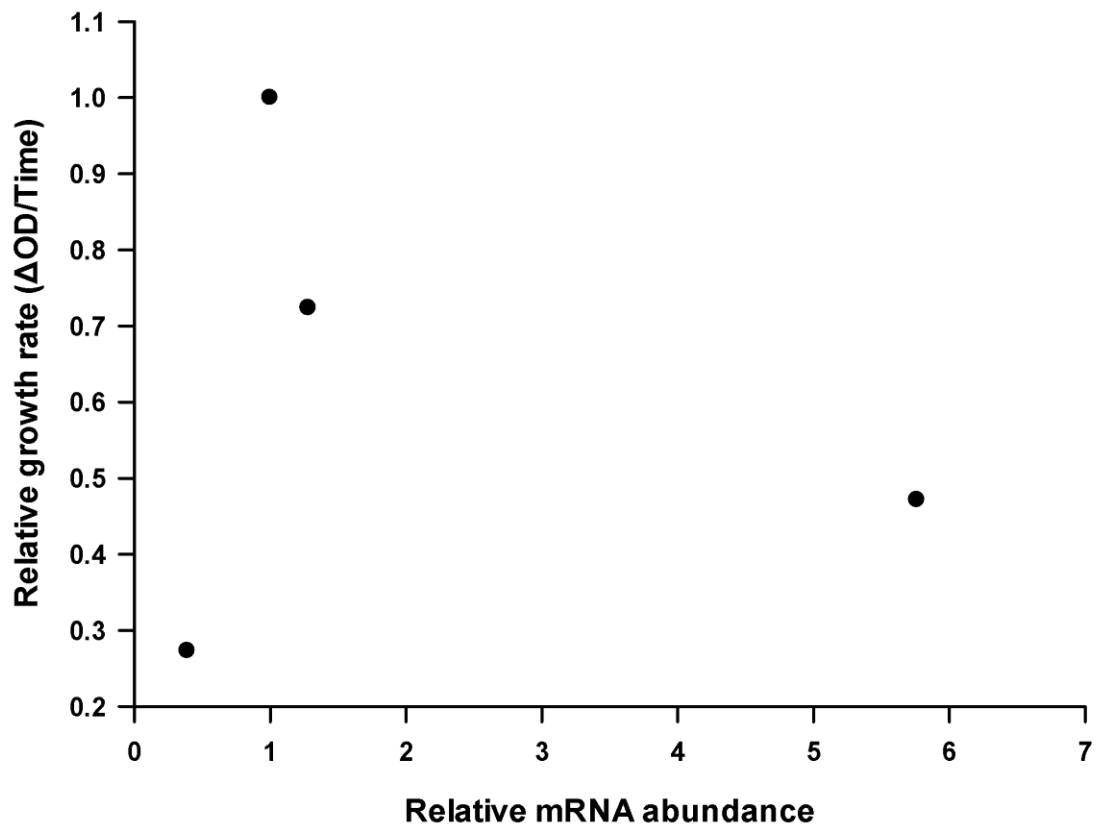
Growth rate titration of *E.coli* strain *As-fabI* was achieved by induction with increasing concentrations of IPTG (50, 55 and 65 μM). No significant correlation was revealed between protein molecules per cell or growth rate decline. Pearson product correlation revealed no significant correlation ( $n = 4$ ,  $P > 0.05$ ). Graph shows average protein per cell plotted with average relative growth rate against IPTG induction with standard error bars ( $n=3$ ).



### **3.3.3 MTL<sub>50</sub> and MPL<sub>50</sub> for evaluating stringency of the essential *E.coli* gene *fabI***

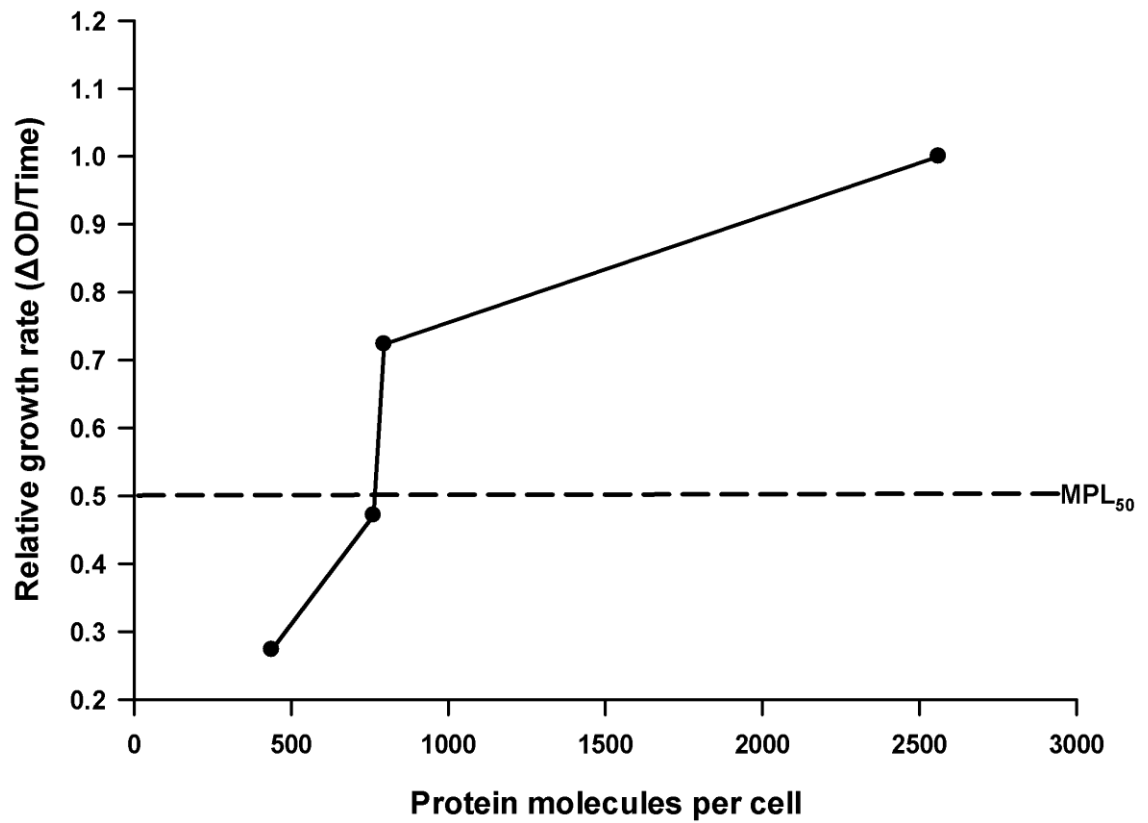
In this study the stringency of the essential *E.coli* gene *fabI* has been assessed using the concept of an MTL<sub>50</sub> value (Figure 3.37). An MTL<sub>50</sub> of 5 relative value or 500% of normal growth was determined for *fabI*, suggesting a large number of mRNA transcripts are required to maintain cell viability, and thus provides a measure of how stringency required the gene is.

An alternative measure to MTL<sub>50</sub> values for determining gene stringency can be obtained by substituting relative mRNA abundance for protein molecules per cell. Relative growth rate can then be expressed as a function of protein molecules per cell to create a Minimum Protein Level (MPL<sub>50</sub>), whereby the number of protein molecules required for maintaining cell viability at 50% growth can be estimated. Application of this measure to *fabI* reveals a MPL<sub>50</sub> value of approximately  $7.5 \times 10^2$  molecules per cell (Figure 3.38).



**Figure 3.37 Determining  $MTL_{50}$  value for the essential gene *fabI*.**

Average relative mRNA was plotted against corresponding average relative growth rate to generate the  $MTL_{50}$ . This enables an estimation of the amount of mRNA required at the point where growth is reduced by 50% ( $y=0.5$ ). This value represents the  $MTL_{50}$  which serves as a measure of gene stringency. Due to the increase in relative mRNA abundance at approximately 50% and 75% of normal growth rate, no linear relationship could be established; hence  $MTL_{50}$  could not be estimated in this case.



**Figure 3.38 Determining MPL<sub>50</sub> value for the essential gene *fabI*.**

Average protein molecules per cell were plotted against corresponding average relative growth rate to generate the MPL<sub>50</sub>. The resulting curve enables an estimation of the protein required at the point where growth is reduced by 50% (y=0.5). This value represents the MPL<sub>50</sub> which provides an alternative measure of essential gene stringency.

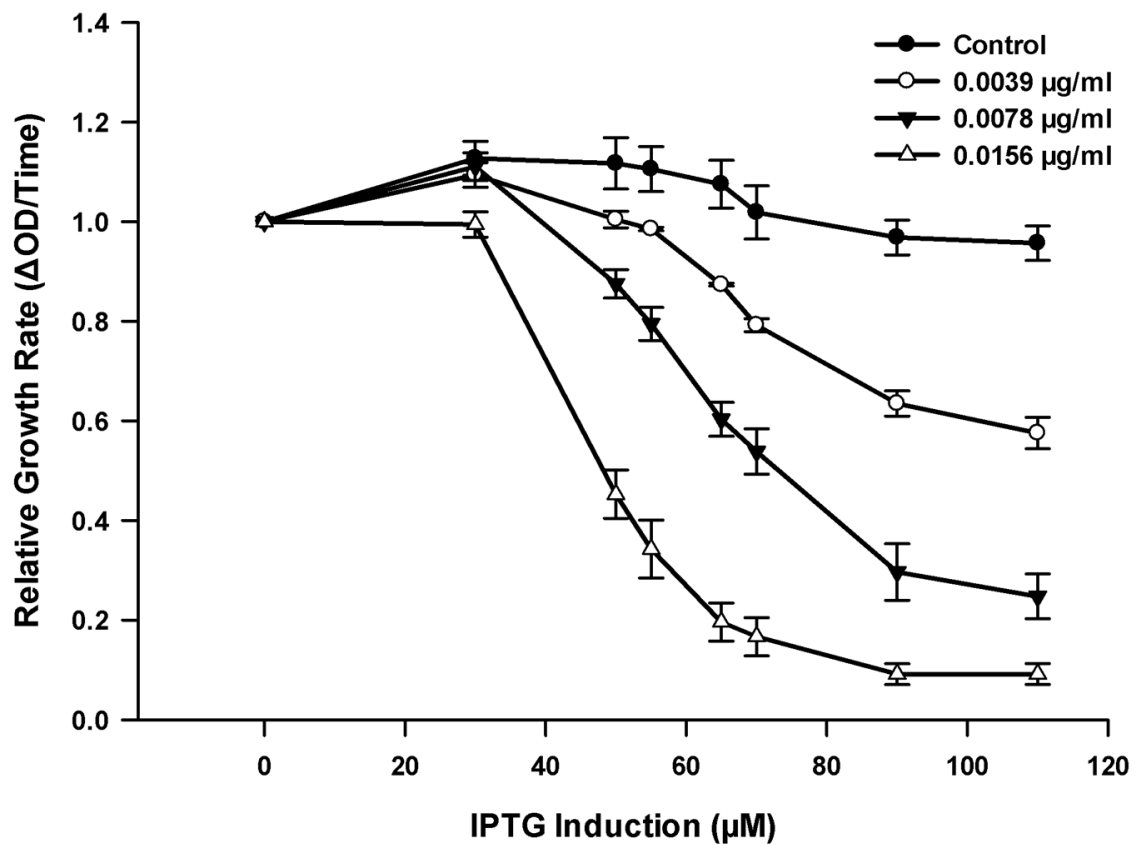
### **3.3.4 Antisense RNA based whole-cell screening assays using triclosan and phosphomycin**

In addition to identifying essential gene targets,  $MPL_{50}$  values could be utilised for the optimisation of antisense RNA based whole cell screening assays. Appropriate levels of IPTG induction for antisense RNA expression required to achieve a specific level of sensitivity could therefore be defined. This is considered critical requirement for assays when screening for low abundance natural products (Baltz, 2008).

As the initial phase of this experiment, the level of antisense RNA expression required to achieve a decrease in the MIC of target specific antibiotics was investigated. Having found a level of IPTG induction required to decrease the MIC for example 2-fold, cellular protein would then be quantified using the quantitative LC-MS/MS protocol incorporating the FabI protein internal standard.

The MIC of strains sensitized for FabI (Figure 3.39) and MurA (Figure 3.40) were determined by liquid growth assay (see methods section: 2.6.10). The FabI inhibitor triclosan and MurA inhibitor phosphomycin were used since both compounds directly inhibit their respective encoded targets. Sub-inhibitory concentrations of each antibiotic were used based on previously determined MIC's for the susceptibility of wild-type *E.coli* to triclosan (Bailey *et al.*, 2009) and phosphomycin (Takahata *et al.*, 2010).

Expressed RNA silencing of *fabI*, resulted in a significant difference between relative growth rates at each triclosan concentration (Kruskal-Wallis,  $H= 13.705$ , 3 df,  $P<0.05$ ). At the highest level of IPTG induction (110  $\mu$ M) an approximate 7 fold increase in triclosan sensitivity was observed. In the case of *murA* silencing, no significant difference was observed between relative growth rates at selected phosphomycin concentrations (Kruskal-Wallis,  $H= 0.628$ , 3 df,  $P>0.05$ ).



**Figure 3.39** Triclosan sensitivity in *E.coli* following expressed antisense RNA silencing of *fabI*.

IPTG was used to induce antisense RNA expression in order to sensitize *E.coli* As-*fabI* to sub-inhibitory concentrations of triclosan at 0.0339, 0.0078 and 0.0156 μg/ml. This was intended demonstrate how MIC changes with increased gene silencing. Graph shows average relative growth rates with standard error bars (n=3).

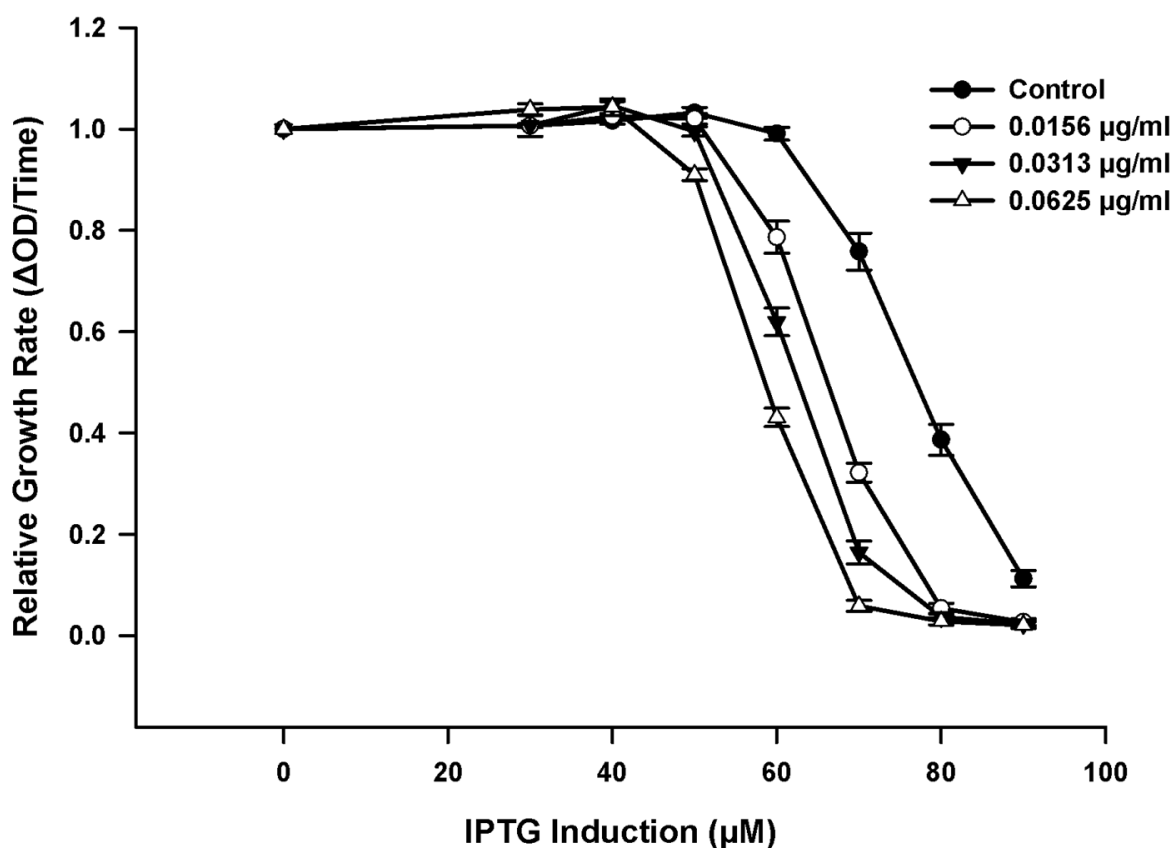


Figure 3.40. Phosphomycin sensitivity in *E.coli* following expressed antisense RNA silencing of *murA*.

IPTG was used to induce antisense RNA expression in order to sensitize *E.coli* *As-murA* to sub-inhibitory concentrations of phosphomycin at 0.0156, 0.0313 and 0.0625 μg/ml. As with the assay for triclosan, this was intended demonstrate how MIC changes with increased gene silencing. Graph shows average relative growth rates with standard error bars (n=3).

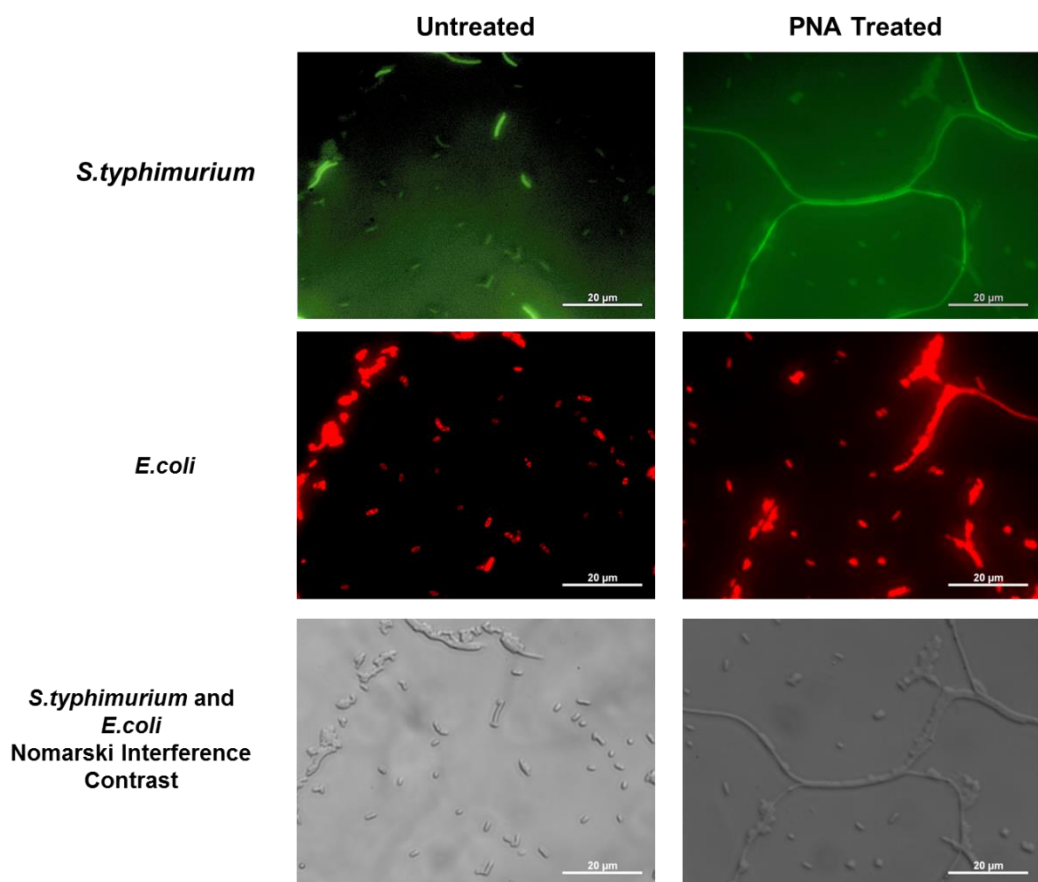
### 3.4 Species-specificity of peptide-PNA conjugates

#### 3.4.1 Evaluation of PNA to discriminate between bacterial species in a mixed culture

The capacity of peptide PNA conjugates to induce gene silencing has been assessed directly with expressed antisense RNA from plasmids and found to be highly comparable (Goh *et al.*, 2009). In addition to defining gene stringency, the inherent stability of PNA's *in vivo* has facilitated research into their development as species-specific antimicrobials, in an effort to address antibiotic resistance (Good and Stach, 2011). The use of MPL<sub>50</sub> values could therefore be applied to in the context of PNA's to ascertain sufficient dose concentrations required to achieve bactericidal activity. Indeed bactericidal activity has previously been demonstrated against individual gene targets in *E.coli* (Good *et al.*, 2001b). To date however, the capacity for PNA's to promote bactericidal activity in an individual species within a mixed culture has not been evaluated. To examine the discriminatory potential of PNA's further, a bioinformatics approach (see methods section: 2.7.1), was employed to identify the +5 to -5 translation initiation region (TIR) of essential genes conserved between *S.typhimurium* and *E.coli*. A total of 93 orthologous genes were identified, 47 of which possessed TIR's with >2 base-pair mismatches necessary for effective silencing (Dryselius *et al.*, 2006a). The essential gene *ftsZ* was selected for further investigation. In the majority of prokaryotes *ftsZ* encodes an FTPase that functions as a cytoskeletal element, analogous to the mammalian homologue tubulin in the regulation of cytokinesis (Bi and Lutkenhaus, 1991; De Boer *et al.*, 1992). Following chromosome replication, FtsZ self-assembles into protofilaments that form a Z-ring structure via isodesmic mechanisms. Once assembled the Z-ring localizes to the interior of the cytoplasmic membrane at the midcell, where it is stabilised by FtsA and ZipA and recruits other cell division proteins in the formation of a division septum (Margolin, 2005).

Mutations that reduce the expression of *ftsZ* cause a characteristic change in cell morphology, whereby highly elongated cells are formed (Bi and Lutkenhaus, 1992). Therefore PNA inhibition of *ftsZ* would enable phenotypic differentiation between *S.typhimurium*, which would be expected to form elongated cells compared to *E.coli*, where normal cell size would be maintained thereby allowing microscopic observation of PNA specificity within a mixed

culture. To provide contrast, *E.coli* AC01 and *S.typhimurium* AC02 were transfected with L-arabinose inducible plasmids, expressing DS-red and Green Fluorescent Protein (GFP) respectively. Mixed cultures were prepared, induced with L-arabinose and treated with 1.25  $\mu$ M of Peptide-PNA Se0002. Samples were taken after 6 hours incubation and viewed using fluorescent microscopy (Figure 3.41). The phenotype of *E.coli* AC01 treated with Se0002 was consistent with untreated controls, indicating the absence of gene silencing. In contrast *S.typhimurium* AC02 displayed cell elongation, indicating a failure in cellular division associated with the inhibition of *ftsZ*. These results demonstrate the capacity for PNA's to discriminate between species in a mixed culture.



**Figure 3.41 Effects of species-specific PNA essential gene *ftsZ* on *E.coli* and *S.typhimurium*.**

Mixed cultures of *E.coli* AC01 expressing DS-Red and *S.typhimurium* AC02 expressing GFP were treated with 1.25  $\mu$ M Peptide PNA Se0002 targeting *ftsZ* and observed with fluorescent microscopy after 6 hours incubation. A filamentous phenotype associated with *ftsZ* silencing was observed for *S.typhimurium* AC02, but not *E.coli* AC01.



## Chapter 4 Discussion

### 4.1 Introduction

The threat of increasing antibiotic resistance has created an urgent requirement for the development of targeted screening strategies for identifying novel antibiotics. Critical for such assays is the identification of suitable molecular targets that sustain cellular growth. Essential genes have previously been identified as potential candidates using various genetic strategies. However, only recently has a concerted effort been made to ascertain the minimally required amount of gene expression necessary to sustain bacterial growth. A differential growth requirement (stringency) has been observed among essential genes. To date stringency has been assessed using mRNA abundance to represent expressed protein, yet issues have been raised concerning the large variation in mRNA-protein correlations for many genes. The lack of correlation between mRNA and protein is attributed to distinct post-transcriptional and post translational processing mechanisms, but also gene stochasticity. This study aimed to evaluate the stringency of essential gene targets in *E.coli*; firstly by examining operon based targets *fusA* and *rplE* and secondly; developing an LC-MS/MS strategy for measuring stringency at the protein level, using the essential gene *fabI* to validate the approach.

### 4.2 Specificity of antisense constructs

The concept of antisense RNA based regulation in prokaryotes has been adapted to provide practical tools for studying gene expression (Rasmussen *et al.*, 2007). Typical strategies involve the plasmid based expression of natural RNA transcripts within cells. Gene silencing occurs through a *trans*-mechanism, whereby expressed antisense RNA hybridizes to cognate mRNA, inducing transcript degradation and steric hindrance, and subsequently reducing the amount of translated protein. Using this strategy two essential gene targets in *E.coli*; *fusA* encoding elongation factor G (EF-G), a translational GTPase involved in protein synthesis (Rodnina *et al.*, 1997) and *rplE* encoding the 5S rRNA ribosome accessory protein L5 (Korepanov *et al.*, 2007), in addition to previous gene targets *fabI*, *murA*, *ftsZ* and *acpP*, have been evaluated. Growth assays revealed that expressed antisense RNA silencing of both essential gene targets *fusA* and *rplE*, causes a significant decline in cell growth rate. Notably a

reduction in growth rate was achieved at lower levels of IPTG induction, in comparison to previously assessed essential genes (Goh *et al.*, 2009).

Experimental conditions may have exerted significant influence in this study since the specificity of *fusA* and *rpIE* was examined using MH media compared to LB media used in the assessment of *fabI*, *murA*, *ftsZ* and *acpP* (Goh *et al.*, 2009). In LB media carbon sources are limited and promote a change in the physiological state of *E.coli*, which switches metabolism to utilize amino acids causing a diauxic growth profile (Sezonov *et al.*, 2007). In comparison, the use of MH media may support a higher rate of growth and subsequent antisense expression, resulting in higher silencing efficiency. A comparison between *fabI*, *murA*, *ftsZ* and *acpP* grown in MH and LB media however, revealed similar growth profiles over the same induction range of IPTG. An additional source of variability may arise from exogenous small RNA's present in complex growth media, that arise from either the environment or as a by-product of RNA biogenesis from cells grown in the media. It has been determined that the presence of such exogenous sRNA's exerts a significant effect on protein profiles (Pavankumar *et al.*, 2012). Whether the presence of such entities would significantly enhance or suppress the effects of expressed antisense silencing is unknown. To validate any potential effects of exogenous sRNA's would require growth profile comparisons of each strain using media pre-treated with RNase to prevent extraneous interference, ideally supported by transcript analyses.

With regards to the difference in growth rate to previously examined essential gene targets, It is more plausible that the low level of IPTG induction required for growth inhibition of *fusA* and *rpIE* compared to *acpP*, *ftsZ*, *fabI* or *murA*, can be ascribed to antisense sequence design. Silencing efficiency is dependent upon mRNA turnover, efficiency of translation initiation and accessibility of the paired termini-antisense sequence to mRNA (Nakashima and Tamura, 2009). Such factors could be assessed using an array of bioinformatics frameworks that incorporate structural fluctuation and the transition state mRNA undergoes according to environmental conditions to predict silencing efficiency (Johnson and Srivastava, 2013). Critically a BLAST alignment of the *fusA* antisense sequence reveals an alignment with the 3' translated region of *rpsG*, which encodes the 30S ribosomal subunit protein S7. Similarly the *rpIE* antisense sequence is complementary to the 3' translated region of the 50S ribosomal

subunit protein L24. Consequently off target effects may have occurred, resulting in reduced transcription of *rpsG* and *rplX*.

Despite the significant growth inhibition which indicated a high degree of specificity for *fusA* and *rpIE* paired constructs, speculation regarding gene stringency should be avoided in the absence of transcript or protein data.

#### **4.3 Validation of *fusA* and *rpIE* expressed antisense RNA specificity**

Transcomplementation strategies are an established method to validate target specificity and mechanisms of action in gene silencing studies, since the expression of a complete ORF accounts for all transcript variations (Goh *et al.*, 2009). Although the specificity of *rpIE* could be validated by transcomplementation, cell growth in *fusA* silenced *E.coli* could not be rescued. Currently no published data exists regarding the transcomplementation of either *rpIE* of *fusA* in *E.coli*, following expressed antisense silencing. However transcomplementation has been used successfully to validate the specificity of expressed antisense RNA constructs for *acpP*, *ftsZ*, *fabI* and *murA* (Goh *et al.*, 2009).

The overexpression of *fusA* appeared to contribute towards further growth reduction with increasing concentrations of L-arabinose. Furthermore, growth curves exhibited an extended lag phase in addition to a final cell density that was considerably lower compared to *rpIE*. The absence of rescue may reflect a number of phenomena associated with gratuitous protein expression. The overexpression of certain genes is known to be toxic and detrimentally affects the growth rate of *E.coli*, due to a decrease in protein synthesis and accumulation of heat shock proteins (Dong *et al.*, 1995). In addition the perturbation of normal cellular protein concentrations can disrupt the homeostasis of protein-protein interactions, thereby altering the rate of protein degradation and post-translational modification. Consequently protein half-life can be extended culminating in an accumulation of extraneous protein, which localizes at critical intracellular sites and disturbs normal cell functions (Koller *et al.*, 2000). Excessive protein expression can induce an internal starvation response, resulting in a depletion of nutrients which through an unknown

mechanism, initiates the destruction of rRNA and ribosomes, thereby reducing cellular capacity for protein synthesis (Kurland and Dong, 1996). The breakdown of rRNA also increases competition between the mRNA species of clones and native proteins for functional ribosomes, contributing towards further growth reduction (Dong *et al.*, 1995). Although not examined further, microscopic examination of *E.coli* under these conditions would confirm if protein aggregation was occurring during transcomplementation of *fusA*. This would suggest if production was excessive and prompt the redesign of transcomplementation plasmid, possibly to incorporate a weaker promoter to reduce expression.

Alternatively the reduced growth rate could be due to the metabolic burden associated with the simultaneous expression of plasmids with different selection markers. This state arises as a consequence of excess requirements for cell resources (energy and biosynthetic precursors), associated with the transcription and translation of encoded genes (Glick, 1995). Previous studies have reported growth defects arising from the expression of two strong promoter gene cassettes from multicopy plasmids that increase the metabolic burden on cells promoting plasmid loss (Anthony *et al.*, 2009). Furthermore plasmid maintenance can alter the intracellular concentrations of cAMP-crP, disrupting the transcription of many host regulons (Diaz Ricci and Hernández, 2000). However *rpIE* did not reveal a similar trend using the same plasmids and selection marker genes, which suggests that *fusA* itself, may have been contributory factor in the failure to rescue cell growth.

Although the *fusA* encoded protein functions as a translational GTPase in protein synthesis, a secondary role has been suggested as a molecular chaperone in facilitating the stabilization and folding of denatured proteins (Caldas *et al.*, 2000). This purported role has been supported by increases amounts of FusA in response to exogenous stressors in *E.coli* (Han *et al.*, 2008). Consequently, the excess of FusA synthesized following induction may exceed a critical level required for protein synthesis. Excess FusA may therefore have been available to function inappropriately as a chaperone and bind non-specifically with cytosol proteins to detrimental effect. This could be evaluated by examining cell lysate via SDS-PAGE or by using western blotting to detect increased levels of FusA.

The inability to validate antisense function by transcomplementation in the case of *fusA* may reflect a disruption in protein homeostasis, by altering protein-protein interactions and stoichiometry. Such changes may promote the misfolding and aggregation of numerous cellular proteins and alteration in protein turnover. Where excessive synthesis occurs, chaperone proteins and proteases that constitute the intracellular quality control system can no longer compensate (Mogk *et al.*, 2011). Alternatively *fusA* expression for cell viability may be dependant upon a fine equilibrium between mRNA transcripts and translated protein. This could be attributed to the IPTG and L-arabinose based expression systems, which may lack the capacity to provide sufficiently refined induction ranges to maintain optimal expression. Indeed cross talk has been observed between  $P_{Bad}$  and  $P_{Lac}$  promoters through an undefined mechanism, which was found to be alleviated through directed evolution of the arabinose transcriptional activator AraC (Sung *et al.*, 2007). Incidentally the detrimental effects on cellular growth observed from transcomplementation would suggest that *fusA* may be a stringently required target. Due to the discrepancies observed with transcomplementation however, in particular with *fusA*, this technique may be limited to the validation of expressed antisense specificity of individually transcribed genes.

#### **4.4 Growth inhibitory effects of silencing *rpIE* and *fusA* in *E.coli***

Essential gene targets that display bactericidal activity upon inhibition are desirable due to the reduced prospects of resistance developing and increased therapeutic efficacy (Stratton, 2003). Therefore characterizing the activity of molecular targets is a pre-requisite for the selection of suitable targets as part of antibiotic screening assay development.

The inhibitory effects of silencing both *fusA* and *rpIE* in this study were determined to be bacteriostatic, since viable cell counts did not show a 3 log reduction in cfu/ml, representing the benchmark for bactericidal activity. The bacteriostatic nature of *fusA* is supported by the documented activity of known inhibitors such as fusidic acid in sensitive *E.coli* strains (Harvey *et al.*, 1966). To date there is no known antibiotic that specifically targets *rpIE*, however, inhibition following the silencing of ribosomal genes *rpIJ* and *rpsL*, (encoding 50S ribosomal protein L10 and 30S ribosomal subunit protein S12 respectively) in *M. smegmatis*, have been characterized as bacteriostatic (Kaur *et al.*, 2009).

Further support is evident in the mechanism of macrolide antibiotics, which are characteristically bacteriostatic and have a speculated capacity to interact with the L5 subunit (Schlünzen *et al.*, 2003). Bacteriostatic inhibition arising from targeting of the ribosome has been linked to the inhibition of the *in vitro* folding capacity of the 23S rRNA, preventing the peptidyl transferase reaction rather than ribosome destruction (Chattopadhyay *et al.*, 1996). This is especially prevalent with antibiotics that target the 50S subunit exclusively (Chattopadhyay *et al.*, 1999), where the L5 ribosome protein encoded by *rplE* is assembled.

Despite the specificity and rapid decline in growth rate observed following silencing of *fusA* and *rplE*, the experimental findings characterize both genes as bacteriostatic targets. Whether the use of expressed antisense provides an accurate characterization of target inhibition is unclear, since inhibition of the same gene targets by different entities can cause variable effects. In the case of antibiotics, chloramphenicol is bacteriostatic in *E.coli*, but bactericidal in *H.influenza*. Furthermore, the inhibitory effects of PNA's on gene targets are bactericidal, despite bacteriostatic inhibition observed with target specific antibiotics.

The bactericidal activity observed with ribosome targeting has been attributed to the mistranslation and misfolding of membrane proteins which initiates a stress response. Antibiotic induced conformational changes in the ribosome, causes tRNA mismatching resulting in mistranslated proteins, which are bound to chaperone proteins and directed towards the membrane. At this stage defective proteins undergo translocation across the inner membrane which activates the envelope two-component stress response sensor CpxA. CpxA phosphorylates CpxR which in turn activates the stress response proteins including the redox responsive two-component transcription factor ArcA which upregulates both metabolic and respiratory systems leading to oxidative stress (Kohanski *et al.*, 2008). In contrast antisense mediated inhibition reduces target mRNA abundance through decay or steric hindrance, neither of which initiates a secondary pathway capable of initiating oxidative stress. Consequently the use of antisense silencing may not be predictive of the mode of action utilised by target specific inhibitors. Although the silencing of one essential gene may be insufficient to generate bactericidal activity, the silencing of two essential genes simultaneously may prove synergistic. Dual silencing of the *ackA-pta* operon in

*E.coli*, demonstrated increased silencing compared to individual expression (Nakashima and Tamura, 2009). Therefore essential genes within the same operon may be assessed in tandem to determine if bactericidal activity is evident.

#### 4.5 Evaluation of stringency in essential genes *rpIE* and *fusA*

Evaluating the stringency of essential genes supports gene characterization, network analyses and the identification of suitable molecular targets for antibiotic screening assays. This study has evaluated the stringency of essential genes *rpIE* and *fusA* in *E.coli*, using expressed antisense RNA silencing to titrate down the growth rate and measure the associated transcript abundance. Gene silencing of both *rpIE* and *fusA* although causing a significant decline in growth rate, failed to cause a significant decrease in mRNA abundance. In *E.coli* expressing antisense *rpIE*, the transcript abundance initially increased upon induction and remained stable as relative growth rate decreased. Silencing of *fusA* revealed an initial decrease in mRNA abundance, prior to a large increase in mRNA at the lowest growth rate. The standard error for relative growth rate showed significant variation, especially for two data points in *fusA*. This may reflect variation in cDNA synthesis despite batch reactions being performed. These results contradict previous determinations of essential gene stringency for *fabI*, *murA*, *acpP* and *ftsZ*, where mRNA abundance decreased in parallel with growth rate (Goh *et al.*, 2009).

For direct comparisons of stringency with previously validated essential targets, MTL<sub>50</sub> values were calculated. An inverse trend for both *fusA* and *rpIE* was observed, whereby expressed antisense silencing causes an increase in transcription, resulting in higher target mRNA abundance. The transcript abundance required to maintain a 50% growth rate was higher than the level for normal growth rate. This finding initially suggests that *rpIE* and *fusA* are not stringently required for cell viability. However, the apparent increase and stability of mRNA abundance could be ascribed to partial degradation of mRNA, resulting in non-functional transcripts that were still viable templates for amplification during QPCR. If true, then a reduction in growth rate would be expected, while mRNA abundance remains high. The location of amplified regions used by primers in QPCR would likely play a significant role. If amplified regions were located at the 5' end of the mRNA transcript, then the possibility of viable template persisting is likely, since enzyme mediated destruction of antisense bound transcripts occurs primarily from the 3' end (Condon, 2007).

Unlike *fusA* and *rpIE*, previously assessed essential genes were individually transcribed (Goh *et al.*, 2009). In contrast both *rpIE* and *fusA* are located within



ribosomal operons, which are subject to an autogenous feedback mechanism from genes encoding ribosomal subunit proteins (Yates *et al.*, 1980). The *spc* operon encodes *rpIE* in addition to nine other genes encoding sequential subunits; *rpIN* (L14), *rpIX* (L24), *rpIE* (L5), *rpsN* (S14), *rpsH* (S8), *rpIF* (L6), *rpIR* (L18), *rpsE* (S5), *rpmD* (L30), and *rpIO* (L15), (Cerretti *et al.*, 1983). Within this operon the ribosomal protein S8, functions as a translational repressor (Dean *et al.*, 1981a), through binding in proximity to the translation initiation region of *rpIE*, inhibiting its translation and that of distal proteins. Additionally S8 also governs the expression of proximal proteins (L14 and L24) via a retro-regulation mechanism, involving exonuclease degradation following translation repression of L5 (Mattheakis *et al.*, 1989). These regulatory mechanism may account for the discoordinate expression of mRNA, which has been observed following mutations in the start codon region of *rpIE*, which reduce the synthesis of distal proteins 20-fold, yet exhibit little effect on mRNA synthesis. In addition overproduction of the S8 repressor causes a 3-fold reduction in protein synthesis, but not mRNA synthesis (Mattheakis and Nomura, 1988). A separate ribosomal operon *str* encodes both ribosomal protein subunits; *rpsL* (S12), *rpsG* (S7), elongation factors *fusA* (EF-G) and *tufA* (EF-Tu). Within this operon, S7 acts as a translational repressor and regulates the synthesis of EF-G and EF-Tu by translational coupling (Dean *et al.*, 1981b), and S12 by retro regulation (Saito and Nomura, 1994).

Unlike individual genes, the transcription of polycistronic mRNA and subsequent translation is discoordinated (Dryselius *et al.*, 2006a). Evidence For this phenomena is derived from a study of the *lac* operon in *E.coli* using targeted PNA's to the structural genes *lacZ*, *lacY* and *lacA*, which revealed a directed inhibitory effect. PNA mediated inhibition of *lacZ* reduces expression of downstream *lacY* and *lacA*, yet inhibition of *lacY* does not affect the expression of *lacZ*, only *lacA* (Dryselius *et al.*, 2006a). A further discrepancy was observed with the number of *lacZ* transcripts that were 3-4 fold higher, due to cleavage by RNaseE within an intergenic region between *lacZ* and *lacY*, resulting in increased stability of *lacZ* transcripts and decay of *lacA* (Li and Altman, 2004).

The lack of decline in mRNA abundance observed following gene silencing of *fusA* and *rpIE*, may reflect a disturbance in normal feedback regulation within each operon due to translational coupling of distal genes. For *rpIE* in particular,

the regulation of r-protein expression is founded on the interaction between rRNA and mRNA, due to structural features that recognize homologous sequences. A single subunit can therefore coordinately regulate the synthesis of other subunits via translational feedback (Nomura *et al.*, 1980). In the context of these results a reduction in *rpIE* encoded L5 protein may alter the stoichiometry of ribosomal subunits, consequently reducing repressor mediated effects of the S8 regulatory protein. Parallel examination of both mRNA and protein abundance would aid in elucidating these effects. Indeed disproportionate reductions of mRNA and protein have been observed for genes within operons (Dryselius *et al.*, 2006a).

The lack of consistent mRNA decline with increased antisense RNA silencing may also reflect the inherent heterogeneous nature of isogenic (genetically identical) cell populations. Within these populations, sub-populations exist that exhibit variations in physiologically state, manifesting as in growth rate, cell age, metabolic state or stage in the cell cycle. These variations arise due to intracellular differences in resources and cell machinery, which are amplified by cellular cascade mechanisms (Lidstrom and Konopka, 2010). These differences underlie the phenomena of gene stochasticity (noise), whereby the expression of genes occurs at irregular intervals and in short burst of transcription and translation to generate variable numbers of proteins (McAdams and Arkin, 1997). Early studies of gene stochasticity identifying key causes, which were attributed to Intrinsic noise; associated with the expression of a gene sequence, and extrinsic noise arising from the interactions of other cellular components (Elowitz *et al.*, 2002). From an evolutionary perspective, these stochastic events have been speculated to function as a survival strategy, that confers a selective advantage, by priming regulatory pathways for the onset of sudden environmental changes (Fraser and Kærn, 2009). Subsequent studies into gene stochasticity have lead to the development of fluorescent based techniques that permit the measurement of mRNA in individual bacterial cells (Golding *et al.*, 2005). Such techniques represent an alternative to QPCR and provide a more direct and visible means of quantifying transcript abundance.

From an experimental perspective, the abnormally high MTL<sub>50</sub> values could also reflect the narrow range of IPTG used to induce silencing. For *fusA* and *rpIE*, this ranged from 5 – 25  $\mu$ M in comparison to wider range of inducer

concentrations for *fabI*, *murA*, *ftsZ*, *acpP* (Goh *et al.*, 2009). In retrospect, both *rplE* and *fusA* represent poor targets and highlight the limitations of assessing gene stringency based upon mRNA abundance, especially for gene targets encoded within operons with regulatory feedback mechanisms.

Recently a method for evaluating operon genes in isolation was developed to address such discoordinate expression. A dynamic transcriptional control on/off switch was constructed using the Lambda repressor, which was inserted into the chromosomal position of the *lac* repressor. A plasmid expressing *fusA* under control of the lambda promoter was then used for constitutive expression. The addition of IPTG, subsequently enabled gene expression to be switched on and off (Min *et al.*, 2012). In summary the determined MTL<sub>50</sub> values for *fusA* and *rplE* suggest neither gene is stringently required for cell viability, based on the present definition.

These results emphasize the complexities of evaluating operon based essential genes due to translation coupling and regulatory feedback mechanisms. Consequently the MTL<sub>50</sub> value may be limited as a measure of gene stringency and supports the case for evaluating essential genes at the level of protein expression.

#### 4.6 Evaluation of gene stringency in essential gene *fabI* by proteomics

Previous measures of essential gene stringency have assumed that relative mRNA transcripts are directly related to the amount of translated protein. However a lack of correlation between mRNA and protein due to gene organization and regulatory mechanisms is well documented. Consequently the direct measurement of expressed protein provides the most appropriate determination of cell physiology and therefore gene stringency. This study aimed to address the limitations of the MTL<sub>50</sub> value used for measuring essential gene stringency. An LC-MS/MS assay was devised to quantify the abundance of *fabI* protein in *E.coli* in parallel with mRNA abundance, following expressed antisense RNA silencing.

The LC-MS/MS assay revealed a significant decrease in the abundance of FabI following gene silencing; however no correlation with mRNA abundance was observed. Indeed relative mRNA abundance increased when the relative growth rate reached 50% of normal growth. Although transcriptome and proteome analyses often reveal divergent profiles (Jayapal *et al.*, 2008), the silencing of *fabI* in a previous study did show an overall decline in mRNA abundance with IPTG induction ranging from 20 to 80  $\mu$ M. At induction concentrations above 80  $\mu$ M however, mRNA abundance was seen to increase marginally (Goh *et al.*, 2009). The lack of definitive decrease in mRNA abundance observed in this study may reflect the narrow range of IPTG concentrations used to induce antisense expression (50 – 65  $\mu$ M). Over this range, decreases in mRNA abundance may be obscured by inherent noise associated with stochastic gene expression (Raj and van Oudenaarden, 2008). However the observed growth reduction suggests expressed antisense is functioning. As in the case of *rpIE* and *fusA*, those transcripts detected in QPCR analyses may be non functional, yet provide viable templates for amplification, generating mRNA profiles.

The disproportionate correlation between mRNA and protein abundance may also reflect the dynamic relationship between their respective rates of synthesis and degradation (Vogel and Marcotte, 2012). The synthesis of mRNA is dictated by factors that effect recruitment of RNA polymerase and associated transcription factors to the transcription start site, such as chromosome structure and promoter strength (Balleza *et al.*, 2009). The stability of mRNA

transcripts is governed by RNaseE and the length of mRNA. Although mRNA turnover contributes to mRNA-protein correlation, the greatest influence is attributed to post-transcriptional and post-translational mechanisms that regulate protein turnover (Maier *et al.*, 2009). The secondary structure of transcribed mRNA, strength of the Shine Dalgarno sequence, codon bias and ribosome density all contribute to translational efficiency (Maier *et al.*, 2009). Following translation mRNA-protein ratios are influenced further by protein half-life, which is dictated by protein stability and post-translational processes (Maier *et al.*, 2009). Consequently examining mRNA and protein turnover may provide a more comprehensive assessment of gene stringency. Attempts at quantifying mRNA and protein turnover have been attempted in the minimal genome bacterium *Mycoplasma pneumonia*, where protein abundance ranged over three orders of magnitude (Maier *et al.*, 2011). Furthermore the ratio of mRNA:protein varied during the growth phase and was highly uncorrelated for genes existing within operons. Notably, a higher correlation was observed if operons were short and genes were not situated at the 3' end (Maier *et al.*, 2011). However a global analysis of mRNA decay and abundance of 4288 predicted mRNA's in *E.coli*, suggested that generalizations concerning the decay of transcripts was limited in applicability. This conclusion arose from the finding of an inverse relationship between transcript abundance and stability. A similar inverse trend was found following analysis in *M.tuberculosis*, where a global stabilization of transcripts was observed in response to cellular stress (Rustad *et al.*, 2013).

An alternative to the use of QPCR would be to employ RNA sequencing to quantitate expression of silenced genes. The approach utilizes deep sequencing technologies based on the conversion of mRNA to cDNA which is ligated to adapters and sequenced. RNA sequencing displays a high level of sensitivity and is not susceptible to background interference resulting in strong signal:noise ratios. Furthermore, unlike QPCR RNA sequencing does not require normalization of data sets (Wang *et al.*, 2009). The quantitative capacity of RNA sequencing has been demonstrated, and shows strong correlation with both QPCR (Nagalakshmi *et al.*, 2008) and label-free LC-MS/MS methods (Fu *et al.*, 2009).

A comparison of FabI molecules per cell between *E.coli* As-*fabI*, AG1 ME5305 and a wild-type control revealed a significant difference in protein abundance. The number of FabI molecules per cell was higher in the antisense control, which may be evidence of high basal promoter (*P<sub>trc</sub>*) activity associated with the paired termini vector in the absence of IPTG (Nakashima and Tamura, 2009). To date limited published data exists regarding the protein abundance of *fabI* quantified using LC-MS/MS following antisense expressed silencing. In *E.coli* a value 12,500 FabI copies per cell has been determined (Ishihama *et al.*, 2008). A basal expression level of 12,000 copies per cell has also been determined in *S.aureus* (Slater-Radosti *et al.*, 2001). These estimations are approximately 5-fold higher than this study's observations in the *E.coli* As-*fabI* control (2561 FabI molecules per cell) and approximately 17 fold higher than the basal level observed in wild-type *E.coli* (718 FabI copies per cell). An accurate comparison is limited however, since abundance measurements in *E.coli* was determined using a linear ion trap rather than an orbitrap mass spectrometer, resulting in faster scan cycles and increased *m/z* range. The use of additional protein fractionation steps to increase identification coverage, in addition to the use of an emPAI approach for quantitative analysis (Ishihama *et al.*, 2008), would also account for the variation observed with this study. With regards to *S.aureus*, genetic differences such as the presence of homologues and the semi-quantification of protein abundance using western blotting, also limit a direct comparison (Slater-Radosti *et al.*, 2001).

The quantification of the <sup>15</sup>N FabI internal standard revealed a significant difference in the fmol amount after extrapolation from independent standard curves generated for EGAELAFTYQNDK and ILVTGVASK. Experimental error is a feasible explanation due to the low volumes of standard spiked into pre-trypsin digests. However, steps were introduced to limit potential inaccuracies, such as the preparation of serial dilutions at fixed ratio rather than fixed interval (Han and Higgs, 2008). Further variation in the quantitative analysis may be accounted for by the effects of <sup>15</sup>N incorporation. Absolute quantification using a <sup>15</sup>N labelled internal standard is reliant upon the isotope dilution principle. This concept is based on the assumption that physiochemical properties between light and heavy labelled peptides are equivalent. The concept also assumes that the effects of isotope incorporation on protein structure and function are

negligible. This notion has been disputed following a recent study of  $^{15}\text{N}$  labelling on protein expression in *E.coli*. where incorporation of  $^{15}\text{N}$  was found to effect the expression of over 40 cytoplasmic proteins. Critically the proteins affected included key metabolic enzymes, which accounted for significantly reduced growth rates (Filiou *et al.*, 2012). The direct incorporation of a heavy isotope may result in decreased enzyme function due to conformational changes in key residues such as the active site. It is known that  $^{15}\text{N}$  can alter structural integrity of proteins, through changes in peptide backbone torsion, side-chain orientation of residues and hydrogen bonding between neighbouring peptides (Xu and Case, 2002). This phenomenon has been validated by studies of copper metallothioneins in *S.cerevisiae*, which show aberrant spectroscopic profiles, indicating the distortion of normal protein architecture with  $^{15}\text{N}$  labelling (Hartmann *et al.*, 2003).

The incorporation effects of  $^{15}\text{N}$  may therefore influence the quantification process in particular the tryptic digestion step, as trypsin has been found to cleave light proteins at a faster rate compared to heavy labelled proteins (Konopka *et al.*, 2012). False L/H ratios could therefore be generated compromising accurate quantification of protein abundance. Despite this risk, the effects of  $^{15}\text{N}$  incorporation have been limited to physiochemical properties of peptides, such as chromatographic retention time and found to exert no influence on quantification accuracy (Webhofer, 2013). Nonetheless, incorporation effects on trypsin digestion should be evaluated as part of the LC-MS/MS validation. Ideally this would be incorporated as part of the internal standard validation process and would be evaluated by analysing tryptic digests of equal quantities of  $^{15}\text{N}$  labelled and unlabelled FabI protein over varying incubation times. LC-MS/MS could then be used to examine the degree of variance in the L:H ratio, which would be expected to be equal if the effects of  $^{15}\text{N}$  incorporation were negligible.

The absence of correlation between transcripts and expressed FabI in *E.coli* as demonstrated in this study, highlight the limitations of using mRNA as a direct proxy for expressed protein. The obtained results demonstrate the capability of an LC-MS/MS strategy incorporating a fully  $^{15}\text{N}$  labelled internal standard, for measuring protein concentration per cell. From the quantitative data, essential gene stringency has been established using the  $\text{MPL}_{50}$  value to define the

absolute growth requirement of the essential gene *fabI*. These findings therefore support the argument for quantifying expressed protein, which provides a more accurate reflection of essential gene stringency.



#### 4.7 Species specificity of peptide-PNA conjugates in mixed bacterial cultures

Alternative strategies for the treatment of bacterial infections have been pursued to address the limitations posed by the development of multiple antibiotic resistance mechanisms. Gene silencing using peptide-PNA conjugates demonstrates the potential to discriminate between gene sequences, since they can be designed to exploit the degeneracy of the genetic code, thereby allowing gene silencing to be used in a discriminatory capacity..

This study has examined this potential of peptide-PNA conjugates as a species-specific antimicrobial, by designing a PNA sequence incorporating a 2 base-pair mismatch, to delineate the effects of silencing of the essential gene *ftsZ* in *S.typhimurium* and *E.coli*. Treatment of mixed cultures with PNA revealed no effect on *E.coli*, however elongated cells of *S.typhimurium* were observed. This phenotype is consistent with mutations that reduce expression of *ftsZ* causing a characteristic change in cell morphology to form highly elongated cells (Bi and Lutkenhaus, 1992). Further validation is provided by similar results in previous silencing studies of *ftsZ* using peptide-PNA conjugates (Goh *et al.*, 2009). The results confirm that a peptide-PNA conjugate with sequences incorporating a 2 base-pair mismatch were sufficient to selectively bind to and inhibit translation of *ftsZ* thereby interrupting cell division causing cell elongation in *S.typhimurium* but not *E.coli*.

## Chapter 5 Conclusion and Future Work

The sustained threat of multiple antibiotic resistance has invoked interest in empirical target based screening assays, for the discovery of novel antibiotic compounds. An integral component of target based strategies is the selection of an encoded molecular target, such as essential genes which provide suitable candidates. Defining essential genes has been previously founded on systematic gene deletion studies, where cessation of cell growth in the absence of a gene defines essentiality. The aim of this study was to expand on previous findings that a differential growth requirement or stringency exists among essential genes and is reflected in both the transcriptome and proteome profiles of *E.coli*, following gene silencing

The unpredictable effects of silencing operon based genes presents a significant limitation with the antisense silencing strategy for evaluating gene stringency, as observed in this study with divergent transcript and protein profiles. Consequently, alternative approaches have been sought to provide temporal gene regulation. The development of inducible protein degradation systems represents one such example. This strategy is founded upon adaptor mediated proteolysis, which regulates protein turnover in prokaryotes. The central component is the protease complex ClpXP consisting of an ATPase (ClpP) and a peptidase (ClpX), which recognises and binds to unstructured peptide sequences at the N or C terminus of proteins that function as degradation tags (degrons) (Baker and Sauer, 2012). A known degron tag is the short peptide SsrA which is translated and added to incomplete proteins, following ribosome stalling, due to the absence of stop codons or premature transcription termination (Keiler *et al.*, 1996). Adapting this process enabled an assessment of antibiotic inhibition required to inactivate encoded gene products (termed vulnerability) (Wei *et al.*, 2011). In this study, phage homologous recombination was used to insert a sequence encoding a modified SsrA tag fused to a protecting peptide, into chromosome regions encoding individual antibiotic targets in *M.smegmatis*. A virus-specific protease targeting the protecting peptide was put under the control of a tetracycline promoter and also integrated. Expression of the viral protease removed the protecting peptide and exposed the SsrA tag to ClpXP mediated degradation of individual target proteins causing an increase in antibiotic sensitivity. This strategy also enabled

the differentiation between bactericidal and bacteriostatic antibiotic targets (Wei *et al.*, 2011).

More recently an alternative inducible protein degradation system has also been described in *E.coli*. Strains were constructed using homologous recombination to insert a proteolytic system consisting of a promoter (*lac*) driven ClpA gene for inducible degradation and constitutive promoter driven ubiquitin protease. A modified tryptophan promoter was combined with the *trpR* gene encoding tryptophan repressor and replaced the tryptophanase *tnaA* gene. The ORF for *ubi4* (target for ubiquitin protease) was then inserted upstream of the target gene. This system enabled biphasic control of target protein and also delineation between bactericidal and bacteriostatic activity (Yoshida *et al.*, 2012).

Unlike expressed antisense RNA silencing, inducible protein degradation systems are capable of maintaining basal expression levels, while avoiding directional effects associated with operon silencing, including the disruption of feedback regulation. Protein half-life can also be strictly regulated to prevent extraneous accumulation and potential toxicity. The experimental approach in this study could therefore be improved by combining inducible protein degradation with a quantitative LC-MS/MS strategy, to provide a comprehensive determination of gene stringency.

Whether using antisense RNA or protein degradation systems for studying gene stringency, consideration should be given towards the unknown consequences of silencing a single gene on the expression of other genes, either directly or through feedback regulation. Due to the lack of knowledge concerning how cellular pathways interact, a pre-requisite to determining gene stringency, would therefore be to validate the effects of silencing strategies on the global transcriptome and proteome of model organisms such as *E.coli*.

An intended aim of evaluating essential gene stringency was to facilitate the pre-screening of genes to enable the prioritization of targets for natural product screening assays and for designing species specific PNA's (Goh *et al.*, 2009). Further applications have since become apparent in the emerging research field of synthetic biology, which aims to combine investigative biology techniques with engineering concepts such as standardization, decoupling and abstraction

(Endy, 2005). This multidisciplinary approach is envisaged to facilitate the rationale design and systematic construction of a library of compatible modular devices, which can be assembled to form functional metabolic pathways and networks. This would support the elucidation of a minimal genome chassis, and ultimately the development of synthetic organisms (Purnick and Weiss, 2009).

In order to facilitate the assembly of complex cell systems, there is a requirement for a library of characterized and standardized elements to enable interchangeability between modules (Güttinger, 2013). With regards to the construction of a minimal genome, a pre-requisite would be the characterization of minimal gene sets for cell viability. Previous efforts to define minimal genes have generated inconsistent results, due to inherent limitations in the experimental techniques used. Transposon mutagenesis overestimates the number of essential genes by considering those that slow growth, but do not stop it as essential. Antisense RNA silencing is also limited to genes that can be inhibited by sufficiently expressed levels of antisense inhibitor and does account for redundant genes (Moya *et al.*, 2009). Consequently misinterpretations arise as evident from genome reductions of up to 30% which do not decrease cell viability (Fehér *et al.*, 2007), despite many deleted genes having previously been characterized as essential (Gerdes *et al.*, 2003).

Given these discrepancies, the evaluation of essential gene stringency would support characterization efforts and enable the creation of an essentiality threshold. Knowledge of stringently required genes would therefore minimise biological complexity through the removal of non-stringent genes from minimal gene sets, facilitating the construction of a minimal genome chassis and supporting the classification of modular functionality (Esvelt and Wang, 2013). Furthermore, essential gene stringency would aid the development of *in silico* modelling especially for metabolic reconstitutions (Chavali *et al.*, 2012) and genome scale engineering (Esvelt and Wang, 2013).

## References

- Aebersold, R. and Mann, M. (2003) 'Mass spectrometry-based proteomics', *Nature*, 422(6928), pp. 198-207.
- Akerley, B.J., Rubin, E.J., Camilli, A., Lampe, D.J., Robertson, H.M. and Mekalanos, J.J. (1998) 'Systematic identification of essential genes by in vitro mariner mutagenesis', *Proceedings of the National Academy of Sciences of the United States of America*, 95(15), pp. 8927-8932.
- Alekshun, M.N. and Levy, S.B. (2007) 'Molecular mechanisms of antibacterial multidrug resistance', *Cell*, 128(6), pp. 1037-50.
- Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, K.A., Davies, J. and Handelsman, J. (2010) 'Call of the wild: antibiotic resistance genes in natural environments', *Nat Rev Microbiol*, 8(4), pp. 251-9.
- Almeida, R. and Allshire, R.C. (2005) 'RNA silencing and genome regulation', *Trends in Cell Biology*, 15(5), pp. 251-258.
- Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) 'Basic local alignment search tool', *Journal of Molecular Biology*, 215(3), pp. 403-410.
- Ames, B.N. and Martin, R.G. (1964) 'BIOCHEMICAL ASPECTS OF GENETICS: THE OPERON', *Annual review of biochemistry*, 33, pp. 235-258.
- Andersson, D.I. and Hughes, D. (2010) 'Antibiotic resistance and its cost: is it possible to reverse resistance?', *Nat Rev Microbiol*, 8(4), pp. 260-71.
- Andersson, D.I. and Levin, B.R. (1999) 'The biological cost of antibiotic resistance', *Current Opinion in Microbiology*, 2(5), pp. 489-493.
- Andre, G., Even, S., Putzer, H., Burguiere, P., Croux, C., Danchin, A., Martin-Verstraete, I. and Soutourina, O. (2008) 'S-box and T-box riboswitches and antisense RNA control a sulfur metabolic operon of *Clostridium acetobutylicum*', *Nucleic Acids Res*, 36(18), pp. 5955-69.
- Andrews, J.M. (2001) 'The development of the BSAC standardized method of disc diffusion testing', *Journal of Antimicrobial Chemotherapy*, 48, pp. 29-42.
- Andrews, J.M. (2009) *BSAC Methods for Antimicrobial Susceptibility Testing*. Available at: [http://www.bsac.org.uk/\\_db/\\_documents/Version\\_8\\_-\\_January\\_2009.pdf](http://www.bsac.org.uk/_db/_documents/Version_8_-_January_2009.pdf) (Accessed: 17.06.2009).

- Anthony, J.R., Anthony, L.C., Nowroozi, F., Kwon, G., Newman, J.D. and Keasling, J.D. (2009) 'Optimization of the mevalonate-based isoprenoid biosynthetic pathway in *Escherichia coli* for production of the anti-malarial drug precursor amorpha-4,11-diene', *Metabolic Engineering*, 11(1), pp. 13-19.
- Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Tomita, M., Wanner, B.L. and Mori, H. (2006) 'Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection', *Mol Syst Biol*, 2, p. 2006 0008.
- Badarinarayana, V., Estep, P.W., Shendure, J., Edwards, J., Tavazoie, S., Lam, F. and Church, G.M. (2001) 'Selection analyses of insertional mutants using subgenic-resolution arrays', *Nature Biotechnology*, 19(11), pp. 1060-1065.
- Baerenfaller, K., Grossmann, J., Grobei, M.A., Hull, R., Hirsch-Hoffmann, M., Yalovsky, S., Zimmermann, P., Grossniklaus, U., Gruissem, W. and Baginsky, S. (2008) 'Genome-scale proteomics reveals *Arabidopsis thaliana* gene models and proteome dynamics', *Science*, 320(5878), pp. 938-941.
- Bailey, A.M., Constantinidou, C., Ivens, A., Garvey, M.I., Webber, M.A., Coldham, N., Hobman, J.L., Wain, J., Woodward, M.J. and Piddock, L.J. (2009) 'Exposure of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium to triclosan induces a species-specific response, including drug detoxification', *J Antimicrob Chemother*, 64(5), pp. 973-85.
- Baker, T.A. and Sauer, R.T. (2012) 'ClpXP, an ATP-powered unfolding and protein-degradation machine', *Biochimica et Biophysica Acta - Molecular Cell Research*, 1823(1), pp. 15-28.
- Balleza, E., López-Bojorquez, L.N., Martínez-Antonio, A., Resendis-Antonio, O., Lozada-Chávez, I., Balderas-Martínez, Y.I., Encarnación, S. and Collado-Vides, J. (2009) 'Regulation by transcription factors in bacteria: Beyond description', *FEMS Microbiology Reviews*, 33(1), pp. 133-151.
- Baltz, R.H. (2007) 'Antimicrobials from actinomycetes: Back to the future', *Microbe*, 2(3), pp. 125-131.
- Baltz, R.H. (2008) 'Renaissance in antibacterial discovery from actinomycetes', *Current Opinion in Pharmacology*, 8(5), pp. 557-563.

- Baltz, R.H. (2010) 'Streptomyces and Saccharopolyspora hosts for heterologous expression of secondary metabolite gene clusters', *Journal of Industrial Microbiology and Biotechnology*, 37(8), pp. 759-772.
- Bandow, J.E., Brotz, H., Leichert, L.I.O., Labischinski, H. and Hecker, M. (2003) 'Proteomic Approach to Understanding Antibiotic Action', *Antimicrobial Agents and Chemotherapy*, 47(3), pp. 948-955.
- Bantscheff, M., Schirle, M., Sweetman, G., Rick, J. and Kuster, B. (2007) 'Quantitative mass spectrometry in proteomics: a critical review', *Anal Bioanal Chem*, 389(4), pp. 1017-31.
- Baulcombe, D. (2004) 'RNA silencing in plants', *Nature*, 431(7006), pp. 356-363.
- Beck, M., Claassen, M. and Aebersold, R. (2011) 'Comprehensive proteomics', *Current Opinion in Biotechnology*, 22(1), pp. 3-8.
- Becker, D., Selbach, M., Rollenhagen, C., Ballmaier, M., Meyer, T.F., Mann, M. and Bumann, D. (2006) 'Robust Salmonella metabolism limits possibilities for new antimicrobials', *Nature*, 440(7082), pp. 303-7.
- Becker, G.W. (2008) 'Stable isotopic labeling of proteins for quantitative proteomic applications', *Brief Funct Genomic Proteomic*, 7(5), pp. 371-82.
- Belasco, J.G. (2010) 'All things must pass: contrasts and commonalities in eukaryotic and bacterial mRNA decay', *Nat Rev Mol Cell Biol*, 11(7), pp. 467-78.
- Beppu, T. (1992) 'Secondary metabolites as chemical signals for cellular differentiation', *Gene*, 115(1-2), pp. 159-165.
- Bérdy, J. (2005) 'Bioactive microbial metabolites: A personal view', *Journal of Antibiotics*, 58(1), pp. 1-26.
- Bergmann, S., Schümann, J., Scherlach, K., Lange, C., Brakhage, A.A. and Hertweck, C. (2007) 'Genomics-driven discovery of PKS-NRPS hybrid metabolites from *Aspergillus nidulans*', *Nature Chemical Biology*, 3(4), pp. 213-217.
- Beveridge, T.J. (1999) 'Structures of gram-negative cell walls and their derived membrane vesicles', *Journal of Bacteriology*, 181(16), pp. 4725-4733.
- Bi, E. and Lutkenhaus, J. (1991) 'FtsZ ring structure associated with division in *Escherichia coli*', *Nature*, 354(6349), pp. 161-164.

- Bi, E. and Lutkenhaus, J. (1992) 'Isolation and characterization of ftsZ alleles that affect septal morphology', *Journal of Bacteriology*, 174(16), pp. 5414-5423.
- Bi, J., Wang, H. and Xie, J. (2011) 'Comparative genomics of NAD(P) biosynthesis and novel antibiotic drug targets', *J Cell Physiol*, 226(2), pp. 331-40.
- Black, M.T. and Hodgson, J. (2005) 'Novel target sites in bacteria for overcoming antibiotic resistance', *Adv Drug Deliv Rev*, 57(10), pp. 1528-38.
- Bode, H.B., Bethe, B., Höfs, R. and Zeeck, A. (2002) 'Big effects from small changes: Possible ways to explore nature's chemical diversity', *ChemBioChem*, 3(7), pp. 619-627.
- Bondarenko, P.V., Chelius, D. and Shaler, T.A. (2002) 'Identification and relative quantitation of protein mixtures by enzymatic digestion followed by capillary reversed-phase liquid chromatography - Tandem mass spectrometry', *Analytical Chemistry*, 74(18), pp. 4741-4749.
- Bouvier, M. and Carpousis, A.J. (2011) 'A tale of two mRNA degradation pathways mediated by RNase E', *Mol Microbiol*, 82(6), pp. 1305-10.
- Brandt, O. and Hoheisel, J.D. (2004) 'Peptide nucleic acids on microarrays and other biosensors', *Trends in Biotechnology*, 22(12), pp. 617-622.
- Brantl, S. (2007) 'Regulatory mechanisms employed by cis-encoded antisense RNAs', *Curr Opin Microbiol*, 10(2), pp. 102-9.
- Brinster, S., Lamberet, G., Staels, B., Trieu-Cuot, P., Gruss, A. and Poyart, C. (2009) 'Type II fatty acid synthesis is not a suitable antibiotic target for Gram-positive pathogens', *Nature*, 458(7234), pp. 83-6.
- Bronsema, K.J., Bischoff, R. and Van de Merbel, N.C. (2012) 'Internal standards in the quantitative determination of protein biopharmaceuticals using liquid chromatography coupled to mass spectrometry', *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 893-894, pp. 1-14.
- Bronsema, K.J., Bischoff, R., van de Merbel, N.C. (2013) 'High-Sensitivity LC-MS/MS Quantification of Peptides and Proteins in Complex Biological Samples: The Impact of Enzymatic Digestion and Internal Standard Selection on Method Performance', *Analytical Chemistry*, 85, pp. 9528-9535.



- Brotz-Oesterhelt, H., Bandow, J.E. and Labischinski, H. (2005) 'Bacterial proteomics and its role in antibacterial drug discovery', *Mass Spectrom Rev*, 24(4), pp. 549-65.
- Brötz-Oesterhelt, H. and Brunner, N.A. (2008) 'How many modes of action should an antibiotic have?', *Current Opinion in Pharmacology*, 8(5), pp. 564-573.
- Brown, J.R. and Warren, P.V. (1998) 'Antibiotic discovery: Is it all in the genes?', *Drug Discovery Today*, 3(12), pp. 564-566.
- Brun, V., Dupuis, A., Adrait, A., Marcellin, M., Thomas, D., Court, M., Vandenesch, F. and Garin, J. (2007) 'Isotope-labeled protein standards: toward absolute quantitative proteomics', *Mol Cell Proteomics*, 6(12), pp. 2139-49.
- Brun, V., Masselon, C., Garin, J. and Dupuis, A. (2009) 'Isotope dilution strategies for absolute quantitative proteomics', *J Proteomics*, 72(5), pp. 740-9.
- Bugg, T.D., Braddick, D., Dowson, C.G. and Roper, D.I. (2011) 'Bacterial cell wall assembly: still an attractive antibacterial target', *Trends Biotechnol*, 29(4), pp. 167-73.
- Bull, A.T., Ward, A.C. and Goodfellow, M. (2000) 'Search and Discovery Strategies for Biotechnology: the Paradigm Shift', *Microbiology and Molecular Biology Reviews*, 64(3), pp. 573-606.
- Bumann, D. (2008) 'Has nature already identified all useful antibacterial targets?', *Curr Opin Microbiol*, 11(5), pp. 387-92.
- Butler, A. (1998) 'Acquisition and utilization of transition metal ions by marine organisms', *Science*, 281(5374), pp. 207-210.
- Caldas, T., Laalami, S. and Richarme, G. (2000) 'Chaperone properties of bacterial elongation factor EF-G and initiation factor IF2', *Journal of Biological Chemistry*, 275(2), pp. 855-860.
- Carpousis, A.J. (2007) 'The RNA degradosome of Escherichia coli: an mRNA-degrading machine assembled on RNase E', *Annu Rev Microbiol*, 61, pp. 71-87.
- Carpousis, A.J., Vanzo, N.F. and Raynal, L.C. (1999) 'mRNA degradation: A tale of poly(A) and multiprotein machines', *Trends in Genetics*, 15(1), pp. 24-28.
- Casadevall, A. (2006) 'The third age of antimicrobial therapy', *Clinical Infectious Diseases*, 42(10), pp. 1414-1416.

- Casadevall, A. (2009) 'The case for pathogen-specific therapy', *Expert Opin. Pharmacother.*, 10(11), pp. 1699-1703.
- Cerretti, D.P., Dean, D., Davis, G.R., Bedwell, D.M. and Nomura, M. (1983) 'The spc ribosomal protein operon of *Escherichia coli*: Sequence and cotranscription of the ribosomal protein genes and a protein export gene', *Nucleic Acids Research*, 11(9), pp. 2599-2616.
- Chan, P.F., Holmes, D.J. and Payne, D.J. (2004) 'Finding the gems using genomic discovery: Antibacterial drug discovery strategies - The successes and the challenges', *Drug Discovery Today: Therapeutic Strategies*, 1(4), pp. 519-527.
- Charlebois, R.L. and Doolittle, W.F. (2004) 'Computing prokaryotic gene ubiquity: Rescuing the core from extinction', *Genome Research*, 14(12), pp. 2469-2477.
- Chattopadhyay, S., Das, B. and Dasgupta, C. (1996) 'Reactivation of denatured proteins by 23S ribosomal RNA: Role of domain V', *Proceedings of the National Academy of Sciences of the United States of America*, 93(16), pp. 8284-8287.
- Chattopadhyay, S., Pal, S., Pal, D., Sarkar, D., Chandra, S. and Das Gupta, C. (1999) 'Protein folding in *Escherichia coli*: Role of 23S ribosomal RNA', *Biochimica et Biophysica Acta - Protein Structure and Molecular Enzymology*, 1429(2), pp. 293-298.
- Chaudhuri, R.R., Allen, A.G., Owen, P.J., Shalom, G., Stone, K., Harrison, M., Burgis, T.A., Lockyer, M., Garcia-Lara, J., Foster, S.J., Pleasance, S.J., Peters, S.E., Maskell, D.J. and Charles, I.G. (2009) 'Comprehensive identification of essential *Staphylococcus aureus* genes using Transposon-Mediated Differential Hybridisation (TMDH)', *BMC Genomics*, 10.
- Chavali, A.K., D'Auria, K.M., Hewlett, E.L., Pearson, R.D. and Papin, J.A. (2012) 'A metabolic network approach for the identification and prioritization of antimicrobial drug targets', *Trends in Microbiology*, 20(3), pp. 113-123.
- Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Marsh, T., Garrity, G.M. and Tiedje, J.M. (2009) 'The Ribosomal Database Project: improved alignments and new tools for rRNA analysis', *Nucleic Acids Research*, 37, pp. D141-D145.

- Condon, C. (2007) 'Maturation and degradation of RNA in bacteria', *Current Opinion in Microbiology*, 10(3), pp. 271-278.
- Covey, T.R., Huang, E.C. and Henion, J.D. (1991) 'Structural characterization of protein tryptic peptides via liquid chromatography/mass spectrometry and collision-induced dissociation of their doubly charged molecular ions', *Analytical Chemistry*, 63(13), pp. 1193-1200.
- Cox, J. and Mann, M. (2007) 'Is Proteomics the New Genomics?', *Cell*, 130(3), pp. 395-398.
- Craig, R., Cortens, J.P. and Beavis, R.C. (2004) 'Open source system for analyzing, validating, and storing protein identification data', *Journal of Proteome Research*, 3(6), pp. 1234-1242.
- Crampton, N., Bonass, W.A., Kirkham, J., Rivetti, C. and Thomson, N.H. (2006) 'Collision events between RNA polymerases in convergent transcription studied by atomic force microscopy', *Nucleic Acids Research*, 34(19), pp. 5416-5425.
- Davies, J. (1994) 'Inactivation of antibiotics and the dissemination of resistance genes', *Science*, 264(5157), pp. 375-382.
- De Boer, P., Crossley, R. and Rothfield, L. (1992) 'The essential bacterial cell-division protein FtsZ is a GTPase', *Nature*, 359(6392), pp. 254-256.
- Dean, D., Yates, J.L. and Nomura, M. (1981a) 'Escherichia coli ribosomal protein S8 feedback regulates part of spc operon', *Nature*, 289(5793), pp. 89-91.
- Dean, D., Yates, J.L. and Nomura, M. (1981b) 'Identification of ribosomal protein S7 as a repressor of translation within the str operon of E. coli', *Cell*, 24(2), pp. 413-419.
- Deana, A., Celesnik, H. and Belasco, J.G. (2008) 'The bacterial enzyme RppH triggers messenger RNA degradation by 5' pyrophosphate removal', *Nature*, 451(7176), pp. 355-8.
- Delilhas, N. and Forst, S. (2001) 'MicF: An antisense RNA gene involved in response of Escherichia coli to global stress factors', *Journal of Molecular Biology*, 313(1), pp. 1-12.
- Demidov, V.V., Potaman, V.N., Frank-Kamenetskii, M.D., Egholm, M., Buchard, O., Sonnichsen, S.H. and Nielsen, P.E. (1994) 'Stability of peptide nucleic acids

in human serum and cellular extracts', *Biochemical Pharmacology*, 48(6), pp. 1310-1313.

Dervan, P.B. (2001) 'Molecular recognition of DNA by small molecules', *Bioorganic & Med. Chem.*, 9(9), pp. 2215-2235.

DeVito, J.A., Mills, J.A., Liu, V.G., Agarwal, A., Sizemore, C.F., Yao, Z., Stoughton, D.M., Cappiello, M.G., Barbosa, M.D.F.S., Foster, L.A. and Pompliano, D.L. (2002) 'An array of target-specific screening strains for antibacterial discovery', *Nature Biotechnology*, 20(5), pp. 478-483.

Diaz Ricci, J.C. and Hernández, M.E. (2000) 'Plasmid effects on Escherichia coli metabolism', *Critical Reviews in Biotechnology*, 20(2), pp. 79-108.

Domon, B. and Aebersold, R. (2010) 'Options and considerations when selecting a quantitative proteomics strategy', *Nat Biotechnol*, 28(7), pp. 710-21.

Donadio, S., Carrano, L., Brandi, L., Serina, S., Soffientini, A., Raimondi, E., Montanini, N., Sosio, M. and Gualerzi, C.O. (2002) 'Targets and assays for discovering novel antibacterial agents', *Journal of Biotechnology*, 99(3), pp. 175-185.

Dong, H., Nilsson, L. and Kurland, C.G. (1995) 'Gratuitous overexpression of genes in Escherichia coli leads to growth inhibition and ribosome destruction', *Journal of Bacteriology*, 177(6), pp. 1497-1504.

Dornenburg, J.E., DeVita, A.M., Palumbo, M.J. and Wade, J.T. (2010) 'Widespread antisense transcription in Escherichia coli', *mBio*, 1(1).

Dreyfus, M. and Régnier, P. (2002) 'The poly(A) tail of mRNAs: Bodyguard in eukaryotes, scavenger in bacteria', *Cell*, 111(5), pp. 611-613.

Dryselius, R., Aswasti, S.K., Rajarao, G.K., Nielsen, P.E. and Good, L. (2003) 'The translation start codon region is sensitive to antisense PNA inhibition in *Escherichia coli*', *Oligonucleotides*, 13(6), pp. 427-433.

Dryselius, R., Nikravesh, A., Kulyté, A., Goh, S. and Good, L. (2006a) 'Variable coordination of cotranscribed genes in Escherichia coli following antisense repression', *BMC Microbiology*, 6.

Dryselius, R., Nikravesh, A., Kulyté, A., Goh, S. and Good, L. (2006b) 'Variable coordination of cotranscribed genes in *Escherichia coli* following antisense repression', *BMC Microbiol.*, 6:97.

Duhring, U., Axmann, I.M., Hess, W.R. and Wilde, A. (2006) 'An internal antisense RNA regulates expression of the photosynthesis gene *isiA*', *Proc Natl Acad Sci U S A*, 103(18), pp. 7054-8.

Durfee, T., Nelson, R., Baldwin, S., Plunkett, G., Burland, V., Mau, B., Petrosino, J.F., Qin, X., Muzny, D.M., Ayele, M., Gibbs, R.A., Csorgo, B., Posfai, G., Weinstock, G.M. and Blattner, F.R. (2008a) 'The complete genome sequence of *Escherichia coli* DH10B: Insights into the biology of a laboratory workhorse', *Journal of Bacteriology*, 190(7), pp. 2597-2606.

Durfee, T., Nelson, R., Baldwin, S., Plunkett, G., Burland, V., Mau, B., Petrosino, J.F., Qin, X., Muzny, D.M., Ayele, M., Gibbs, R.A., Csörgo, B., Pósfai, G., Weinstock, G.M. and Blattner, F.R. (2008b) 'The complete genome sequence of *Escherichia coli* DH10B: Insights into the biology of a laboratory workhorse', *Journal of Bacteriology*, 190(7), pp. 2597-2606.

East, S.P. and Silver, L.L. (2013) 'Multitarget ligands in antibacterial research: Progress and opportunities', *Expert Opinion on Drug Discovery*, 8(2), pp. 143-156.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S.R., Nelson, K.E. and Relman, D.A. (2005) 'Diversity of the human intestinal microbial flora', *Science*, 308(5728), pp. 1635-1638.

Eckert, R., He, J., Yarbrough, D.K., Qi, F., Anderson, M.H. and Shi, W. (2006a) 'Targeted killing of *Streptococcus mutans* by a pheromone-guided "smart" antimicrobial peptide.', *Antimicrob. Agents and Chemother.*, 50(11), pp. 3651-3657.

Eckert, R., Qi, F., Yarbrough, D.K., He, J., Anderson, M.H. and Shi, W. (2006b) 'Adding selectivity to antimicrobial peptides: rational design of a multidomain peptide against *Pseudomonas* spp.', *Antimicrob. Agents and Chemother.*, 50(4), pp. 1480-1488.

Eguchi, Y. (1991) 'Antisense RNA', *Annual Review of Biochemistry*, 60, pp. 631-652.

Elowitz, M.B., Levine, A.J., Siggia, E.D. and Swain, P.S. (2002) 'Stochastic gene expression in a single cell', *Science*, 297(5584), pp. 1183-1186.

- Endy, D. (2005) 'Foundations for engineering biology', *Nature*, 438(7067), pp. 449-453.
- Epstein, W. and Beckwith, J. (1968) 'Regulation of gene expression', *Annual Review of Biochemistry*, 37, pp. 411-436.
- Eriksson, M., Nielsen, P.E. and Good, L. (2002) 'Cell permeabilization and uptake of antisense peptide-peptide nucleic acid (PNA) into *Escherichia coli*', *J Biol Chem*, 277(9), pp. 7144-7.
- Eschenburg, S., Priestman, M. and Schönbrunn, E. (2005) 'Evidence that the fosfomycin target Cys115 in UDP-N-acetylglucosamine Enolpyruvyl Transferase (MurA) is essential for product release', *Journal of Biological Chemistry*, 280(5), pp. 3757-3763.
- Esvelt, K.M. and Wang, H.H. (2013) 'Genome-scale engineering for systems and synthetic biology', *Molecular Systems Biology*, 9.
- Fabbretti, A., Gualerzi, C.O. and Brandi, L. (2011) 'How to cope with the quest for new antibiotics', *FEBS Lett*, 585(11), pp. 1673-81.
- Fajardo, A., Linares, J.F. and Martínez, J.L. (2009) 'Towards an ecological approach to antibiotics and antibiotic resistance genes', *Clinical Microbiology and Infection*, 15(SUPPL. 1), pp. 14-16.
- Fajardo, A. and Martinez, J.L. (2008) 'Antibiotics as signals that trigger specific bacterial responses', *Curr Opin Microbiol*, 11(2), pp. 161-7.
- Faridani, O.R., Nikraves, A., Pandey, D.P., Gerdes, K. and Good, L. (2006) 'Competitive inhibition of natural antisense Sok-RNA interactions activates Hok-mediated cell killing in *Escherichia coli*', *Nucleic Acids Res.*, 34(20), pp. 5915-22.
- Fehér, T., Papp, B., Pál, C. and Pósfai, G. (2007) 'Systematic genome reductions: Theoretical and experimental approaches', *Chemical Reviews*, 107(8), pp. 3498-3513.
- Fenn, J.B., Mann, M., Meng, C.K., Wong, S.F. and Whitehouse, C.M. (1989) 'Electrospray ionization for mass spectrometry of large biomolecules', *Science*, 246(4926), pp. 64-71.

- Fields, P.I., Swanson, R.V., Haidaris, C.G. and Heffron, F. (1986) 'Mutants of *Salmonella*-Typhimurium That Cannot Survive within the Macrophage Are Avirulent', *Proc. Natl. Acad. Sci. U.S.A.*, 83(14), pp. 5189-5193.
- Filiou, M.D., Varadarajulu, J., Teplytska, L., Reckow, S., Maccarrone, G. and Turck, C.W. (2012) 'The <sup>15</sup>N isotope effect in *Escherichia coli*: A neutron can make the difference', *Proteomics*, 12(21), pp. 3121-3128.
- Fischbach, M.A. (2009) 'Antibiotics from microbes: converging to kill', *Curr Opin Microbiol*, 12(5), pp. 520-7.
- Fischbach, M.A. and Walsh, C.T. (2006) 'Assembly-line enzymology for polyketide and nonribosomal peptide antibiotics: Logic machinery, and mechanisms', *Chemical Reviews*, 106(8), pp. 3468-3496.
- Fischbach, M.A. and Walsh, C.T. (2009) 'Antibiotics for emerging pathogens', *Science*, 325(5944), pp. 1089-93.
- Fleischmann, R.D., Adams, M.D., White, O., Clayton, R.A., Kirkness, E.F., Kerlavage, A.R., Bult, C.J., Tomb, J.F., Dougherty, B.A., Merrick, J.M., McKenney, K., Sutton, G., FitzHugh, W., Fields, C., Gocayne, J.D., Scott, J., Shirley, R., Liu, L.I. and Venter, J.C. (1995) 'Whose-genome random sequencing and assembly of *Haemophilus influenzae* Rd', *Science*, 269(5223), pp. 496-521.
- Foliaki, S., Pearce, N., Bjorksten, B., Mallol, J., Montefort, S. and von Mutius, E. (2009) 'Antibiotic use in infancy and symptoms of asthma, rhinoconjunctivitis, and eczema in children 6 and 7 years old: International Study of Asthma and Allergies in Childhood Phase III', *Journal of Allergy and Clinical Immunology*, 124(5), pp. 982-989.
- Forsyth, R.A., Haselbeck, R.J., Ohlsen, K.L., Yamamoto, R.T., Xu, H., Trawick, J.D., Wall, D., Wang, L., Brown-Driver, V., Froelich, J.M., Kedar, G.C., King, P., McCarthy, M., Malone, C., Misiner, B., Robbins, D., Tan, Z., Zhu, Z.Y., Carr, G., Mosca, D.A., Zamudio, C., Foulkes, J.G. and Zyskind, J.W. (2002) 'A genome-wide strategy for the identification of essential genes in *Staphylococcus aureus*', *Molecular Microbiology*, 43(6), pp. 1387-1400.
- Fowler, S., Webber, A., Cooper, B.S., Phimister, A., Price, K., Carter, Y., Kibbler, C.C., Simpson, A.J.H. and Stone, S.R. (2007) 'Successful use of feedback to improve antibiotic prescribing and reduce *Clostridium difficile*

infection: a controlled interrupted time series', *Journal of Antimicrobial Chemotherapy*, 59(5), pp. 990-995.

Franch, T., Petersen, M., Wagner, E.G.H., Jacobsen, J.P. and Gerdes, K. (1999) 'Antisense RNA regulation in prokaryotes: Rapid RNA/RNA interaction facilitated by a general U-turn loop structure', *Journal of Molecular Biology*, 294(5), pp. 1115-1125.

Fraser, D. and Kærn, M. (2009) 'A chance at survival: Gene expression noise and phenotypic diversification strategies', *Molecular Microbiology*, 71(6), pp. 1333-1340.

Freiberg, C., Brotz-Oesterhelt, H. and Labischinski, H. (2004) 'The impact of transcriptome and proteome analyses on antibiotic drug discovery', *Curr Opin Microbiol*, 7(5), pp. 451-9.

Friedman, L., Alder, J.D. and Silverman, J.A. (2006) 'Genetic changes that correlate with reduced susceptibility to daptomycin in *Staphylococcus aureus*.', *Antimicrob. Agents and Chemother.*, 50(6), pp. 2137-2145.

Fu, X., Fu, N., Guo, S., Yan, Z., Xu, Y., Hu, H., Menzel, C., Chen, W., Li, Y., Zeng, R. and Khaitovich, P. (2009) 'Estimating accuracy of RNA-Seq and microarrays with proteomics', *BMC Genomics*, 10.

Gadebusch, H.H., Stapley, E.O. and Zimmerman, S.B. (1992) 'Discovery of cell wall active antibacterial antibiotics', *Critical Reviews in Biotechnology*, 12(3), pp. 225-243.

Gallop, M.A., Barrett, R.W., Dower, W.J., Fodor, S.P.A. and Gordon, E.M. (1994) 'Applications of combinatorial technologies to drug discovery. 1. Background and peptide combinatorial libraries', *Journal of Medicinal Chemistry*, 37(9), pp. 1233-1251.

Gama-Castro, S., Jiménez-Jacinto, V., Peralta-Gil, M., Santos-Zavaleta, A., Peñaloza-Spinola, M.I., Contreras-Moreira, B., Segura-Salazar, J., Muñiz-Rascado, L., Martínez-Flores, I., Salgado, H., Bonavides-Martínez, C., Abreu-Goodger, C., Rodríguez-Penagos, C., Miranda-Ríos, J., Morett, E., Merino, E., Huerta, A.M., Treviño-Quintanilla, L. and Collado-Vides, J. (2008) 'RegulonDB (version 6.0): Gene regulation model of *Escherichia coli* K-12 beyond transcription, active (experimental) annotated promoters and Textpresso navigation', *Nucleic Acids Research*, 36(SUPPL. 1), pp. D120-D124.



Gao, F. and Zhang, R.R. (2011) 'Enzymes are enriched in bacterial essential genes', *PLoS ONE*, 6(6).

Georg, J. and Hess, W.R. (2011) 'cis-antisense RNA, another level of gene regulation in bacteria', *Microbiol Mol Biol Rev*, 75(2), pp. 286-300.

Gerber, S.A., Rush, J., Stemman, O., Kirschner, M.W. and Gygi, S.P. (2003) 'Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS', *Proc Natl Acad Sci U S A*, 100(12), pp. 6940-5.

Gerdes, S., Edwards, R., Kubal, M., Fonstein, M., Stevens, R. and Osterman, A. (2006) 'Essential genes on metabolic maps', *Curr Opin Biotechnol*, 17(5), pp. 448-56.

Gerdes, S.Y., Scholle, M.D., Campbell, J.W., Balazsi, G., Ravasz, E., Daugherty, M.D., Somera, A.L., Kyrpides, N.C., Anderson, I., Gelfand, M.S., Bhattacharya, A., Kapatral, V., D'Souza, M., Baev, M.V., Grechkin, Y., Mseeh, F., Fonstein, M.Y., Overbeek, R., Barabasi, A.L., Oltvai, Z.N. and Osterman, A.L. (2003) 'Experimental Determination and System Level Analysis of Essential Genes in Escherichia coli MG1655', *Journal of Bacteriology*, 185(19), pp. 5673-5684.

Ghosal, A., Vitali, A., Stach, J.E.M. and Nielsen, P.E. (2013) 'Role of SbmA in the uptake of peptide nucleic acid (PNA)-peptide conjugates in E. coli', *ACS Chemical Biology*, 8(2), pp. 360-367.

Giesen, U., Kleider, W., Berding, C., Geiger, A., Orum, H. and Nielsen, P. (1998) 'A formula for thermal stability (T<sub>m</sub>) prediction of PNA/DNA duplexes', *Nucleic Acids Res.*, 26(21), p. 5004.

Gillings, M.R. and Stokes, H.W. (2012) 'Are humans increasing bacterial evolvability?', *Trends in Ecology and Evolution*, 27(6), pp. 346-352.

Glasner, J.D., Liss, P., Plunkett Iii, G., Darling, A., Prasad, T., Rusch, M., Byrnes, A., Gilson, M., Biehl, B., Blattner, F.R. and Perna, N.T. (2003) 'ASAP, a systematic annotation package for community analysis of genomes', *Nucleic Acids Research*, 31(1), pp. 147-151.

Glass, J.I., Assad-Garcia, N., Alperovich, N., Yooseph, S., Lewis, M.R., Maruf, M., Hutchison Iii, C.A., Smith, H.O. and Venter, J.C. (2006) 'Essential genes of

a minimal bacterium', *Proceedings of the National Academy of Sciences of the United States of America*, 103(2), pp. 425-430.

Glick, B.R. (1995) 'Metabolic load and heterologous gene expression', *Biotechnology Advances*, 13(2), pp. 247-261.

Goh, S., Boberek, J.M., Nakashima, N., Stach, J. and Good, L. (2009) 'Concurrent growth rate and transcript analyses reveal essential gene stringency in *Escherichia coli*', *PLoS One*, 4(6), p. e6061.

Golding, I., Paulsson, J., Zawilski, S.M. and Cox, E.C. (2005) 'Real-time kinetics of gene activity in individual bacteria', *Cell*, 123(6), pp. 1025-1036.

Good, L. (2002) 'Antisense Inhibition of Bacterial Gene Expression and Cell Growth', in Nielsen, P. (ed.) *Peptide Nucleic Acids: Protocols and Applications (Methods in Molecular Biology)*. New York: Springer, pp. 237-248.

Good, L., Awasthi, S.K., Dryselius, R., Larsson, O. and Nielsen, P.E. (2001a) 'Bactericidal antisense effects of peptide-PNA conjugates', *Nat. Biotechnol.*, 19(4), pp. 360-4.

Good, L., Awasthi, S.K., Dryselius, R., Larsson, O. and Nielsen, P.E. (2001b) 'Bactericidal antisense effects of peptide - PNA conjugates', *Nature Biotechnology*, 19(4), pp. 360-364.

Good, L. and Nielsen, P.E. (1997) 'Progress in developing PNA as a gene-targeted drug', *Antisense and Nucleic Acid Drug Development*, 7(4), pp. 431-437.

Good, L. and Nielsen, P.E. (1998) 'Inhibition of translation and bacterial growth by peptide nucleic acid targeted to ribosomal RNA', *Proc. Natl. Acad. Sci. U.S.A.*, 95(5), pp. 2073-6.

Good, L., Sandberg, R., Larsson, O., Nielsen, P.E. and Wahlestedt, C. (2000a) 'Antisense PNA effects in *Escherichia coli* are limited by the outer-membrane LPS layer', *Microbiology*, 146(10), pp. 2665-2670.

Good, L., Sandberg, R., Larsson, O., Nielsen, P.E. and Wahlestedt, C. (2000b) 'Antisense PNA effects in *Escherichia coli* are limited by the outer-membrane LPS layer', *Microbiol. UK*, 146, pp. 2665-70.

Good, L. and Stach, J.E. (2011) 'Synthetic RNA silencing in bacteria - antimicrobial discovery and resistance breaking', *Front Microbiol*, 2, p. 185.

- Goodfellow, M. and Fiedler, H.P. (2010) 'A guide to successful bioprospecting: informed by actinobacterial systematics', *Antonie Van Leeuwenhoek*, 98(2), pp. 119-42.
- Goodfellow, M. and Williams, S.T. (1983) 'Ecology of actinomycetes', *Annual Review of Microbiology*, 37, pp. 189-216.
- Granieri, L., Miller, O.J., Griffiths, A.D. and Merten, C.A. (2009) 'A competition-based assay for the screening of species-specific antibiotics', *Journal of Antimicrobial Chemotherapy*, 64(1), pp. 62-68.
- Gripenland, J., Netterling, S., Loh, E., Tiensuu, T., Toledo-Arana, A. and Johansson, J. (2010) 'RNAs: regulators of bacterial virulence', *Nat Rev Microbiol*, 8(12), pp. 857-66.
- Gross, H., Stockwell, V.O., Henkels, M.D., Nowak-Thompson, B., Loper, J.E. and Gerwick, W.H. (2007) 'The Genomisotopic Approach: A Systematic Method to Isolate Products of Orphan Biosynthetic Gene Clusters', *Chemistry and Biology*, 14(1), pp. 53-63.
- Güttinger, S. (2013) 'Creating parts that allow for rational design: Synthetic biology and the problem of context-sensitivity', *Studies in History and Philosophy of Science Part C :Studies in History and Philosophy of Biological and Biomedical Sciences*, 44(2), pp. 199-207.
- Guzman, L.M., Belin, D., Carson, M.J. and Beckwith, J. (1995) 'Tight regulation, modulation, and high-level expression by vectors containing the arabinose P(BAD) promoter', *Journal of Bacteriology*, 177(14), pp. 4121-4130.
- Gygi, S.P., Rist, B., Gerber, S.A., Turecek, F., Gelb, M.H. and Aebersold, R. (1999) 'Quantitative analysis of complex protein mixtures using isotope-coded affinity tags', *Nature Biotechnology*, 17(10), pp. 994-999.
- Hacek, D.M., Dressel, D.C. and Peterson, L.R. (1999) 'Highly reproducible bactericidal activity test results by using a modified National Committee for Clinical Laboratory Standards broth macrodilution technique', *Journal of Clinical Microbiology*, 37(6), pp. 1881-4.
- Hale, C.A. and De Boer, P.A.J. (1997) 'Direct binding of FtsZ to ZipA, an essential component of the septal ring structure that mediates cell division in *E. coli*', *Cell*, 88(2), pp. 175-185.

- Han, B. and Higgs, R.E. (2008) 'Proteomics: from hypothesis to quantitative assay on a single platform. Guidelines for developing MRM assays using ion trap mass spectrometers', *Brief Funct Genomic Proteomic*, 7(5), pp. 340-54.
- Han, K.Y., Park, J.S., Seo, H.N., Ann, K.Y. and Lee, J. (2008) 'Multiple stressor-Induced proteome responses of escherichia coli BL21(DE3)', *Journal of Proteome Research*, 7(5), pp. 1891-1903.
- Hancock, R.E.W. (1997) 'Peptide antibiotics', *Lancet*, 349(9049), pp. 418-422.
- Handelsman, J., Rondon, M.R., Brady, S.F., Clardy, J. and Goodman, R.M. (1998) 'Molecular biological access to the chemistry of unknown soil microbes: A new frontier for natural products', *Chemistry and Biology*, 5(10), pp. R245-R249.
- Harth, G., Zamecnik, P.C., Tang, J.Y., Tabatadze, D. and Horwitz, M.A. (2000) 'Treatment of *Mycobacterium tuberculosis* with antisense oligonucleotides to glutamine synthetase mRNA inhibits glutamine synthetase activity, formation of the poly-L-glutamate/glutamine cell wall structure, and bacterial replication', *Proc. Natl. Acad. Sci. U.S.A.*, 97(1), pp. 418-423.
- Hartmann, H.J., Kaup, Y. and Weser, U. (2003) 'Does <sup>13</sup>C-or <sup>15</sup>N-labeling affect Cu(I)-thiolate cluster arrangement in yeast copper-metallothionein?', *BioMetals*, 16(3), pp. 379-382.
- Harvey, C.L., Knight, S.G. and Sih, C.J. (1966) 'On the mode of action of fusidic acid', *Biochemistry*, 5(10), pp. 3320-3327.
- Hatamoto, M., Nakai, K., Ohashi, A. and Imachi, H. (2009) 'Sequence-specific bacterial growth inhibition by peptide nucleic acid targeted to the mRNA binding site of 16S rRNA', *Appl. Microbiol. Biot*, 84(6), pp. 1161-1168.
- Hatamoto, M., Ohashi, A. and Imachi, H. (2010) 'Peptide nucleic acids (PNAs) antisense effect to bacterial growth and their application potentiality in biotechnology', *Applied Microbiology and Biotechnology*, 86(2), pp. 397-402.
- Heath, R.J. and Rock, C.O. (2000) 'Microbiology: A triclosan-resistant bacterial enzyme', *Nature*, 406(6792), pp. 145-146.
- Heath, R.J., Rubin, J.R., Holland, D.R., Zhang, E., Snow, M.E. and Rock, C.O. (1999) 'Mechanism of triclosan inhibition of bacterial fatty acid synthesis', *Journal of Biological Chemistry*, 274(16), pp. 11110-11114.

- Hirokawa, G., Nijman, R.M., Raj, V.S., Kaji, H., Igarashi, K. and Kaji, A. (2005) 'The role of ribosome recycling factor in dissociation of 70S ribosomes into subunits', *RNA*, 11(8), pp. 1317-1328.
- Hoban, D.J. (2003) 'Antibiotics and collateral damage', *Clin. Cornerstone Suppl.*, 3, pp. S12-20.
- Holmes, E., Kinross, J., Gibson, G.R., Burcelin, R., Jia, W., Pettersson, S. and Nicholson, J.K. (2012) 'Therapeutic Modulation of Microbiota-Host Metabolic Interactions', *Science Translational Medicine*, 4(137).
- Howden, B.P. and Grayson, M.L. (2006) 'Dumb and dumber - The potential waste of a useful antistaphylococcal agent: Emerging fusidic acid resistance in *Staphylococcus aureus*', *Clinical Infectious Diseases*, 42(3), pp. 394-400.
- Hu, Q., Noll, R.J., Li, H., Makarov, A., Hardman, M. and Graham Cooks, R. (2005) 'The Orbitrap: a new mass spectrometer', *J Mass Spectrom*, 40(4), pp. 430-43.
- Huang, C.H., Hsiang, T. and Trevors, J.T. (2012) 'Comparative bacterial genomics: defining the minimal core genome', *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, pp. 1-14.
- Hunt, D.F., Yates Iii, J.R., Shabanowitz, J., Winston, S. and Hauer, C.R. (1986) 'Protein sequencing by tandem mass spectrometry', *Proceedings of the National Academy of Sciences of the United States of America*, 83(17), pp. 6233-6237.
- Huttlin, E.L., Hegeman, A.D. and Sussman, M.R. 52 (2008) 'Chapter 20 Metabolic Labeling Approaches for the Relative Quantification of Proteins' Whitelegge, J.P., pp. 479-513,529-530. Available at: <http://www.scopus.com/inward/record.url?eid=2-s2.0-67149101455&partnerID=40&md5=fb5e2ee3c5544129fdf826f1a6290424>.
- Imlay, J.A. (2003) 'Pathways of oxidative damage', *Annu Rev Microbiol*, 57, pp. 395-418.
- Ishihama, Y., Oda, Y., Tabata, T., Sato, T., Nagasu, T., Rappsilber, J. and Mann, M. (2005) 'Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of

sequenced peptides per protein', *Molecular and Cellular Proteomics*, 4(9), pp. 1265-1272.

Ishihama, Y., Schmidt, T., Rappsilber, J., Mann, M., Hartl, F.U., Kerner, M.J. and Frishman, D. (2008) 'Protein abundance profiling of the Escherichia coli cytosol', *BMC Genomics*, 9, p. 102.

Jacob, F. and Monod, J. (1961) 'Genetic regulatory mechanisms in the synthesis of proteins', *Journal of molecular biology*, 3, pp. 318-356.

Jakobsson, H.E., Jernberg, C., Andersson, A.F., Sjolund-Karlsson, M., Jansson, J.K. and Engstrand, L. (2010) 'Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome', *PLoS One*, 5(3), p. e9836.

Jayapal, K.P., Philp, R.J., Kok, Y.J., Yap, M.G.S., Sherman, D.H., Griffin, T.J. and Hu, W.S. (2008) 'Uncovering genes with divergent mRNA-protein dynamics in Streptomyces coelicolor', *PLoS ONE*, 3(5).

Jayaraman, R. (2009) 'Antibiotic resistance: an overview of mechanisms and a paradigm shift', *Current Science*, 96(11), pp. 1475-1484.

Jeon, B. and Zhang, Q. (2009a) 'Sensitization of Campylobacter jejuni to fluoroquinolone and macrolide antibiotics by antisense inhibition of the CmeABC multidrug efflux transporter', *Journal of Antimicrobial Chemotherapy*, 63(5), pp. 946-948.

Jeon, B. and Zhang, Q.J. (2009b) 'Sensitization of Campylobacter jejuni to fluoroquinolone and macrolide antibiotics by antisense inhibition of the CmeABC multidrug efflux transporter', *Journal of Antimicrobial Chemotherapy*, 63(5), pp. 946-948.

Johnson, E. and Srivastava, R. (2013) 'Volatility in mRNA secondary structure as a design principle for antisense', *Nucleic Acids Research*, 41(3).

Jordan, I.K., Rogozin, I.B., Wolf, Y.I. and Koonin, E.V. (2002) 'Essential Genes Are More Evolutionarily Conserved Than Are Nonessential Genes in Bacteria', *Genome Research*, 12(6), pp. 962-968.

Jousset, A. (2012) 'Ecological and evolutive implications of bacterial defences against predators', *Environmental Microbiology*, 14(8), pp. 1830-1843.

- Juhas, M., Eberl, L. and Glass, J.I. (2011) 'Essence of life: essential genes of minimal genomes', *Trends Cell Biol*, 21(10), pp. 562-8.
- Kaern, M., Elston, T.C., Blake, W.J. and Collins, J.J. (2005) 'Stochasticity in gene expression: from theories to phenotypes', *Nat Rev Genet*, 6(6), pp. 451-64.
- Kaur, P., Agarwal, S. and Datta, S. (2009) 'Delineating bacteriostatic and bactericidal targets in mycobacteria using IPTG inducible antisense expression', *PLoS ONE*, 4(6).
- Kawano, M., Aravind, L. and Storz, G. (2007) 'An antisense RNA controls synthesis of an SOS-induced toxin evolved from an antitoxin', *Mol Microbiol*, 64(3), pp. 738-54.
- Keiler, K.C., Waller, P.R.H. and Sauer, R.T. (1996) 'Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA', *Science*, 271(5251), pp. 990-993.
- Keller, M. and Zengler, K. (2004) 'Tapping into microbial diversity', *Nat Rev Microbiol*, 2(2), pp. 141-50.
- Kirkpatrick, D.S., Gerber, S.A. and Gygi, S.P. (2005) 'The absolute quantification strategy: a general procedure for the quantification of proteins and post-translational modifications', *Methods*, 35(3), pp. 265-73.
- Kitagawa, M., Ara, T., Arifuzzaman, M., Ioka-Nakamichi, T., Inamoto, E., Toyonaga, H. and Mori, H. (2005a) 'Complete set of ORF clones of Escherichia coli ASKA library (A complete set of E. coli K-12 ORF archive): unique resources for biological research', *DNA Research*, 12(5), pp. 291-299.
- Kitagawa, M., Ara, T., Arifuzzaman, M., Ioka-Nakamichi, T., Inamoto, E., Toyonaga, H. and Mori, H. (2005b) 'Complete set of ORF clones of Escherichia coli ASKA library (a complete set of E. coli K-12 ORF archive): unique resources for biological research', *DNA Res.*, 12(5), pp. 291-299.
- Kittle, J.D., Simons, R.W., Lee, J. and Kleckner, N. (1989) 'Insertion sequence IS10 anti-sense pairing initiates by an interaction between the 5' end of the target RNA and a loop in the anti-sense RNA', *Journal of Molecular Biology*, 210(3), pp. 561-572.

Knight, R. (2010) 'Translational medicine and the human microbiome', *Genome Biol.*, 11, p. (Suppl 1):l15.

Knudsen, H. and Nielsen, P.E. (1996) 'Antisense properties of duplex- and triplex-forming PNAs', *Nucleic Acids Research*, 24(3), pp. 494-500.

Knuth, K., Niesalla, H., Hueck, C.J. and Fuchs, T.M. (2004) 'Large-scale identification of essential *Salmonella* genes by trapping lethal insertions', *Molecular Microbiology*, 51(6), pp. 1729-1744.

Kobayashi, K., Ehrlich, S.D., Albertini, A., Amati, G., Andersen, K.K., Arnaud, M., Asai, K., Ashikaga, S., Aymerich, S., Bessieres, P., Boland, F., Brignell, S.C., Bron, S., Bunai, K., Chapuis, J., Christiansen, L.C., Danchin, A., Debarbouille, M., Dervyn, E., Deuerling, E., Devine, K., Devine, S.K., Dreesen, O., Errington, J., Fillinger, S., Foster, S.J., Fujita, Y., Galizzi, A., Gardan, R., Eschevins, C., Fukushima, T., Haga, K., Harwood, C.R., Hecker, M., Hosoya, D., Hullo, M.F., Kakeshita, H., Karamata, D., Kasahara, Y., Kawamura, F., Koga, K., Koski, P., Kuwana, R., Imamura, D., Ishimaru, M., Ishikawa, S., Ishio, I., Le Coq, D., Masson, A., Mauel, C., Meima, R., Mellado, R.P., Moir, A., Moriya, S., Nagakawa, E., Nanamiya, H., Nakai, S., Nygaard, P., Ogura, M., Ohanan, T., O'Reilly, M., O'Rourke, M., Pragai, Z., Pooley, H.M., Rapoport, G., Rawlins, J.P., Rivas, L.A., Rivolta, C., Sadaie, A., Sadaie, Y., Sarvas, M., Sato, T., Saxild, H.H., Scanlan, E., Schumann, W., Seegers, J.F.M.L., Sekiguchi, J., Sekowska, A., Seror, S.J., Simon, M., Stragier, P., Studer, R., Takamatsu, H., Tanaka, T., Takeuchi, M., Thomaidis, H.B., Vagner, V., van Dijl, J.M., Watabe, K., Wipat, A., Yamamoto, H., Yamamoto, M., Yamamoto, Y., Yamane, K., Yata, K., Yoshida, K., Yoshikawa, H., Zuber, U. and Ogasawara, N. (2003a) 'Essential *Bacillus subtilis* genes', *Proc. Natl. Acad. Sci. U.S.A.*, 100(8), pp. 4678-4683.

Kobayashi, K., Ehrlich, S.D., Albertini, A., Amati, G., Andersen, K.K., Arnaud, M., Asai, K., Ashikaga, S., Aymerich, S., Bessieres, P., Boland, F., Brignell, S.C., Bron, S., Bunai, K., Chapuis, J., Christiansen, L.C., Danchin, A., Débarbouillé, M., Dervyn, E., Deuerling, E., Devine, K., Devine, S.K., Dreesen, O., Errington, J., Fillinger, S., Foster, S.J., Fujita, Y., Galizzi, A., Gardan, R., Eschevins, C., Fukushima, T., Haga, K., Harwood, C.R., Hecker, M., Hosoya, D., Hullo, M.F., Kakeshita, H., Karamata, D., Kasahara, Y., Kawamura, F., Koga, K., Koski, P., Kuwana, R., Imamura, D., Ishimaru, M., Ishikawa, S., Ishio, I., le Coq, D., Masson, A., Mauël, C., Meima, R., Mellado, R.P., Moir, A.,



Moriya, S., Nagakawa, E., Nanamiya, H., Nakai, S., Nygaard, P., Ogura, M., Ohanan, T., O'Reilly, M., O'Rourke, M., Pragai, Z., Pooley, H.M., Rapoport, G., Rawlins, J.P., Rivas, L.A., Rivolta, C., Sadaie, A., Sadaie, Y., Sarvas, M., Sato, T., Saxild, H.H., Scanlan, E., Schumann, W., Seegers, J.F.M.L., Sekiguchi, J., Sekowska, A., Séror, S.J., Simon, M., Stragier, P., Studer, R., Takamatsu, H., Tanaka, T., Takeuchi, M., Thomaidis, H.B., Vagner, V., van Dijl, J.M., Watabe, K., Wipat, A., Yamamoto, H., Yamamoto, M., Yamamoto, Y., Yamane, K., Yata, K., Yoshida, K., Yoshikawa, H., Zuber, U. and Ogasawara, N. (2003b) 'Essential *Bacillus subtilis* genes', *Proceedings of the National Academy of Sciences of the United States of America*, 100(8), pp. 4678-4683.

Koehn, F.E. and Carter, G.T. (2005) 'The evolving role of natural products in drug discovery', *Nat Rev Drug Discov*, 4(3), pp. 206-20.

Kohanski, M.A., Dwyer, D.J. and Collins, J.J. (2010) 'How antibiotics kill bacteria: from targets to networks', *Nat Rev Microbiol*, 8(6), pp. 423-35.

Kohanski, M.A., Dwyer, D.J., Wierzbowski, J., Cottarel, G. and Collins, J.J. (2008) 'Mistranslation of membrane proteins and two-component system activation trigger antibiotic-mediated cell death', *Cell*, 135(4), pp. 679-90.

Kolb, F.A., Engdahl, H.M., Slagter-Jäger, J.G., Ehresmann, B., Ehresmann, C., Westhof, E., Wagner, E.G.H. and Romby, P. (2000) 'Progression of a loop-loop complex to a four-way junction is crucial for the activity of a regulatory antisense RNA', *EMBO Journal*, 19(21), pp. 5905-5915.

Koller, E., Gaarde, W.A. and Monia, B.P. (2000) 'Elucidating cell signaling mechanisms using antisense technology', *Trends in Pharmacological Sciences*, 21(4), pp. 142-148.

Konopka, A., Boehm, M.E., Rohmer, M., Baeumlisberger, D., Karas, M. and Lehmann, W.D. (2012) 'Improving the precision of quantitative bottom-up proteomics based on stable isotope-labeled proteins', *Analytical and Bioanalytical Chemistry*, 404(4), pp. 1079-1087.

Koonin, E.V. (2003) 'Comparative genomics, minimal gene-sets and the last universal common ancestor', *Nat Rev Microbiol*, 1(2), pp. 127-36.

Koppelhus, U. and Nielsen, P.E. (2003) 'Cellular delivery of peptide nucleic acid (PNA)', *Advanced Drug Delivery Reviews*, 55(2), pp. 267-280.

- Korepanov, A.P., Gongadze, G.M., Garber, M.B., Court, D.L. and Bubunenko, M.G. (2007) 'Importance of the 5 S rRNA-binding ribosomal proteins for cell viability and translation in *Escherichia coli*', *J Mol Biol*, 366(4), pp. 1199-208.
- Korepanov, A.P., Korobeinikova, A.V., Shestakov, S.A., Garber, M.B. and Gongadze, G.M. (2012) 'Protein L5 is crucial for in vivo assembly of the bacterial 50S ribosomal subunit central protuberance', *Nucleic Acids Research*, 40(18), pp. 9153-9159.
- Kühner, S., Van Noort, V., Betts, M.J., Leo-Madas, A., Batische, C., Rode, M., Yamada, T., Maier, T., Bader, S., Beltran-Alvarez, P., Castaño-Diez, D., Chen, W.H., Devos, D., Güell, M., Norambuena, T., Racke, I., Rybin, V., Schmidt, A., Yus, E., Aebersold, R., Herrmann, R., Böttcher, B., Frangakis, A.S., Russell, R.B., Serrano, L., Bork, P. and Gavin, A.C. (2009) 'Proteome organization in a genome-reduced bacterium', *Science*, 326(5957), pp. 1235-1240.
- Kulyté, A., Dryselius, R., Karlsson, J. and Good, L. (2005) 'Gene selective suppression of nonsense termination using antisense agents', *Biochimica et Biophysica Acta - Gene Structure and Expression*, 1730(3), pp. 165-172.
- Kurien, B.T. and Scofield, R.H. (2006) 'Western blotting', *Methods*, 38(4), pp. 283-93.
- Kurland, C.G. and Dong, H. (1996) 'Bacterial growth inhibition by overproduction of protein', *Molecular Microbiology*, 21(1), pp. 1-4.
- Kurtboke, D.I. (2012) 'Biodiscovery from rare actinomycetes: an ecotaxonomical perspective', *Appl Microbiol Biotechnol*, 93(5), pp. 1843-52.
- Kurupati, P., Tan, K.S.W., Kumarasinghe, G. and Poh, C.L. (2007) 'Inhibition of gene expression and growth by antisense peptide nucleic acids in a multiresistant beta-lactamase-producing *Klebsiella pneumoniae* strain', *Antimicrob. Agents Chemother.*, 51(3), pp. 805-11.
- Lane, A.L. and Moore, B.S. (2011) 'A sea of biosynthesis: marine natural products meet the molecular age', *Nat Prod Rep*, 28(2), pp. 411-28.
- Lange, R.P., Locher, H.H., Wyss, P.C. and Then, R.L. (2007) 'The targets of currently used antibacterial agents: Lessons for drug discovery', *Current Pharmaceutical Design*, 13(30), pp. 3140-3154.

- Lapidot, M. and Pilpel, Y. (2006) 'Genome-wide natural antisense transcription: coupling its regulation to its different regulatory mechanisms', *EMBO Rep*, 7(12), pp. 1216-22.
- Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J. and Higgins, D.G. (2007) 'Clustal W and Clustal X version 2.0', *Bioinformatics*, 23(21), pp. 2947-2948.
- Larsen, H.J., Bentin, T. and Nielsen, P.E. (1999) 'Antisense properties of peptide nucleic acid', *Biochimica et Biophysica Acta - Gene Structure and Expression*, 1489(1), pp. 159-166.
- Laurent, J.M., Vogel, C., Kwon, T., Craig, S.A., Boutz, D.R., Huse, H.K., Nozue, K., Walia, H., Whiteley, M., Ronald, P.C. and Marcotte, E.M. (2010) 'Protein abundances are more conserved than mRNA abundances across diverse taxa', *Proteomics*, 10(23), pp. 4209-12.
- Lechevalier, H.A. and Lechevalier, M.P. (1967) 'Biology of actinomycetes', *Annual Review of Microbiology*, 21, pp. 71-100.
- Lee, E.J. and Groisman, E.A. (2010) 'An antisense RNA that governs the expression kinetics of a multifunctional virulence gene', *Mol Microbiol*, 76(4), pp. 1020-33.
- Lemon, K.P., Armitage, G.C., Relman, D.A. and Fischbach, M.A. (2012) 'Microbiota-targeted therapies: an ecological perspective', *Science translational medicine*, 4(137), pp. 137rv5-137rv5.
- Levin, P.A. 31 (2002a) '6 Light microscopy techniques for bacterial cell biology'. pp. 115-132. Available at: <http://www.scopus.com/inward/record.url?eid=2-s2.0-27944456245&partnerID=40&md5=1f0046740cc85b95d04cc328d4422624>.
- Levin, P.A. (2002b) 'Light microscopy techniques for bacterial cell biology', in Sansonetti, P. and Zychlinsky, A. (eds.) *Methods in Microbiology: Molecular Cellular Microbiology*. London: Academic Press Limited, pp. 115-132.
- Levine, E. and Hwa, T. (2008) 'Small RNAs establish gene expression thresholds', *Curr Opin Microbiol*, 11(6), pp. 574-9.
- Levine, E., Zhang, Z., Kuhlman, T. and Hwa, T. (2007) 'Quantitative characteristics of gene regulation by small RNA', *PLoS Biol*, 5(9), p. e229.

- Li, J.W. and Vederas, J.C. (2009) 'Drug discovery and natural products: end of an era or an endless frontier?', *Science*, 325(5937), pp. 161-5.
- Li, Y. and Altman, S. (2004) 'Polarity effects in the lactose operon of *Escherichia coli*', *Journal of Molecular Biology*, 339(1), pp. 31-39.
- Lidstrom, M.E. and Konopka, M.C. (2010) 'The role of physiological heterogeneity in microbial population behavior', *Nature Chemical Biology*, 6(10), pp. 705-712.
- Lipinski, C.A., Lombardo, F., Dominy, B.W. and Feeney, P.J. (2012) 'Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings', *Advanced Drug Delivery Reviews*, 64(SUPPL.), pp. 4-17.
- Liu, H., Sadygov, R.G. and Yates lii, J.R. (2004) 'A model for random sampling and estimation of relative protein abundance in shotgun proteomics', *Analytical Chemistry*, 76(14), pp. 4193-4201.
- Livak, K.J. and Schmittgen, T.D. (2001) 'Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method', *Methods*, 25(4), pp. 402-8.
- Lock, R.L. and Harry, E.J. (2008) 'Cell-division inhibitors: new insights for future antibiotics', *Nat Rev Drug Discov*, 7(4), pp. 324-38.
- Lonkar, P., Kim, K.H., Kuan, J.Y., Chin, J.Y., Rogers, F.A., Knauert, M.P., Kole, R., Nielsen, P.E. and Glazer, P.M. (2009) 'Targeted correction of a thalassemia-associated  $\beta$ -globin mutation induced by pseudo-complementary peptide nucleic acids', *Nucleic Acids Research*, 37(11), pp. 3635-3644.
- Lovering, A.L., Safadi, S.S. and Strynadka, N.C.J. 81 (2012) 'Structural perspective of peptidoglycan biosynthesis and assembly'. pp. 451-478. Available at: <http://www.scopus.com/inward/record.url?eid=2-s2.0-84861892432&partnerID=40&md5=9b1dabe66be341c89fd117fd3ca231cc>.
- Lu, P., Vogel, C., Wang, R., Yao, X. and Marcotte, E.M. (2007) 'Absolute protein expression profiling estimates the relative contributions of transcriptional and translational regulation', *Nature Biotechnology*, 25(1), pp. 117-124.
- Lundin, K.E., Good, L., Strömberg, R., Gräslund, A. and Smith, C.I.E. 56 (2006) 'Biological Activity and Biotechnological Aspects of Peptide Nucleic Acid' Hall,

- J.C., Dunlap, J.C., Friedmann, T. and Heyningen, V., pp. 1-51. Available at: <http://www.scopus.com/inward/record.url?eid=2-s2.0-33745928141&partnerID=40&md5=d9e447de1cac3e1d6f55b27d64228e5f>.
- MacBeath, G. (2002) 'Protein microarrays and proteomics', *Nature Genetics*, 32(5 SUPPL. DEC.), pp. 526-532.
- Magnuson, K., Jackowski, S., Rock, C.O. and Cronan Jr, J.E. (1993) 'Regulation of fatty acid biosynthesis in Escherichia coli', *Microbiological Reviews*, 57(3), pp. 522-542.
- Maier, T., Guell, M. and Serrano, L. (2009) 'Correlation of mRNA and protein in complex biological samples', *FEBS Lett*, 583(24), pp. 3966-73.
- Maier, T., Schmidt, A., Guell, M., Kuhner, S., Gavin, A.C., Aebersold, R. and Serrano, L. (2011) 'Quantification of mRNA and protein and integration with protein turnover in a bacterium', *Mol Syst Biol*, 7, p. 511.
- Makarov, A. (2000) 'Electrostatic axially harmonic orbital trapping: A high-performance technique of mass analysis', *Analytical Chemistry*, 72(6), pp. 1156-1162.
- Makarov, A. and Scigelova, M. (2010) 'Coupling liquid chromatography to Orbitrap mass spectrometry', *J Chromatogr A*, 1217(25), pp. 3938-45.
- Maki, H. (2002) 'Origins of spontaneous mutations: specificity and directionality of base-substitution, frameshift, and sequence-substitution mutageneses', *Annu Rev Genet*, 36, pp. 279-303.
- Malmström, J., Beck, M., Schmidt, A., Lange, V., Deutsch, E.W. and Aebersold, R. (2009) 'Proteome-wide cellular protein concentrations of the human pathogen *Leptospira interrogans*', *Nature*, 460(7256), pp. 762-765.
- Mann, M. (2008) 'Can proteomics retire the western blot?', *Journal of Proteome Research*, 7(8), p. 3065.
- Maplestone, R.A., Stone, M.J. and Williams, D.H. (1992) 'The evolutionary role of secondary metabolites - A review', *Gene*, 115(1-2), pp. 151-157.
- Margolin, W. (2005) 'FtsZ and the division of prokaryotic cells and organelles', *Nature Reviews Molecular Cell Biology*, 6(11), pp. 862-871.
- Martinez, J.L. and Baquero, F. (2000) 'Mutation frequencies and antibiotic resistance', *Antimicrobial Agents and Chemotherapy*, 44(7), pp. 1771-1777.

- Martinez, J.L., Fajardo, A., Garmendia, L., Hernandez, A., Linares, J.F., Martinez-Solano, L. and Sanchez, M.B. (2009) 'A global view of antibiotic resistance', *FEMS Microbiol Rev*, 33(1), pp. 44-65.
- Mattheakis, L., Vu, L., Sor, F. and Nomura, M. (1989) 'Retroregulation of the synthesis of ribosomal proteins L14 and L24 by feedback repressor S8 in *Escherichia coli*', *Proceedings of the National Academy of Sciences of the United States of America*, 86(2), pp. 448-452.
- Mattheakis, L.C. and Nomura, M. (1988) 'Feedback regulation of the *spc* operon in *Escherichia coli*: translational coupling and mRNA processing', *Journal of Bacteriology*, 170(10), pp. 4484-4492.
- Matuszewski, B.K., Constanzer, M.L. and Chavez-Eng, C.M. (2003) 'Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS', *Analytical Chemistry*, 75(13), pp. 3019-3030.
- Mazodier, P. and Davies, J. (1991) 'Gene transfer between distantly related bacteria', *Annual Review of Genetics*, 25, pp. 147-171.
- McAdams, H.H. and Arkin, A. (1997) 'Stochastic mechanisms in gene expression', *Proceedings of the National Academy of Sciences of the United States of America*, 94(3), pp. 814-819.
- McClerren, A.L., Cooper, L.E., Quan, C., Thomas, P.P., Kelleher, N.L. and Van Der Donk, W.A. (2006) 'Discovery and in vitro biosynthesis of haloduracin, a two-component lantibiotic', *Proceedings of the National Academy of Sciences of the United States of America*, 103(46), pp. 17243-17248.
- McDaniel, R., Ebert-Khosla, S., Hopwood, D.A. and Khosla, C. (1995) 'Rational design of aromatic polyketide natural products by recombinant assembly of enzymatic subunits', *Nature*, 375(6532), pp. 549-554.
- McDevitt, D. and Rosenberg, M. (2001) 'Exploiting genomics to discover new antibiotics', *Trends in Microbiology*, 9(12), pp. 611-617.
- McGinnis, S. and Madden, T. (2004a) 'BLAST: at the core of a powerful and diverse set of sequence analysis tools', *Nucleic Acids Res.*, 32, pp. W20-W25.
- McGinnis, S. and Madden, T.L. (2004b) 'BLAST: At the core of a powerful and diverse set of sequence analysis tools', *Nucleic Acids Research*, 32(WEB SERVER ISS.), pp. W20-W25.

- McLeod, S.M., Dougherty, T.J. and Pucci, M.J. (2012) 'Novel Antibacterial Targets/Identification of New Targets by Comparative Genomics', pp. 881-900.
- McMurry, L.M., Oethinger, M. and Levy, S.B. (1998) 'Triclosan targets lipid synthesis [4]', *Nature*, 394(6693), pp. 531-532.
- Meng, J., Kanzaki, G., Meas, D., Lam, C.K., Crummer, H., Tain, J. and Xu, H.H. (2012) 'A genome-wide inducible phenotypic screen identifies antisense RNA constructs silencing Escherichia coli essential genes', *FEMS Microbiol Lett*, 329(1), pp. 45-53.
- Menossi, M., Cremonese, N., Maron, L.G. and Arruda, P. (2000) 'Making colony PCR easier by adding gel-loading buffer to the amplification reaction', *Biotechniques*, 28(3), pp. 424-426.
- Miesel, L., Greene, J. and Black, T.A. (2003) 'Genetic strategies for antibacterial drug discovery', *Nat Rev Genet*, 4(6), pp. 442-56.
- Mills, S.D. and Dougherty, T.J. (2012) 'Cell-Based Screening in Antibacterial Discovery', pp. 901-929.
- Min, B.E., Seo, S.W. and Jung, G.Y. (2012) 'Switching control of an essential gene for reprogramming of cellular phenotypes in Escherichia coli', *Biotechnology and Bioengineering*, 109(7), pp. 1875-1880.
- Miyajima, Y., Ishizuka, T., Yamamoto, Y., Sumaoka, J. and Komiyama, M. (2009) 'Origin of high fidelity in target-sequence recognition by PNA Ce(IV)/EDTA combinations as site-selective DNA cutters', *Journal of the American Chemical Society*, 131(7), pp. 2657-2662.
- Mogk, A., Huber, D. and Bukau, B. (2011) 'Integrating protein homeostasis strategies in prokaryotes', *Cold Spring Harbor perspectives in biology*, 3(4).
- Møller, T., Franch, T., Udesen, C., Gerdes, K. and Valentin-Hansen, P. (2002) 'Spot 42 RNA mediates discoordinate expression of the E. coli galactose operon', *Genes and Development*, 16(13), pp. 1696-1706.
- Montange, R.K. and Batey, R.T. (2008) 'Riboswitches: emerging themes in RNA structure and function', *Annu Rev Biophys*, 37, pp. 117-33.
- Moya, A., Gil, R., Latorre, A., Peretó, J., Pilar Garcillán-Barcia, M. and De La Cruz, F. (2009) 'Toward minimal bacterial cells: Evolution vs. design', *FEMS Microbiology Reviews*, 33(1), pp. 225-235.

- Mruk, I., Liu, Y., Ge, L. and Kobayashi, I. (2011) 'Antisense RNA associated with biological regulation of a restriction-modification system', *Nucleic Acids Res*, 39(13), pp. 5622-32.
- Mushegian, A.R. and Koonin, E.V. (1996) 'A minimal gene set for cellular life derived by comparison of complete bacterial genomes', *Proceedings of the National Academy of Sciences of the United States of America*, 93(19), pp. 10268-10273.
- Nagalakshmi, U., Wang, Z., Waern, K., Shou, C., Raha, D., Gerstein, M. and Snyder, M. (2008) 'The transcriptional landscape of the yeast genome defined by RNA sequencing', *Science*, 320(5881), pp. 1344-1349.
- Nakashima, N. and Tamura, T. (2009) 'Conditional gene silencing of multiple genes with antisense RNAs and generation of a mutator strain of Escherichia coli', *Nucleic Acids Research*, 37(15).
- Nakashima, N., Tamura, T. and Good, L. (2006) 'Paired termini stabilize antisense RNAs and enhance conditional gene silencing in Escherichia coli', *Nucleic Acids Res*, 34(20), p. e138.
- Nekhotiaeva, N., Awasthi, S.K., Nielsen, P.E. and Good, L. (2004) 'Inhibition of Staphylococcus aureus gene expression and growth using antisense peptide nucleic acids', *Molecular Therapy*, 10(4), pp. 652-659.
- Nielsen, P.E. (2010) 'Peptide Nucleic Acids (PNA) in chemical biology and drug discovery', *Chemistry and Biodiversity*, 7(4), pp. 786-804.
- Nielsen, P.E., Egholm, M., Berg, R.H. and Buchardt, O. (1991) 'Sequence-selective recognition of DNA by strand displacement with thymine-substituted polyamide', *Science*, 254(5037), pp. 1497-1500.
- Nikaido, H. (2003) 'Molecular Basis of Bacterial Outer Membrane Permeability Revisited', *Microbiology and Molecular Biology Reviews*, 67(4), pp. 593-656.
- Nikraves, A., Dryselius, R., Faridani, O.R., Goh, S., Sadeghizadeh, M., Behmanesh, M., Ganyu, A., Klok, E.J., Zain, R. and Good, L. (2007) 'Antisense PNA accumulates in Escherichia coli and mediates a long post-antibiotic effect', *Molecular Therapy*, 15(8), pp. 1537-1542.
- Nolan, T., Hands, R.E. and Bustin, S.A. (2006) 'Quantification of mRNA using real-time RT-PCR', *Nat Protoc*, 1(3), pp. 1559-82.



- Nomura, M., Yates, J.L., Dean, D. and Post, L.E. (1980) 'Feedback regulation of ribosomal protein gene expression in *Escherichia coli*: Structural homology of ribosomal RNA and ribosomal protein mRNA', *Proceedings of the National Academy of Sciences of the United States of America*, 77(12 II), pp. 7084-7088.
- O'Shea, R. and Moser, H.E. (2008) 'Physicochemical properties of antibacterial compounds: Implications for drug discovery', *Journal of Medicinal Chemistry*, 51(10), pp. 2871-2878.
- Olsen, J.V., Ong, S.E. and Mann, M. (2004) 'Trypsin cleaves exclusively C-terminal to arginine and lysine residues', *Mol Cell Proteomics*, 3(6), pp. 608-14.
- Omura, S., Ikeda, H., Ishikawa, J., Hanamoto, A., Takahashi, C., Shinose, M., Takahashi, Y., Horikawa, H., Nakazawa, H., Osonoe, T., Kikuchi, H., Shiba, T., Sakaki, Y. and Hattori, M. (2001) 'Genome sequence of an industrial microorganism *Streptomyces avermitilis*: Deducing the ability of producing secondary metabolites', *Proceedings of the National Academy of Sciences of the United States of America*, 98(21), pp. 12215-12220.
- Ong, S.E., Blagoev, B., Kratchmarova, I., Kristensen, D.B., Steen, H., Pandey, A. and Mann, M. (2002) 'Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics', *Molecular & cellular proteomics : MCP*, 1(5), pp. 376-386.
- Opdyke, J.A., Kang, J.G. and Storz, G. (2004) 'GadY, a small-RNA regulator of acid response genes in *Escherichia coli*', *J Bacteriol*, 186(20), pp. 6698-705.
- Oyamada, Y., Ito, H., Fujimoto-Nakamura, M., Tanitame, A., Iwai, N., Nagai, K., Yamagishi, J.I. and Wachi, M. (2006) 'Anucleate cell blue assay: A useful tool for identifying novel type II topoisomerase inhibitors', *Antimicrobial Agents and Chemotherapy*, 50(1), pp. 348-350.
- Pathom-aree, W., Stach, J.E.M., Ward, A.C., Horikoshi, K., Bull, A.T. and Goodfellow, M. (2006) 'Diversity of actinomycetes isolated from Challenger Deep sediment (10,898 m) from the Mariana Trench', *Extremophiles*, 10(3), pp. 181-189.
- Patterson, S.D. and Aebersold, R.H. (2003) 'Proteomics: The first decade and beyond', *Nature Genetics*, 33(SUPPL.), pp. 311-323.

- Pavankumar, A.R., Ayyappasamy, S.P. and Sankaran, K. (2012) 'Small RNA fragments in complex culture media cause alterations in protein profiles of three species of bacteria', *BioTechniques*, 52(3), pp. 167-172.
- Payne, D.J., Gwynn, M.N., Holmes, D.J. and Pompliano, D.L. (2007) 'Drugs for bad bugs: confronting the challenges of antibacterial discovery', *Nat Rev Drug Discov*, 6(1), pp. 29-40.
- Pfeiffer, V., Sittka, A., Tomer, R., Tedin, K., Brinkmann, V. and Vogel, J. (2007) 'A small non-coding RNA of the invasion gene island (SPI-1) represses outer membrane protein synthesis from the *Salmonella* core genome', *Molecular Microbiology*, 66(5), pp. 1174-1191.
- Picotti, P. and Aebersold, R. (2012) 'Selected reaction monitoring-based proteomics: workflows, potential, pitfalls and future directions', *Nat Methods*, 9(6), pp. 555-66.
- Purnick, P.E.M. and Weiss, R. (2009) 'The second wave of synthetic biology: From modules to systems', *Nature Reviews Molecular Cell Biology*, 10(6), pp. 410-422.
- Qiu, X.-Q., Wang, H., Lu, X.-F., Zhang, J., Li, S.-F., Cheng, G., Wan, L., Yang, L., Zuo, J.-Y., Zhou, Y.-Q., Wang, H.-Y., Cheng, X., Zhang, S.-H., Ou, Z.-R., Zhong, Z.-C., Cheng, J.-Q., Li, Y.-P. and Wu, G.Y. (2003) 'An engineered multidomain bactericidal peptide as a model for targeted antibiotics against specific bacteria.', *Nature Biotechnology*, 21(12), pp. 1480-1485.
- Qiu, X.-Q., Zhang, J., Wang, H. and Wu, G.Y. (2005) 'A novel engineered peptide, a narrow-spectrum antibiotic, is effective against vancomycin-resistant *Enterococcus faecalis*.', *Antimicrob. Agents and Chemother.*, 49(3), pp. 1184-1189.
- Qiu, X., Janson, C.A., Court, R.I., Smyth, M.G., Payne, D.J. and Abdel-Meguid, S.S. (1999) 'Molecular basis for triclosan activity involves a flipping loop in the active site', *Protein Science*, 8(11), pp. 2529-2532.
- Raj, A. and van Oudenaarden, A. (2008) 'Nature, Nurture, or Chance: Stochastic Gene Expression and Its Consequences', *Cell*, 135(2), pp. 216-226.

- Raju, R.M., Goldberg, A.L. and Rubin, E.J. (2012) 'Bacterial proteolytic complexes as therapeutic targets', *Nature Reviews Drug Discovery*, 11(10), pp. 777-789.
- Rappsilber, J., Ryder, U., Lamond, A.I. and Mann, M. (2002) 'Large-scale proteomic analysis of the human spliceosome', *Genome Research*, 12(8), pp. 1231-1245.
- Rasmussen, L.C., Sperling-Petersen, H.U. and Mortensen, K.K. (2007) 'Hitting bacteria at the heart of the central dogma: sequence-specific inhibition', *Microb Cell Fact*, 6, p. 24.
- Reich, K.A., Chovan, L. and Hessler, P. (1999) 'Genome scanning in *Haemophilus influenzae* for identification of essential genes', *Journal of Bacteriology*, 181(16), pp. 4961-4968.
- Rodnina, M.V., Savelsbergh, A., Katunin, V.I. and Wintermeyer, W. (1997) 'Hydrolysis of GTP by elongation factor G drives tRNA movement on the ribosome', *Nature*, 385(6611), pp. 37-41.
- Rustad, T.R., Minch, K.J., Brabant, W., Winkler, J.K., Reiss, D.J., Baliga, N.S. and Sherman, D.R. (2013) 'Global analysis of mRNA stability in *Mycobacterium tuberculosis*', *Nucleic Acids Research*, 41(1), pp. 509-517.
- Rutherford, K., Parkhill, J., Crook, J., Horsnell, T., Rice, P., Rajandream, M.A. and Barrell, B. (2000) 'Artemis: Sequence visualization and annotation', *Bioinformatics*, 16(10), pp. 944-945.
- Sadygov, R.G., Cociorva, D. and Yates, J.R., 3rd (2004) 'Large-scale database searching using tandem mass spectra: looking up the answer in the back of the book', *Nat Methods*, 1(3), pp. 195-202.
- Saito, K. and Nomura, M. (1994) 'Post-transcriptional regulation of the *str* operon in *Escherichia coli*. Structural and mutational analysis of the target site for translational repressor S7', *Journal of Molecular Biology*, 235(1), pp. 125-139.
- Saleh, S., Haddadin, R.N., Baillie, S. and Collier, P.J. (2011) 'Triclosan - an update', *Lett Appl Microbiol*, 52(2), pp. 87-95.
- Sapan, C.V., Lundblad, R.L. and Price, N.C. (1999) 'Colorimetric protein assay techniques', *Biotechnology and Applied Biochemistry*, 29(2), pp. 99-108.

Schlünzen, F., Harms, J.M., Franceschi, F., Hansen, H.A.S., Bartels, H., Zarivach, R. and Yonath, A. (2003) 'Structural basis for the antibiotic activity of ketolides and azalides', *Structure*, 11(3), pp. 329-338.

Schrimpf, S.P., Weiss, M., Reiter, L., Ahrens, C.H., Jovanovic, M., Malmström, J., Brunner, E., Mohanty, S., Lercher, M.J., Hunziker, P.E., Aebersold, R., von Mering, C. and Hengartner, M.O. (2009) 'Comparative functional analysis of the *Caenorhabditis elegans* and *Drosophila melanogaster* proteomes', *PLoS biology*, 7(3).

Sezonov, G., Joseleau-Petit, D. and D'Ari, R. (2007) 'Escherichia coli physiology in Luria-Bertani broth', *Journal of Bacteriology*, 189(23), pp. 8746-8749.

Shajani, Z., Sykes, M.T. and Williamson, J.R. 80 (2011) 'Assembly of bacterial ribosomes'. pp. 501-526. Available at: <http://www.scopus.com/inward/record.url?eid=2-s2.0-79959438183&partnerID=40&md5=7a697ee667696f0fec854a607a6acc91>.

Shen, N., Ko, J.H., Xiao, G., Wesolowski, D., Shan, G., Geller, B., Izadjoo, M. and Altman, S. (2009) 'Inactivation of expression of several genes in a variety of bacterial species by EGS technology', *Proc. Natl. Acad. Sci. U.S.A.*, 106(20), pp. 8163-8.

Shevchenko, A., Tomas, H., Havlis, J., Olsen, J.V. and Mann, M. (2006) 'In-gel digestion for mass spectrometric characterization of proteins and proteomes', *Nat Protoc*, 1(6), pp. 2856-60.

Shimoni, Y., Friedlander, G., Hetzroni, G., Niv, G., Altuvia, S., Biham, O. and Margalit, H. (2007) 'Regulation of gene expression by small non-coding RNAs: a quantitative view', *Mol Syst Biol*, 3, p. 138.

Silver, L. (2012) 'Rational Approaches to Antibacterial Discovery: Pre-Genomic Directed and Phenotypic Screening', in Dougherty, T.J.P., M.J. (ed.) *Antibiotic Discovery and Development*. USA: Springer Science+Business Media, LLC, pp. 33-75.

Silver, L.L. (2007) 'Multi-targeting by monotherapeutic antibacterials', *Nature Reviews Drug Discovery*, 6(1), pp. 41-55.

- Silver, L.L. (2008) 'Are natural products still the best source for antibacterial discovery? The bacterial entry factor', *Expert Opinion on Drug Discovery*, 3(5), pp. 487-500.
- Silver, L.L. (2011) 'Challenges of antibacterial discovery', *Clinical Microbiology Reviews*, 24(1), pp. 71-109.
- Silver, L.L. and Bostian, K.A. (1993) 'Discovery and development of new antibiotics: The problem of antibiotic resistance', *Antimicrobial Agents and Chemotherapy*, 37(3), pp. 377-383.
- Simons, R.W. and Kleckner, N. (1988) 'Biological regulation by antisense RNA in prokaryotes', *Annual Review of Genetics*, 22, pp. 567-600.
- Singh, S.B., Phillips, J.W. and Wang, J. (2007) 'Highly sensitive target-based whole-cell antibacterial discovery strategy by antisense RNA silencing', *Current Opinion in Drug Discovery and Development*, 10(2), pp. 160-166.
- Slater-Radosti, C., Van Aller, G., Greenwood, R., Nicholas, R., Kellerb, P.M., DeWolf Jr, W.E., Fan, F., Payne, D.J. and Jaworskia, D.D. (2001) 'Biochemical and genetic characterization of the action of triclosan on *Staphylococcus aureus*', *Journal of Antimicrobial Chemotherapy*, 48(1), pp. 1-6.
- Snijders, A.P.L., De Koning, B. and Wright, P.C. (2005) 'Perturbation and interpretation of nitrogen isotope distribution patterns in proteomics', *Journal of Proteome Research*, 4(6), pp. 2185-2191.
- Sorek, R., Kunin, V. and Hugenholtz, P. (2008) 'CRISPR - A widespread system that provides acquired resistance against phages in bacteria and archaea', *Nature Reviews Microbiology*, 6(3), pp. 181-186.
- Srinivas, N., Jetter, P., Ueberbacher, B.J., Werneburg, M., Zerbe, K., Steinmann, J., Van der Meijden, B., Bernardini, F., Lederer, A., Dias, R.L.A., Misson, P.E., Henze, H., Zumbunn, J., Gombert, F.O., Obrecht, D., Hunziker, P., Schauer, S., Ziegler, U., Kach, A., Eberl, L., Riedel, K., DeMarco, S.J. and Robinson, J.A. (2010) 'Peptidomimetic Antibiotics Target Outer-Membrane Biogenesis in *Pseudomonas aeruginosa*', *Science*, 327(5968), pp. 1010-1013.
- Stach, J. and Good, L. (2011) 'Synthetic RNA silencing in bacteria – antimicrobial discovery and resistance breaking', *Frontiers in Antimicrobials*, 2 pp. Article 185 1-11.

- Stach, J.E.M. and Bull, A.T. (2005) 'Estimating and comparing the diversity of marine actinobacteria', *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, 87(1), pp. 3-9.
- Steitz, T.A. (2008) 'A structural understanding of the dynamic ribosome machine', *Nature Reviews Molecular Cell Biology*, 9(3), pp. 242-253.
- Stirchak, E.P., Summerton, J.E. and Weller, D.D. (1989) 'Uncharged stereoregular nucleic acid analogs: 2. Morpholino nucleoside oligomers with carbamate internucleoside linkages', *Nucleic Acids Research*, 17(15), pp. 6129-6141.
- Stork, M., Di Lorenzo, M., Welch, T.J. and Crosa, J.H. (2007) 'Transcription termination within the iron transport-biosynthesis operon of *Vibrio anguillarum* requires an antisense RNA', *J Bacteriol*, 189(9), pp. 3479-88.
- Stratton, C.W. (2003) 'Dead bugs don't mutate: Susceptibility issues in the emergence of bacterial resistance', *Emerging Infectious Diseases*, 9(1), pp. 10-16.
- Su, C., Peregrin-Alvarez, J.M., Butland, G., Phanse, S., Fong, V., Emili, A. and Parkinson, J. (2008) 'Bacteriome.org - An integrated protein interaction database for *E. coli*', *Nucleic Acids Research*, 36(SUPPL. 1), pp. D632-D636.
- Summerton, J. and Weller, D. (1997) 'Morpholino antisense oligomers: Design, preparation, and properties', *Antisense and Nucleic Acid Drug Development*, 7(3), pp. 187-195.
- Summerton, J.E. (2006) 'Morpholinos and PNAs Compared', in *Peptide Nucleic Acids, Morpholinos and related Antisense Biomolecules*. Kluwer Academic / Plenum Publishing, pp. 91-113.
- Sung, K.L., Chou, H.H., Pflieger, B.F., Newman, J.D., Yoshikuni, Y. and Keasling, J.D. (2007) 'Directed evolution of AraC for improved compatibility of arabinose- and lactose-inducible promoters', *Applied and Environmental Microbiology*, 73(18), pp. 5711-5715.
- Tahlan, K., Ahn, S.K., Sing, A., Bodnaruk, T.D., Willems, A.R., Davidson, A.R. and Nodwell, J.R. (2007) 'Initiation of actinorhodin export in *Streptomyces coelicolor*', *Molecular Microbiology*, 63(4), pp. 951-961.

Takahata, S., Ida, T., Hiraishi, T., Sakakibara, S., Maebashi, K., Terada, S., Muratani, T., Matsumoto, T., Nakahama, C. and Tomono, K. (2010) 'Molecular mechanisms of fosfomycin resistance in clinical isolates of *Escherichia coli*', *International Journal of Antimicrobial Agents*, 35(4), pp. 333-337.

Tamae, C., Liu, A., Kim, K., Sitz, D., Hong, J., Becket, E., Bui, A., Solaimani, P., Tran, K.P., Yang, H. and Miller, J.H. (2008) 'Determination of antibiotic hypersensitivity among 4,000 single-gene-knockout mutants of *Escherichia coli*', *Journal of Bacteriology*, 190(17), pp. 5981-5988.

Then, R.L. and Sahl, H.-G. (2010) 'Anti-infective strategies of the future: is there room for species-specific antibacterial agents?', *Current Pharmaceutical Design*, 16, pp. 555-566.

Thisted, T. and Gerdes, K. (1992) 'Mechanism of post-segregational killing by the *hok/sok* system of plasmid R1: *Sok* antisense RNA regulates *hok* gene expression indirectly through the overlapping *mok* gene', *Journal of Molecular Biology*, 223(1), pp. 41-54.

Thomason, M.K. and Storz, G. (2010) 'Bacterial antisense RNAs: how many are there, and what are they doing?', *Annu Rev Genet*, 44, pp. 167-88.

Thompson, A., Schäfer, J., Kuhn, K., Kienle, S., Schwarz, J., Schmidt, G., Neumann, T. and Hamon, C. (2003) 'Tandem mass tags: A novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS', *Analytical Chemistry*, 75(8), pp. 1895-1904.

Tilley, L.D., Hine, O.S., Kellogg, J.A., Hassinger, J.N., Weller, D.D., Iversen, P.L. and Geller, B.L. (2006) 'Gene-specific effects of antisense phosphorodiamidate morpholino oligomer-peptide conjugates on *Escherichia coli* and *Salmonella enterica* serovar Typhimurium in pure culture and in tissue culture', *Antimicrob. Agents Chemother.*, 50(8), pp. 2789-96.

Tillotson, G.S. (2008) 'Where does novel antibiotics R&D stand among other pharmaceutical products: An industrial perspective?', *Expert Review of Anti-Infective Therapy*, 6(5), pp. 551-552.

Tomizawa, J., Itoh, T., Selzer, G. and Som, T. (1981) 'Inhibition of ColE1 RNA primer formation by a plasmid-specified small RNA', *Proceedings of the National Academy of Sciences of the United States of America*, 78(3), pp. 1421-1425.

- Tsai, S.P., Hartin, R.J. and Ryu, J. (1989) 'Transformation in restriction-deficient *Salmonella typhimurium* LT2', *Journal of General Microbiology*, 135(9), pp. 2561-2567.
- Turner, P.R. and Denny, W.A. (2000) 'The genome as a drug target: Sequence specific minor groove binding ligands', *Current Drug Targets*, 1(1), pp. 1-14.
- Typas, A., Nichols, R.J., Siegele, D.A., Shales, M., Collins, S.R., Lim, B., Braberg, H., Yamamoto, N., Takeuchi, R., Wanner, B.L., Mori, H., Weissman, J.S., Krogan, N.J. and Gross, C.A. (2008) 'High-throughput, quantitative analyses of genetic interactions in *E. coli*', *Nat. Methods*, 5(9), pp. 781-787.
- Udvary, D.W., Zeigler, L., Asolkar, R.N., Singan, V., Lapidus, A., Fenical, W., Jensen, P.R. and Moore, B.S. (2007) 'Genome sequencing reveals complex secondary metabolome in the marine actinomycete *Salinispora tropica*', *Proceedings of the National Academy of Sciences of the United States of America*, 104(25), pp. 10376-10381.
- Vaara, M. and Porro, M. (1996) 'Group of peptides that act synergistically with hydrophobic antibiotics against gram-negative enteric bacteria', *Antimicrobial Agents and Chemotherapy*, 40(8), pp. 1801-1805.
- Vaillancourt, F.H., Yeh, E., Vosburg, D.A., Garneau-Tsodikova, S. and Walsh, C.T. (2006) 'Nature's inventory of halogenation catalysts: Oxidative strategies predominate', *Chemical Reviews*, 106(8), pp. 3364-3378.
- Van Eeckhaut, A., Lanckmans, K., Sarre, S., Smolders, I. and Michotte, Y. (2009) 'Validation of bioanalytical LC-MS/MS assays: evaluation of matrix effects', *J Chromatogr B Analyt Technol Biomed Life Sci*, 877(23), pp. 2198-207.
- Van Lanen, S.G. and Shen, B. (2006) 'Microbial genomics for the improvement of natural product discovery', *Curr Opin Microbiol*, 9(3), pp. 252-60.
- Vasquez, K.M., Marburger, K., Intody, Z. and Wilson, J.H. (2001) 'Manipulating the mammalian genome by homologous recombination', *Proceedings of the National Academy of Sciences of the United States of America*, 98(15), pp. 8403-8410.



- Velicer, C.M., Heckbert, S.R., Lampe, J.W., Potter, J.D., Robertson, C.A. and Taplin, S.H. (2004) 'Antibiotic use in relation to the risk of breast cancer', *JAMA, J. Am. Med. Assoc.*, 291(7), pp. 827-835.
- Vogel, C. and Marcotte, E.M. (2012) 'Insights into the regulation of protein abundance from proteomic and transcriptomic analyses', *Nat Rev Genet*, 13(4), pp. 227-32.
- Vogel, J. (2009) 'A rough guide to the non-coding RNA world of *Salmonella*', *Molecular Microbiology*, 71(1), pp. 1-11.
- Vogel, J. and Luisi, B.F. (2011) 'Hfq and its constellation of RNA', *Nat Rev Microbiol*, 9(8), pp. 578-89.
- Walsh, C. (2003) *Antibiotics: actions, origins, resistance*. United States of America: ASM Press.
- Wang, J.B., Pan, H.X. and Tang, G.L. (2011) 'Production of doramectin by rational engineering of the avermectin biosynthetic pathway', *Bioorganic and Medicinal Chemistry Letters*, 21(11), pp. 3320-3323.
- Wang, Z., Gerstein, M. and Snyder, M. (2009) 'RNA-Seq: A revolutionary tool for transcriptomics', *Nature Reviews Genetics*, 10(1), pp. 57-63.
- Waters, L.S. and Storz, G. (2009) 'Regulatory RNAs in bacteria', *Cell*, 136(4), pp. 615-28.
- Watve, M.G., Tickoo, R., Jog, M.M. and Bhole, B.D. (2001) 'How many antibiotics are produced by the genus *Streptomyces*?', *Arch Microbiol*, 176(5), pp. 386-90.
- Webhofer, C. (2013) '<sup>15</sup>N metabolic labeling: Evidence for a stable isotope effect on plasma protein levels and peptide chromatographic retention times', *Journal of Proteomics*.
- Wei, J.R., Krishnamoorthy, V., Murphy, K., Kim, J.H., Schnappinger, D., Alber, T., Sasseti, C.M., Rhee, K.Y. and Rubin, E.J. (2011) 'Depletion of antibiotic targets has widely varying effects on growth', *Proceedings of the National Academy of Sciences of the United States of America*, 108(10), pp. 4176-4181.
- Weissman, K.J. and Leadlay, P.F. (2005) 'Combinatorial biosynthesis of reduced polyketides', *Nat Rev Microbiol*, 3(12), pp. 925-36.

- Wenzel, M., Patra, M., Albrecht, D., Chen, D.Y., Nicolaou, K.C., Metzler-Nolte, N. and Bandow, J.E. (2011) 'Proteomic signature of fatty acid biosynthesis inhibition available for in vivo mechanism-of-action studies', *Antimicrob Agents Chemother*, 55(6), pp. 2590-6.
- White, D.G., Maneewannakul, K., vonHofe, E., Zillman, M., Eisenberg, W., Field, A.K. and Levy, S.B. (1997) 'Inhibition of the multiple antibiotic resistance (mar) operon in *Escherichia coli* by antisense DNA analogs', *Antimicrob. Agents Chemother.*, 41(12), pp. 2699-2704.
- Wiese, S., Reidegeld, K.A., Meyer, H.E. and Warscheid, B. (2007) 'Protein labeling by iTRAQ: A new tool for quantitative mass spectrometry in proteome research', *Proteomics*, 7(3), pp. 340-350.
- Williams, D.H., Stone, M.J., Hauck, P.R. and Rahman, S.K. (1989) 'Why are secondary metabolites (natural products) biosynthesized?', *Journal of Natural Products*, 52(6), pp. 1189-1208.
- Williams, P.G. (2009) 'Panning for chemical gold: marine bacteria as a source of new therapeutics', *Trends in Biotechnology*, 27(1), pp. 45-52.
- Wright, G.D. (2005) 'Bacterial resistance to antibiotics: Enzymatic degradation and modification', *Advanced Drug Delivery Reviews*, 57(10), pp. 1451-1470.
- Wright, G.D. (2007) 'The antibiotic resistome: the nexus of chemical and genetic diversity', *Nat Rev Microbiol*, 5(3), pp. 175-86.
- Xie, F., Liu, T., Qian, W.J., Petyuk, V.A. and Smith, R.D. (2011) 'Liquid chromatography-mass spectrometry-based quantitative proteomics', *J Biol Chem*, 286(29), pp. 25443-9.
- Xu, H.H., Real, L. and Bailey, M.W. (2006) 'An array of *Escherichia coli* clones over-expressing essential proteins: a new strategy of identifying cellular targets of potent antibacterial compounds', *Biochem Biophys Res Commun*, 349(4), pp. 1250-7.
- Xu, X.P. and Case, D.A. (2002) 'Probing multiple effects on  $^{15}\text{N}$ ,  $^{13}\text{C}_\alpha$ ,  $^{13}\text{C}_\beta$ , and  $^{13}\text{C}'$  chemical shifts in peptides using density functional theory', *Biopolymers*, 65(6), pp. 408-423.
- Yamaichi, Y., Duigou, S., Shakhnovich, E.A. and Waldor, M.K. (2009) 'Targeting the Replication Initiator of the Second *Vibrio* Chromosome: Towards Generation

of *Vibrionaceae*-Specific Antimicrobial Agents', *Plos Pathogens*, 5(11), p. e1000663.

Yates, C.M., Shaw, D.J., Roe, A.J., Woolhouse, M.E.J. and Amyes, S.G.B. (2006) 'Enhancement of bacterial competitive fitness by apramycin resistance plasmids from non-pathogenic *Escherichia coli*', *Biology Letters*, 2(3), pp. 463-465.

Yates, J.L., Arfsten, A.E. and Nomura, M. (1980) 'In vitro expression of *Escherichia coli* ribosomal protein genes: Autogenous inhibition of translation', *Proceedings of the National Academy of Sciences of the United States of America*, 77(4 I), pp. 1837-1841.

Yoshida, T., Nasu, H. and Yamashita, M. (2012) 'Construction of the control system of target molecule expression in *Escherichia coli*: application to a validation platform for bactericidal and bacteriostatic profiles due to suppression of a target molecule', *FEMS Microbiol Lett*, 331(2), pp. 113-9.

Zerikly, M. and Challis, G.L. (2009) 'Strategies for the discovery of new natural products by genome mining', *ChemBiochem*, 10(4), pp. 625-33.

Zhang, R. and Lin, Y. (2009a) 'DEG 5.0, a database of essential genes in both prokaryotes and eukaryotes', *Nucleic Acids Research*, 37, pp. D455-D458.

Zhang, R. and Lin, Y. (2009b) 'DEG 5.0, a database of essential genes in both prokaryotes and eukaryotes', *Nucleic Acids Research*, 37(SUPPL. 1), pp. D455-D458.

Zipser, D. (1969) 'Polar mutations and operon function', *Nature*, 221(5175), pp. 21-25.

## Appendix A: Growth Media & Buffer compositions

### Luria-Bertani (LB) Agar

Tryptone	-	10.0 g/L
Yeast Extract	-	5.0 g/L
Sodium Chloride	-	5.0 g/L
Agar	-	15.0 g/L

### Muller Hinton Broth

Beef Infusion Solids	-	2.0 g/L
Casein hydrolysate	-	17.5 g/L
Starch	-	1.5 g/L

### SOB medium

Tryptone	-	20g/L
Yeast extract	-	5g/L
Sodium chloride	-	0.5g/L
Potassium chloride	-	10 ml of 250 mM
Magnesium Chloride	-	5ml sterile 2 M prior to use

### SOC Medium

As SOB medium with the addition of 20 ml of 1 M glucose to provide a final 20 mM concentration.

### M9 Minimal media

Disodium phosphate	-	33.78 g/L
Monopotassium phosphate	-	15 g/L
Sodium chloride	-	2.5 g/L

Ammonium chloride	-	5 g/L
Magnesium sulphate	-	2 mM
Glycerol	-	20%
Calcium chloride	-	0.1 mM
Iron Sulphate	-	0.01 mM

## **Appendix B: Publications associated with this study**

Mondhe, M., Ashley Chessher, A., Good, L., Stach, J.E.M (2012). **Species-selective killing of bacteria by "smart" antimicrobial peptide-PNAs.** (In review at time of print).

Chessher, A. (2012). Evaluating the suitability of essential genes as targets for antibiotic screening assays using proteomics. *Protein & Cell*. 3(1): 5–7.

# Species-selective killing of bacteria by "smart" antimicrobial peptide-PNAs

Madhav Mondhe<sup>1†</sup>, Ashley Chessher<sup>1</sup>, Liam Good<sup>2</sup> and James E. M. Stach<sup>1#</sup>

School of Biology, Newcastle University, Newcastle upon Tyne, NE1 7RU, United Kingdom<sup>1</sup>, Department of Pathology and Infectious Diseases, Royal Veterinary College, University of London, London, AL9 7TA, United Kingdom<sup>2</sup>

Running title: Species-specific antibacterials

Keywords: species-specific, species-selective, antibiotic, antimicrobial, antisense, peptide-nucleic acid, narrow-spectrum, microbiome

---

# Corresponding author. Mailing address for J. Stach, School of Biology, Newcastle University, Newcastle-upon-Tyne, NE1 7RU, United Kingdom. Phone: +44 191 222 7709. Fax: +44 191 222 5228. E-mail: jem.stach@ncl.ac.uk. † Present address: Cardiff Institute of Infection & Immunity, Tenovus Building, Heath Park, Cardiff CF14 4XN

Broad-spectrum antimicrobials kill indiscriminately, a property that can lead to negative clinical consequences and an increase in the incidence of resistance. Species-specific antimicrobials that could selectively kill pathogenic bacteria without targeting other species in the microbiome could limit these problems. The pathogen genome presents an excellent target for the development of such antimicrobials. In this study we report the design and evaluation of species-selective peptide nucleic acid (PNA) antibacterials. Selective growth inhibition of *B. subtilis*, *E. coli*, *K. pneumoniae* and *S. enterica* serovar *Typhimurium* in axenic or mixed culture could be achieved with PNAs that exploit species differences in the translation initiation region of essential genes. An *S. Typhimurium*-specific PNA targeting *ftsZ* resulted in a cell elongation phenotype that was not observed in *E. coli*, providing phenotypic evidence of the selectivity of PNA-based antimicrobials. Analysis of the genomes of *E. coli* and *S. Typhimurium* gave a conservative estimate of >150 PNA targets that could potentially discriminate between these two closely related species. This work provides a basis for the development of a single class of antimicrobial with a tuneable spectrum of activity.

## Introduction.

Treatment of bacterial infections with antimicrobial drugs has been clinically effective for over six decades. However, the rise of antimicrobial resistance threatens to limit options for the treatment of life-threatening microbial diseases (Jayaraman, 2009). Arturo Casadevall noted that antimicrobial therapy 'is the only branch of medicine where therapeutic options were better in the mid-20th century than at the beginning of the 21st century (Casadevall, 2006)'. A critical factor behind the rise in resistance is the spectrum of activity of the antibacterial agent; the development of broad-spectrum agents (effective against a number of microbial species) enabled empirical usage (i.e. without the use of diagnostic identification of the pathogen) and rapid treatment of fulminate microbial infections. However, it is this broad-spectrum of activity that is partly responsible for the prevalence of multi-drug resistant bacterial pathogens. Also, the human body plays host to hundreds of microbial species, collectively termed the microbiome (Eckburg et al., 2005), the majority of which are beneficial to health.



Thus, the use of non-specific broad-spectrum antimicrobial agents will have unintended detrimental effects on the microbiome, which can result in colonization of pathogenic microbes. Examples of such opportunistic infections include *Clostridium difficile*-associated diarrhea, antibiotic-associated colitis and candidiasis (Fowler et al., 2007; Casadevall, 2009). Perhaps of greater concern is the link between the microbiome and the normal development of the immune system; there are indications that the use of broad-spectrum agents may increase the risk of diseases such as asthma, eczema, rhinoconjunctivitis and breast cancer (Velicer et al., 2004; Foliaki et al., 2009). Furthermore, broad-spectrum antimicrobial treatment selects for resistance mechanisms in non-target species that are readily transferred to pathogenic species (Hoban, 2003; Yates et al., 2006). The impact of broad-spectrum antimicrobial agents on the gut microflora was highlighted in a recent study by Jakobsson et al (Jakobsson et al., 2010): the authors reported that treatment of *Helicobacter pylori* infection with the macrolide antibiotic clarithromycin, resulted in perturbation of the gut microflora associated with an increase in level of the macrolide antibiotic resistance gene *erm(B)*. These effects were observable four years after antibiotic exposure.

Recent findings demonstrating the importance of the microbiome in host health, have led to a growing interest in the use of microbiota-targeted therapies that can eliminate individual strains of single species (Lemon et al., 2012), for example, through targeting of drugs to microbial genes (Holmes et al., 2012). The increasing incidence of multi-drug resistant bacteria, coupled to declining discovery of novel broad-spectrum antimicrobial compounds, will likely make the development of species-specific antimicrobials a necessity; simultaneously providing the market conditions required for such a change in emphasis within drug development companies. Improved on-site diagnostics should soon enable pathogen/drug pairing in clinics. The development and implementation of any species-specific antimicrobial possess significant clinical challenges: nevertheless, the approach, alongside immunotherapy aimed at improving host responses, is predicted to deliver the “Third Age of antimicrobial therapy (Casadevall, 2006; Casadevall, 2009; Then and Sahl, 2010)”.

Technologies for species-specific pathogen inhibition include immunotherapy, radioimmunotherapy, anti-virulence agents, and phage therapy

(Then and Sahl, 2010, and references therein). Growing interest in narrow-spectrum antimicrobial compounds is evident in recent studies that have developed species-specific antimicrobial screens (Granieri et al., 2009) and through the identification of species-selective antimicrobial compounds (Qiu et al., 2003; Qiu et al., 2005; Eckert et al., 2006a; Eckert et al., 2006b; Yamaichi et al., 2009; Srinivas et al., 2010). Theoretically, the DNA sequence of the genome itself provides the ultimate species-specific target; antimicrobial compounds that bind to nucleic acids in a sequence-specific manner, can be used to define the spectrum of activity. Natural products such as netropsin and distamycin bind to adenine and thymine rich DNA sequences in the minor groove of DNA (Turner and Denny, 2000), with synthetic compounds that recognise all four bases having been described (Dervan, 2001). An alternative approach to targeting specific sequences in the chromosome is silencing of essential genes using antisense mechanisms. Exogenously delivered antisense DNA oligonucleotides, designed to bind to specific mRNA sequences, have been demonstrated to be effective against bacterial targets (White et al., 1997; Harth et al., 2000). Furthermore, DNA mimics such as peptide nucleic acid (PNA) and phosphorodiamidate morpholino (PMO) have uncharged backbone structures that enable delivery across negatively-charged bacterial cell barriers, and offer superior hybridization and stability properties when compared to modified DNA oligonucleotides (Good *et al.*, 2000b). Attachment of carrier peptides to PNAs/PMOs can enhance cell permeation and uptake and improve antisense effects (Good et al., 2001a). PNAs and PMOs targeted to the translation initiation region (TIR) of essential mRNAs are bactericidal and have been successfully applied to a number of different species (Good and Nielsen, 1998; Nekhotiaeva et al., 2004; Kurupati et al., 2007; Shen et al., 2009). Bactericidal PNAs/PMOs are typically 10 bp in length and are more sensitive to target mismatches than equivalent DNA oligonucleotides, properties that make them highly suited to species discrimination.

In this study we tested the hypothesis that the selective binding properties of peptide PNA antimicrobials can be exploited to selectively target certain species in mixed culture based on sequence differences in the translation initiation region of essential genes. We report, for the first time, that

peptide-PNAs can form the basis of a single class of antimicrobial with a tuneable spectrum of activity.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** A list of the strains used in this study is given in Table 1. All strains were grown in Miller's modified Luria broth (MMLB; Sigma-Aldrich, UK) with constant shaking (200 rpm) at 37°C. For mixed-culture growth, MMLB was inoculated with  $1 \times 10^4$  CFU/ml; the proportion of each species needed to give reproducible species counts after 16 h of growth at 35°C was experimentally determined (Table S1 in the supplementary material).

**Design of species-specific peptide PNAs:** General guidelines for the design of antibacterial peptide-PNAs are described elsewhere (Good and Nielsen, 1998; Good *et al.*, 2000b; Good *et al.*, 2001a; Good, 2002). Criteria used for the design of species-specific peptide-PNAs are described in the results section. The Database of Essential Genes (Zhang and Lin, 2009a) and BLAST (Altschul *et al.*, 1990) were used to identify any essential gene homologues present in all four species used in this study. The Artemis program (Rutherford *et al.*, 2000) was used to extract twenty base-pairs (-10 - + 10 bases relative to the start codon) of the TIRs from the genome sequences of *Bacillus subtilis* (Kobayashi *et al.*, 2003a), *Escherichia coli* DH10B (Durfee *et al.*, 2008a), *Klebsiella pneumoniae* and *Salmonella enterica* serovar *Typhimurium* LT2 (Knuth *et al.*, 2004) (GenBank accession numbers: AL009126; CP000948; CP000647; and AL513382 respectively). The 20 bp TIRs from gene homologues were aligned in Clustal X version 2.0 (Larkin *et al.*, 2007) and the number of base-pair mismatches between species was determined. The predicted thermal stability ( $T_m$ ) of PNA/DNA duplexes was determined according to formula of Giesen *et al.* 1998 (Giesen *et al.*, 1998). A genomic analysis of the possible binding sites of the PNAs within their target species was conducted in Artemis, using a cut-off of greater than 2 bp mismatches. Secondly, to comprehensively examine the number of potential antibacterial PNAs that could be used to discriminate

between two closely related species, a semi-automated method was employed: genome sequences were used to identify the start codon positions of essential genes from *E. coli* DH10B and *S. Typhimurium* LT2. A custom PERL script was used to extract the -5 - + 5 bases relative to the start codon of each gene. The 10 bp sequences were used for an all-against-all comparison using standalone BLAST (Altschul *et al.*, 1990; McGinnis and Madden, 2004a) to identify the TIRs of essential genes that were amenable to the design of species specific PNAs. The peptide-PNAs used in this study and their properties are listed in Table 2.

Antimicrobial susceptibility and peptide-PNA minimal inhibitory concentration (MIC) testing. Strains were tested with twenty different antibiotic disks (Oxoid, UK) representing the major classes of antimicrobial compounds. Tests were done according to the standardized disc susceptibility testing method of the British Society for Antimicrobial Chemotherapy (Andrews, 2001; Andrews, 2009). The minimum inhibitory concentration (MIC) of the peptide-PNA conjugants were determined using a method modified from Hacek *et al.* (Hacek *et al.*, 1999) and Friedman *et al.* (Friedman *et al.*, 2006): Peptide-PNA conjugants, obtained as lyophilized powder (Panagene, Korea), were dissolved in ddH<sub>2</sub>O. MIC assays were performed in an ultra low-bind (Costar, UK) polystyrene 96-well plate format in a final volume of 150  $\mu$ l MMLB. An extended gradient of peptide-PNA concentrations was created by combining five sets of twofold serial dilutions from four starting concentrations (10, 4.8, 3.2 and 3 and 2  $\mu$ M); giving 55 final peptide-PNA concentrations which extended over five rows of the 96-well plate. All cultures were incubated at 35°C for 16 h without shaking or agitation. Each peptide-PNA MIC calculation was performed in triplicate, each replicate representing a different starting colony. Mixed-culture experiments were conducted as above with a  $1 \times 10^4$  CFU/ml starting inoculum. For growth curve analysis, 200  $\mu$ l cultures were grown in a BioTek PowerWave HT spectrophotometer, under constant agitation at 37°C in a 96-well plate covered with a breathable film. Growth (OD<sub>550</sub>) was monitored every 5 mins, each experiment was performed in triplicate.

Microscopy. Cells prepared for fluorescence imaging were grown in a BioTek PowerWave HT spectrophotometer as above. After six hours 10  $\mu$ l of culture was removed, washed and resuspended in 1 X PBS by centrifugation (13, 000 x g). Cells were applied to an agarose pad (Levin, 2002b) and viewed using an epifluorescence Leica DMRB microscope. An EXi Aqua CCD camera (QImaging) and Image Pro Plus (MediaCybernetics) were used for image acquisition and processing.

Species identification. In mixed-culture experiments, colonies on MMLB plates were identified using a combination of phenotypic and genotypic properties. In order to rapidly identify species post peptide-PNA treatment, we designed a species-specific PCR-based identification method. Primer sets for peptide deformylase (*def*) were designed that yielded different sized amplicons for each species: *B. subtilis* (352 bp), *E. coli* (394 bp), *K. pneumoniae* (231 bp) and *S. Typhimurium* (280 bp) (see Table S2). All colonies were picked from the plates with the most countable dilution (30-50 cfu per plate) and used directly for colony multiplex PCR in 10  $\mu$ l reaction volumes according to the simplified method of (Menossi et al., 2000). Colonies were identified using standards prepared from pure cultures. Furthermore, in the three-species mixed-culture experiments, colonies were identified using the colony PCR protocol with 16S rRNA gene primers. The sequences of both strands of the resulting amplicons were determined with the BigDye (version 3.1) cycle sequencing kit and a 3730 DNA analyzer (Applied Biosystems, UK). Species identity was confirmed using the SEQMATCH function of the Ribosomal Database Project (Cole et al., 2009)

## RESULTS

Peptide-PNA mediated growth inhibition and species-selectivity. The four bacterial species used in this study were chosen as they have all been reported to be susceptible to antisense antibiotics (Good et al., 2001a; Tilley et al., 2006; Kurupati et al., 2007; Shen et al., 2009). The parameters used in the design of

species-selective peptide-PNAs resulted in a number of potential gene targets that could be used for species-selective growth inhibition. The following criteria were used for the design of species-selective peptide PNAs were: 1) target gene is essential and homologues are present in all four species used in this study; 2) the translation initiation region (TIR) of the mRNA had at least two base-pair differences between species (see below); 3) the TIR sequence was amenable to the design of peptide-PNAs with low melting temperatures; 4) where possible, off-target sites within and between species were not in the TIRs of essential genes; and 5) evidence that gene silencing of the target and/or inhibition of its cognate protein is growth inhibitory. We have previously shown that *murA* and *ftsZ* are good targets for peptide-PNA mediated growth inhibition (Goh et al., 2009), both genes were identified in this study as potential targets for species-selective peptide-PNAs, and thus, were selected for further study. Two base-pair mismatches were selected, as PNAs with one base-pair mismatch will bind to the target, but with reduced affinity (Good et al., 2001a); peptide-PNAs with one base-pair mismatch to their target sites have approximately 33% increase in MIC (Liam Good, unpublished). This is in agreement with the design parameters suggest by Dryselius et al. (Dryselius et al., 2003) in which they suggest 1-bp mismatches within the TIR of off-target genes should be avoided.

The peptide-PNAs designed in this study were assayed for their antibacterial activity against both target and non-target species. The previously reported En108 peptide-PNA (called Ec108 in (Goh et al., 2009)) was used as a control to test the feasibility of species-selectivity at a broad taxonomic level; *E. coli*, *K. pneumoniae* and *S. Typhimurium* (Gram-negative, Enterobacteriaceae) have identical *acpP* TIRs and thus all three species should be susceptible to En108, while the *acpP* TIR of *Bacillus subtilis* (Gram-positive, Bacillaceae) has six base-pair mismatches and should be resistant to En108. Antibacterial assays with En108 proved this to be the case; En108 had an MIC of 1.2, 0.4 and 0.3  $\mu\text{M}$  for *E. coli*, *K. pneumoniae* and *S. Typhimurium* respectively (Table 2), and had no detectable antibacterial activity against *B. subtilis* at concentrations of up to 20  $\mu\text{M}$  (data not shown). Similarly, the species-selective PNAs for *B. subtilis*, *K. pneumoniae* and *S. Typhimurium* were only antibacterial to the intended species (Fig. 1A). The *E. coli*-selective Ec1000 was unexpectedly cross-reactive

with *S. Typhimurium* (discussed below). Table 3 shows the analysis of potential binding sites within the genomes of the target species. Of note is the difference in MIC between *E. coli*, *K. pneumoniae* and *S. Typhimurium* when treated with the acpP-targeting En108; the MIC of this PNA was 3 and 4 fold less in *K. pneumoniae* and *S. Typhimurium*, respectively (Table 2). Analysis of the binding sites of En108, Kp0001 and Se0001 in the genomes of these species revealed that En108 likely binds in the TIR region of other essential genes in *K. pneumoniae* (*mukF* and *ribH*) and *S. Typhimurium* (*yhhM*) each with a 1 bp mismatch. This could account for the decreased MIC in these species, however the relationship is not straightforward as Se0001 is predicted to bind in the TIR of at least three other genes determined to be essential in *E. coli* and *S. Typhimurium* (*hemK*, *Int* and *rluA*) and has an MIC equivalent to that of En108 in *E. coli* (Table 2). Furthermore, there is no obvious relationship between the MIC of a peptide-PNA and the number of off-targets in the genome of the target species, including those that bind in the TIRs of both essential and non-essential genes (Table 3). Reasons for the possible differences between the MICs of the different peptide-PNAs are discussed below.

Use of species-selective PNAs in mixed culture. To test the selectivity of peptide-PNAs in mixed culture, we first used reciprocal treatment in two-species culture (Fig. 1B). When mixed cultures of *E. coli* and *K. pneumoniae* were treated with 3.2  $\mu$ M of *E. coli*-selective Ec1000, after 16 hrs of incubation only *K. pneumoniae* was detectable. Untreated control cultures maintained both species throughout the incubation period. Reciprocal treatment of the same mixed culture with 3.2  $\mu$ M of *K. pneumoniae*-specific Kp0001 showed equivalent selectivity. Species-selective growth inhibition was also observed in mixed culture of *K. pneumoniae* and *S. Typhimurium* treated with 3.2  $\mu$ M Kp0001 or 2.0  $\mu$ M Se0001 (Fig. 1B). *S. Typhimurium* was successfully removed from mixed culture with *E. coli* when treated with 2.0  $\mu$ M Se0001, but reciprocal removal of *E. coli* could not be achieved with Ec1000 (see above). Three-species mixed culture of *B. subtilis*, *K. pneumoniae* and *S. Typhimurium* was used to test the possibility of specifically targeting either one or two species, with a single peptide-PNA or a combination of two. Kp0001 and Se0001 both at 4.5  $\mu$ M were successfully applied to the three-species mixed culture; only *B. subtilis* and *S. Typhimurium* could be detected after 16 hrs incubation with

Kp0001, and only *B. subtilis* and *K. pneumoniae* after Se0001 treatment (Fig. 2). Treatment of the three-species culture with 3.5  $\mu\text{M}$  En108 resulted in the expected selective growth inhibition of both enteric species, with only *B. subtilis* detectable after 16 hrs. Combined use of Kp0001 and Se0001, at 4.5  $\mu\text{M}$  each was also able to specifically remove *K. pneumoniae* and *S. Typhimurium* from the mixed culture, however there was a significant difference in the final *B. subtilis* CFU count between the En108 and dual Kp0001/Se0001 treated cultures. This indicates that, while both Kp0001 and Se0001 had no effect on *B. subtilis* at concentrations of  $> 4.5 \mu\text{M}$ , combined use of the PNAs may have a weak synergistic antibacterial effect (Stach and Good, 2011). Mixed culture experiments with four species were not attempted in this study, however the potential of peptide-PNAs as species-selective antibacterial compounds is highlighted by a comparison of all possible species combinations for the four species tested in this study, alongside the antibiotic spectrum of the peptide-PNAs and twenty known antibiotics (Fig. S1). Only peptide-PNAs are capable of species-selective growth inhibition for the three Gram-negative species. For these species, there are six possible outcomes for species-selective antibacterial treatment of two-species mixed cultures. Treatment with the twenty antibiotics assessed in this study (Table S3), could theoretically achieve four of these outcomes (all combinations except those requiring inhibition of *E. coli* or *K. pneumoniae* in combination with *S. Typhimurium*). Use of peptide-PNAs Ec1000, Se0001 and Kp0001 enabled five of the possible outcomes; unexpected cross-reactivity of Ec1000 prevented selective inhibition of *E. coli* in combination with *S. Typhimurium*. However, it is very likely that evaluation of other *E. coli*-selective peptide PNAs would be able to rectify this result (see below). Furthermore, of the known antibiotics, only streptomycin could select between *E. coli* and *S. Typhimurium*. This would not be the case for most strains of *E. coli* as strain DH10B has an *rpsL* mutation that confers resistance to streptomycin. Unlike the peptide-PNAs, in mixed culture experiments with three species (Fig. 2), no combination of the known antibiotics would be able to selectively kill any of the Gram-negative species tested without killing *B. subtilis*.

Peptide-PNA mediated discrimination of *E. coli* and *S. Typhimurium*. The observation that Ec1000 was antibacterial to the non-target *S. Typhimurium* led us to use a comprehensive genomic analysis to determine the number of



potential targets that could be used to design peptide-PNAs that would discriminate between these two closely related species. For the purposes of defining potential discriminatory target sites, we allowed for 1 bp mismatch difference between the target site of *S. Typhimurium* and *E. coli*; while 1 bp mismatch is not likely to be sufficient to prevent binding, differences in gene silencing activity may be sufficient, in some cases, to enable the development of peptide-PNAs that would be selectively antibacterial to *S. Typhimurium*. Three separate BLAST analyses were done: 1) TIR sequences of genes described as essential in *S. Typhimurium* against the TIRs of *E. coli* essential genes; 2) TIRs of *S. Typhimurium* genes described as essential in *E. coli*, against TIRs of *E. coli* essential genes and 3) the TIRs of *E. coli* essential genes against TIR sequences of genes described as essential in *S. Typhimurium* (Tables S4-6). Using the essential genes of *S. Typhimurium* from the DEG database, and the -5 to +5 TIR region we identified 113 genes that could serve as targets for peptide-PNAs that would be selectively antibacterial to *S. Typhimurium* over *E. coli* (Table S4). Of these, 68 genes had orthologues not identified as essential in *E. coli*, 34 did not have orthologues in *E. coli* and of the 11 genes that had essential orthologues in *E. coli*, 5 of these had TIR sequences with > 2 bp mismatches. The fact that peptide-PNA Se0001, was selectively antibacterial for *S. Typhimurium*, and designed to target a gene not reported to be essential in *S. Typhimurium* indicates that orthologues of genes that are identified as essential in *E. coli* are likely to be essential in *S. Typhimurium*. Furthermore, *E. coli* genes have been identified as essential by failure to construct a specific knockout, whereas those of *S. Typhimurium* were identified by trapping lethal insertions (Knuth et al., 2004). We applied the bioinformatic screening technique described above to identify peptide-PNAs that would discriminate between *S. Typhimurium* and *E. coli* using the essential genes of *E. coli* as the query sequences. This analysis identified a further 93 orthologous genes that could potentially act as targets for discriminatory peptide-PNAs (Table S5). Of these, 47 genes had TIRs with > 2 bp mismatches between the two species, from which *ftsZ* was chosen for further study. This target was chosen for the reasons given above, and because gene silencing of *ftsZ* should result in a cell filamentation phenotype that would enable microscopic evaluation of the specificity of the peptide-PNA in mixed culture. DsRed-labelled *E. coli* AC01, and GFP-labelled *S. Typhimurium* AC02 were exposed to peptide-PNA Se0002

at concentrations  $\leq 5 \mu\text{M}$ . *E. coli* AC01 was unaffected by Se0002 at all concentrations tested, whereas *S. Typhimurium* growth was inhibited at  $1.25 \mu\text{M}$  ( $0.5 \times \text{MIC}$ ), with a lag phase ca. 7.5 hrs longer than that of the untreated sample (Fig. 3a). Growth of *S. Typhimurium* observed in Se0002-treated cultures after 10.5 hrs was not due to the generation of spontaneous resistance mutants, as samples of cells taken after 14 hrs of incubation, passaged into fresh media containing the same concentration of Se0002, exhibited identical growth kinetics to the parent culture. Growth is more likely due to the effective concentration of Se0002 falling below the minimum inhibitory concentration, caused by the peptide-PNA accumulating in non-growing cells, adsorbing to the plastic of the well, or proteolysis of the carrier peptide. Mixed cultures of *E. coli* AC01 and *S. Typhimurium* AC02 prepared as above, treated with  $1.25 \mu\text{M}$  of Se0002 were sampled after 6 hrs of growth and were observed by fluorescence microscopy (Fig. 3b). As predicted, *E. coli* AC01 treated with Se0002 had an identical phenotype to untreated controls, whereas *S. Typhimurium* AC02 cells displayed a distinct filamentous phenotype only upon treatment with Se0002. This phenotype is consistent with previous studies using anti-*ftsZ* peptide-PNAs in *E. coli* (Goh et al., 2009). These results, and those of the mixed culture experiments detailed above, prove that it is possible to employ antisense-based molecules as species-selective antimicrobial agents in mixed culture.

## DISCUSSION

The aim of this study was to test the hypothesis that peptide-PNAs can be applied as species-selective antimicrobial compounds. We demonstrate, for the first time, the design and application of species-selective antisense antibacterials. Antibacterial peptide-PNAs evaluated against *B. subtilis*, *E. coli*, *K. pneumoniae* and *S. Typhimurium*, both in single and mixed cultures, with the exception of Ec1000, displayed detectable antibacterial activity against the intended species only. Peptide-PNA treatment of mixed cultures enabled selective growth inhibition that, in theory, could not be achieved using the twenty antibiotics evaluated in this study. The current requirement for narrow-spectrum antimicrobial agents will likely be met by both small molecule approaches, where examples already exist (Then and Sahl, 2010), and by novel approaches such as the use of antisense agents. Our findings suggest that PNAs are good candidates for narrow-spectrum antimicrobials. For species-selectivity, 16S rRNA would appear to be a logical candidate. However, while peptide-PNAs targeted to 16S rRNA have been demonstrated to be bactericidal and sequence selective (Good and Nielsen, 1998; Hatamoto et al., 2009), prior to this study, species selectivity has not been observed and may be difficult to achieve due to sequence conservation within functional regions of 16S rRNA. Currently, while the mode of action of peptide-PNA antimicrobials is well understood (Good and Nielsen, 1998; Good *et al.*, 2000b; Good *et al.*, 2001a; Good, 2002), differences in uptake of the peptide-PNA (see below), species sensitivity and the affect of non-target binding remain important areas for future experimentation.

The observation that En108 has different activity in three closely related species is intriguing from the perspective of improving the antibacterial activity of peptide-PNAs. Analysis of the binding sites of En108 in the genomes of the three species provides a possible explanation: En108 will bind (allowing for 1 bp mismatch) to the TIRs of off-target essential genes in both, *K. pneumoniae* and *S. enterica*, and only the intended target in *E. coli*. Antisense-based antimicrobials have been shown to have greater growth inhibitory activity when the expression of multiple essential genes is simultaneously inhibited (Harth et

al., 2000). While the efficacy of gene silencing with mismatched PNAs may be reduced, it is reasonable to suggest that silencing of more than one essential gene will lead to lower MICs. En108 will bind to two off-target essential genes in *K. pneumoniae*, and one in *S. Typhimurium*, yet has a lower MIC in the later. This may be explained by mechanistic differences in the gene silencing itself (binding efficacy,  $T_m$  differences caused by mismatches, and location of the off-target relative to TIR) or differences in the stringency of requirement for the target and off-target genes, i.e. a small reduction in the mRNA pool of the two off-targets in *K. pneumoniae* may be better tolerated than that of the off-targets in *S. Typhimurium* (Goh et al., 2009). Differences in the susceptibility of the three species to En108 may also be explained by uptake efficiency: The transporter protein SbmA has recently been identified as required for peptide-PNA uptake in *E. coli* (A, Ghosal, J.E.M. Stach, A. Vitali and P.E. Nielsen, submitted for publication). Mutations in the *sbmA* gene affect uptake kinetics, and as *E. coli* shares 86% and 92% similarity to its orthologues in *K. pneumoniae* and *S. Typhimurium* respectively, the observed difference in sensitivity to En108 in these three species may be explained by differences in SbmA-mediated uptake.

The finding that PNA Ec1000, designed to silence the *murA* gene of *E. coli*, was antibacterial to *S. Typhimurium* was unexpected. The parameters described for the design of species-selective PNAs (see Materials & Methods) should theoretically prevent binding of PNAs to the TIRs of essential genes in non-target species. A study by Dryselius et al. (Dryselius et al., 2003) demonstrated that the TIR of a gene is most sensitive to antisense PNA gene silencing. However, some PNAs that bind intragenically were shown to significantly elevate gene expression. Furthermore, PNAs can repress co-transcribed genes; a PNA binding to the TIR of a nonessential gene, may affect the transcription of an essential downstream gene to a varying degree depending on transcript stability (Dryselius *et al.*, 2006b). Thus, the unexpected antibacterial activity of Ec1000 in *S. Typhimurium* may be due to such events. An analysis of the binding sites (allowing for 1 bp mismatch) for Ec1000 in *S. Typhimurium* shows that it binds intragenically to a number of essential genes (*alaS*, *fusA*, *hemE* and *rplF*) that are reported to be toxic upon overproduction (Kitagawa *et al.*, 2005b). Also, Ec1000 binds (1 bp mismatch) in the intergenic region of *rpsN*

and rplE, genes that are located in a ribosomal protein operon. Thus, it is possible that translation of the transcript rplNXE-rpsNH-rplIFR-rpsE-rpmD-rplO-secY-rpmJ could be repressed for genes downstream of rpsE. The predicted ribosomal binding site for rpsN is 25 bp upstream of the start codon, with the Ec1000 binding site 12 bp upstream, explaining why it was missed in the initial screen for undesirable off-target binding sites. In *S. Typhimurium*, Ec1000 can form a duplex in the TIRs of six genes: pipC, a pathogenicity island-encoded protein; pepP, a metallopeptidase; mrcA, a murein transglycosylase/transpeptidase; STM1127, a putative transcriptional regulator; STM3527, a hypothetical protein and STM4051, a putative outer membrane protein. None of these genes are reported to be essential in *S. Typhimurium* or the other species used in this study. It is also possible that the Ec1000 PNA may bind to a non-coding RNA. Non-coding RNAs are well documented as regulatory elements in *S. Typhimurium* (Vogel, 2009), and while we could identify no potential binding between Ec1000 and known non-coding RNAs in *S. Typhimurium* (Pfeiffer et al., 2007) there may be as yet unidentified non-coding RNAs that are transcribed from, or interact with, intergenic Ec1000 binding sites; disruption of a RNA antitoxin is one possible mechanism that would lead to bactericide (Faridani et al., 2006). It is unlikely that small elevations in essential gene expression, partial silencing of non-essential genes, or disruption of non-coding RNAs could be effective alone, but in combination they may be growth inhibitory. The elucidation of the mechanism responsible for Ec1000-induced growth inhibition in *S. Typhimurium* is required for the continued development of species-selective antibacterial PNAs; understanding of the mechanism will enable the design parameters of peptide-PNAs to be modified to exclude likely off-target effects and/or identification of new targets for gene-silencing antimicrobials. Comparative genomics between sensitive and resistant species, qRT-PCR (Goh et al., 2009) and the introduction of point mutations within putative targets could be applied to identify the cause of the off-target selectivity in *S. Typhimurium*. While the unexpected activity of Ec1000 prevented its use as an *E. coli* species-selective peptide-PNA, our in-silico analysis, and the identification of *E. coli*-specific TIRs (Table S6) suggest that finding targets that are amenable for discriminating *E. coli* from closely related species is readily achievable. There are 46 targets, in the -5 to +5 TIR region of essential genes in *E. coli*, that have >2 bp mismatches with orthologs in *S.*

Typhimurium (Table S5). The *in silico* approach was applied in the successful design of Se0002, and thus a peptide-PNA raised to the same target in *E. coli*, will likely discriminate *E. coli* from *S. Typhimurium*. Furthermore, the number of potential species-selective targets between these two strains is likely an underestimate as we confined our search to a single target region (-5 to +5 of the TIR) and peptide-PNAs with gene silencing activity have been described based on a target region at least twice this size (Dryselius et al., 2003).

As the peptide-PNA mediated growth inhibition is dependent on the silencing of an essential gene or genes, a better understanding of which genes are essential in the specific pathogen, in a relevant (i.e. in host) environment, will facilitate in the design of peptide-PNAs. For example, the silencing of genes that are essential for survival or virulence in the host (Fields et al., 1986) or antimicrobial resistance factors, as peptide-PNAs silencing of such genes can reestablish the antimicrobial sensitive phenotype in resistant strains (Good and Nielsen, 1998; Jeon and Zhang, 2009b). Discovery of synthetically lethal gene combinations in bacteria (Typas et al., 2008) will also broaden the number of targets available for the design of species-selective peptide-PNAs.

As peptide-PNAs are used within environments of increasing species diversity, it is clear that there will be a higher the likelihood of non-target species activity, as a consequence of identical target regions or off-target binding (as is likely the case for Ec1000 in *S. Typhimurium*). Nevertheless, relative to currently used antibiotics, PNAs provide greater opportunity for the design of narrow-spectrum antimicrobials where the primary target is dictated by the nucleic acid sequence. The availability of in-depth microbiome sequencing will enable the assessment (in terms of spectrum of activity) of PNA-based antimicrobial therapy in an animal model to be assessed; aid in the determination of conditions where such strategies may provide alternative or complementary therapy (Knight, 2010), and in the design of species-selective PNAs through exhaustive prediction of binding sites in all species of the microbiome.

While examples of bacterial resistance to peptide-PNAs have not been published, it is reasonable to assume that it may arise through point mutation of the PNA binding site. In this case, activity would be restored by simple modification of the PNA sequence, whereas point mutations that lead to

structural changes in the binding site of proteins and ribosomes require chemical modification of the antimicrobial based on structure activity relationships that are not straightforward to predict. Resistance due to prevention of uptake (as above), degradation of the peptide carrier, or efflux remain possible, and as such, characterization of peptide-PNA resistance mutants and the evaluation of alternative PNA carriers, remain priority areas for the development of antimicrobial PNAs.

Narrow-spectrum antimicrobial therapy has likely been hindered by the need to treat infections quickly without waiting for identification of the pathogen. However, with the advent of cheap sequencing and rapid diagnostics, the benefits of species-selective or narrow spectrum antimicrobials may improve patient outcome and ameliorate the development of antimicrobial resistance. Antimicrobials that act at the gene sequence level, such as PNAs, have great potential in the development of what Casadevall has termed them 'Third-Age antimicrobials' (Casadevall, 2006).

FIG. 1. Species-selective antibacterial peptide-PNAs in two-species mixed culture. *E. coli* (dark grey), *K. pneumoniae* (white) and *S. Typhimurium* (light grey). All cultures were incubated for 16 hrs. A) axenic cultures of the species were treated with *E. coli*- specific Ec1000 at 3.2  $\mu$ M, *K. pneumoniae*-specific Kp0001 at 3.2  $\mu$ M and *S. Typhimurium*-specific Se0001 at 2.0  $\mu$ M. Asterisks indicate species-selective growth inhibition of *E. coli*, *K. pneumoniae* and *S. Typhimurium* respectively. B) Two-species mixed cultures treated with peptide-PNAs as above. The control cultures show the relative proportion of the two species without treatment, the two treatments to the left of the control represent the same mixed culture treated with a peptide-PNA. Black arrows indicate non species-selective growth inhibition of *S. Typhimurium* by Ec1000.

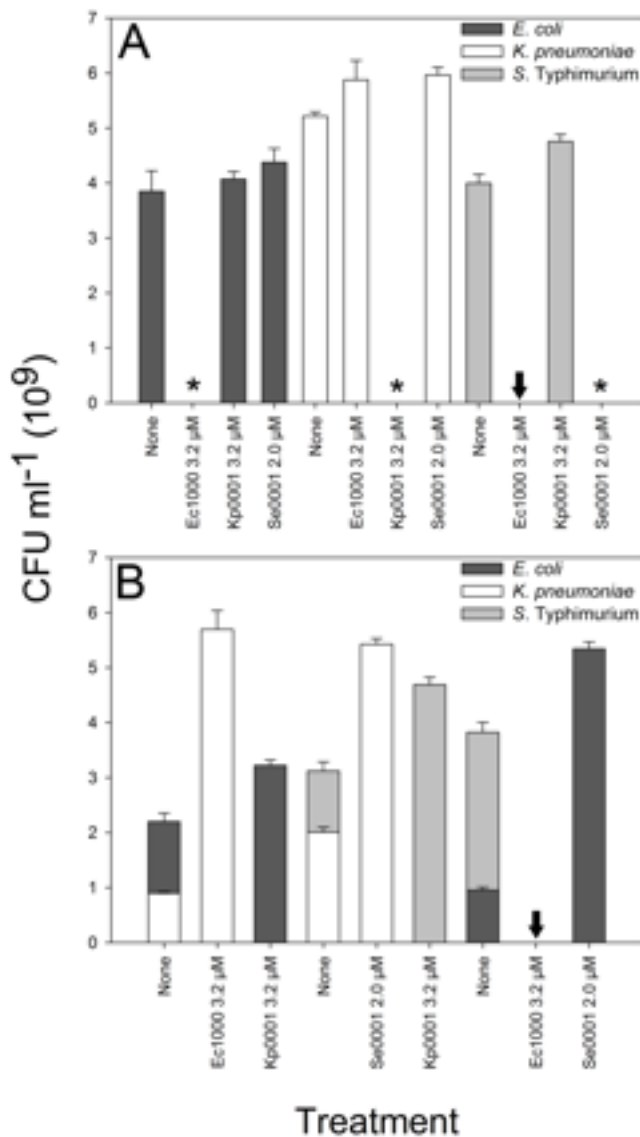




FIG. 2. Species-selective antibacterial peptide-PNAs in three-species mixed culture. *B. subtilis* (dark grey), *K. pneumoniae* (white) and *S. Typhimurium* (light grey) in mixed culture were separately treated with Ec108 at 3.5  $\mu$ M, Kp0001 or Se0001 at 4.5  $\mu$ M or by combined treatment of Kp0001 and Se0001 both at 4.5  $\mu$ M. All cultures were incubated for 16 hrs. Selective inhibition of either *K. pneumoniae* or *S. Typhimurium* individually or together, achieved with the peptide-PNAs, could not theoretically be achieved with any combination of the twenty known antimicrobial compounds tested in this study.

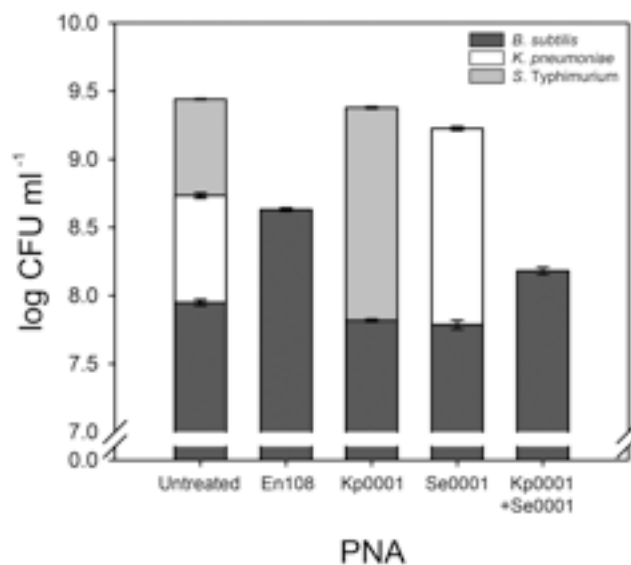
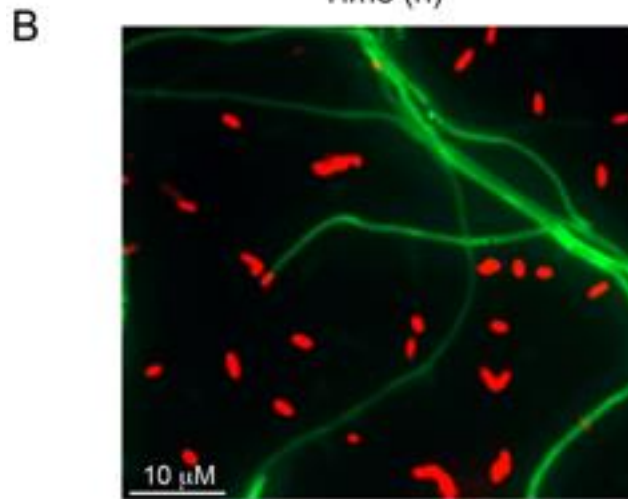
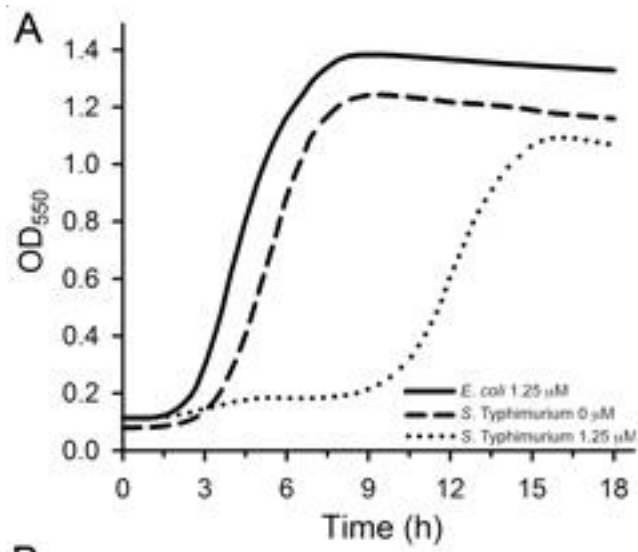


FIG. 3. *S. Typhimurium*-selective growth inhibition. Peptide-PNA Se0002 was designed to target the -5 to +5 region of the translational initiation region (TIR) of *ftsZ* in *S. Typhimurium*. Se0002 has 2 base pair mismatches in the TIR of *ftsZ* in *E. coli*. (A) Growth curve analysis of Se0002 in pure culture. *E. coli* growth in the presence of 1.25  $\mu$ M Se0002 (solid line) was identical to that of untreated controls (not shown). *S. Typhimurium* growth was inhibited in the presence of 1.25  $\mu$ M of Se0002 (dotted line) relative to the untreated control (dashed line). Growth in the treated samples after 10 hrs was not due to resistance (see text for details). (B) Mixed cultures of GFP-labeled *S. Typhimurium* AC02 and DsRed-labeled *E. coli* AC01 were treated with 1.25  $\mu$ M Se0002; and imaged by fluorescence microscopy after 6 hrs of incubation. The

filamentous growth phenotype was only observed in *S. Typhimurium* AC02 and is consistent with silencing of *ftsZ* expression.



**Table 1. Bacterial strains used in this study. a American Type Culture Collection b *Salmonella* Genetic Stock Center**

Strain	Source	Genotype	Characteristic
<i>Bacillus subtilis</i> susp <i>subtilis</i> 168	ATCC <sup>a</sup> 23857	<i>trpC2</i>	Genome sequenced strain
<i>Escherichia coli</i> DH10B	Invitrogen	<i>F endA1, recA1, galE15, galK16, nupG, rpsL, ΔlacX74, Φ80lacZΔM15, araD139, Δ(ara,leu)7697, mcrA, Δ(mrr-hsdRMS-mcrBC), λ<sup>-</sup></i>	Genome sequenced strain, parent of <i>E. coli</i> AC01
<i>E. coli</i> AC01	This study	As above, pDsRed-Express2	Expression of DsRed fluorescent protein
<i>Klebsiella pneumoniae</i> subsp. <i>pneumoniae</i>	ATCC 700721	n/a	Genome sequenced strain
<i>Salmonella enterica</i> serovar Typhimurium LT2	SGSC <sup>b</sup> 1412	n/a	Genome sequenced strain
<i>S. Typhimurium</i> LT2 substr JR501	SGSC 1593	<i>hsdSA29, hsdSB121, hsdL6, metA22, metE55,1 trpC2, ilv-452, H1-b, H2-e,n,x</i> (cured of Fels 2), <i>fla-66, nml, rpsL120, xyl-404, galE719</i>	Restriction-deficient, modification-proficient cloning strain of <i>S. Typhimurium</i> LT2, parent of <i>S. Typhimurium</i> AC02
<i>S. Typhimurium</i> AC02	This study	As above, pGFPuv	Expression of green fluorescent protein

**Table 2. Properties of peptide-PNAs used in this study.**

<sup>a</sup> Code refers to predicted species-specificity Bs = *B. subtilis*, Ec = *E. coli*, En = Enterobacteriaceae, Kp = *K. pneumonia*, Se = *S. enterica* Typhimurium

<sup>b</sup> Indicates if specificity based on bioinformatic prediction was observed.

<sup>c</sup> Target is shown as positions of nucleotides relative to the start codon.

<sup>d</sup> Thermal stability of PNA/DNA duplex.

<sup>e</sup> Ec1000 lacked predicted specificity, see text for details.

Name <sup>a</sup>	Target	Sequence		Minimum Inhibitory Concentration (μM)				Expected specificity <sup>b</sup>	Target site	T <sub>d</sub> <sup>m</sup>	Reference
				<i>B. subtilis</i>	<i>E. coli</i>	<i>K. pneumonia</i>	<i>S. Typhimurium</i>				
Bs0001	<i>ftsZ</i>	(KFF) caacatgcta	3K-eg-	4.0	>10	>10	>10	yes	-4 to +6	53.5	This study
En108	<i>acpP</i>	(KFF) ctcatactct	3K-eg-	>10	1.2	0.4	0.3	yes	-5 to +5	41.5	Goh et al. 2009
Ec1000	<i>murA</i>	(KFF) ccatttagtt	3K-eg-	>10	2.4	>10	3.2	no <sup>e</sup>	-6 to +4	44.0	This study
Kp0001	<i>murA</i>	(KFF) tccattgatt	3K-eg-	>10	>10	2.5	>10	yes	-5 to +5	46.8	This study
Se0001	<i>murA</i>	(KFF) tcattattg	3K-eg-	>10	>10	>10	1.2	yes	-5 to +5	43.5	This study
Se0002	<i>ftsZ</i>	(KFF) aacataatct	3K-eg-	>10	>10	>10	2.5	yes	-5 to +5	46.1	This study

**Table 3. PNA binding site analysis in target species.**

Species	PNA	No. off targets <sup>a</sup>	Off Targets within TIR <sup>b</sup> of gene	
			No. essential (gene) <sup>c</sup>	No. non-essential (gene)
<i>E. coli</i>	En108	128	0	11 ( <i>araH</i> , <i>arpB</i> , <i>cadB</i> , <i>gpp</i> , <i>ssuE</i> , <i>ugpQ</i> , <i>upp</i> , <i>yagW</i> , <i>yhjG</i> , <i>yodC</i> , <i>yqjF</i> )
<i>K. pneumoniae</i>	En108	143	2 ( <i>mukF<sup>d,e</sup></i> , <i>ribH<sup>d,f</sup></i> )	5 ( <i>gppA</i> , <i>kpn_00790</i> , <i>kpn_01124</i> , <i>kpn_04794</i> , <i>nlpA</i> )
<i>S. Typhimurium</i>	En108	138	1 ( <i>yhhM<sup>d</sup></i> )	14 ( <i>dps</i> , <i>hycE</i> , <i>pmgl</i> , STM0566, STM0762, STM1698, STM2137, STM2481, STM3085, STM4218, <i>thiJ</i> , <i>upp</i> , <i>ycdY</i> , <i>ygaC</i> )
<i>E. coli</i>	Ec1000	201	2 ( <i>Int<sup>d,f</sup></i> , <i>rpsN<sup>d,f</sup></i> )	11 ( <i>elaD</i> , <i>hypF</i> , <i>mrcA</i> , <i>ppsR</i> , <i>rhsD</i> , <i>ybfD</i> , <i>ycdC</i> , <i>yfbM</i> , <i>yhaJ</i> , <i>yhhl</i> , <i>yjgH</i> )
<i>S. Typhimurium</i>	Se0001	320	5 ( <i>aspC<sup>f</sup></i> , <i>hemK<sup>d,e,f</sup></i> , <i>Int<sup>d,f</sup></i> , <i>nuoI<sup>f</sup></i> , <i>purD<sup>f</sup></i> , <i>rluA<sup>d,f</sup></i> )	8 ( <i>fucA</i> , <i>ppiC</i> , <i>sodA</i> , STM2343, STM2903, <i>yajB</i> , <i>ybdF</i> , <i>yebB</i> )
<i>S. Typhimurium</i>	Se0002	311	0	11 ( <i>celC</i> , t0363, t0453, t1718, t2652, t4467, <i>tdcE</i> , <i>umuC</i> , <i>yacK</i> , <i>yadI</i> , <i>ybaB</i> )
<i>K. pneumoniae</i>	Kp0001	157	3 <i>ispA<sup>d,f</sup></i> , <i>rpsN<sup>d,f</sup></i> , <i>topB<sup>e</sup></i>	3 (KPN_02027, KPN_04368, <i>rhaT</i> )
<i>B. subtilis</i>	Bs0001	104	0	4 ( <i>yabQ</i> , <i>yeeG</i> , <i>yrul</i> , <i>yvyE</i> )

a includes sites with  $\leq$  1base-pair mismatch with PNA

b Translation Initiation Region

c identified by BLAST searching of the Database of Essential Genes

d essential in *E. coli*

e essential in *S. Typhimurium*

f essential in other prokaryotes

ND Not determine