Survival of non-sporulating *Bacillus subtilis* upon energy limitation

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Abstract

Bacillus subtilis has the capacity to undergo cell differentiation into distinct phenotypes. The most studied phenotype is the dormant cell type named endospore which persists in unfavourable conditions for extended periods of time. Nevertheless sporulation is not adopted by the entire cell population. During nutrient exhaustion a subpopulation of cells survives while the remaining cells either sporulate or lyse. How these surviving cells endure adverse environmental conditions is currently unclear.

Incubation of non-sporulating *B. subtilis* under nutrient starvation revealed their capacity to remain viable for extended periods of time, over 100 days. During the first 14 days sporulation did not provide a survival benefit over the non-sporulating cells. Further characterisation of the non-sporulating cells showed that the cells are metabolically active and undergoing cell growth, albeit at a slow rate. Extracellular proteases, most likely releasing utilisable nutrients from extracellular proteins were shown to play an important role in starvation survival. At last, the cells appeared to be more tolerant to antibiotics and oxidative stress.

The spread of antibiotic resistance and the existence of antibiotic tolerant phenotypes have prompted investigation into new antibiotics and specific modes of action. Dissipating membrane potential, a mode of action of most membrane-targeting antibiotics triggers killing of non-growing *B. subtilis* cells. Deletions of genes involved in oxidative damage prevention and repair (*sodA*, *spx*, *recA* and *perR)* increases the killing, indicating ROS contribute to cell death. Moreover a superoxide scavenger, tiron, resulted in an increase in survival. The two analysed membrane potential dissipating compounds valinomycin and CCCP turned out to trigger cell killing by a different mode of action. The cytochrome *bc1* complex appears to have a key role in cell death triggered by valinomycin, possibly as a source of superoxide. This was indicated by an increase in survival observed for a *qcrA* deletion. This effect was not seen for CCCP. Instead, the majority of the electron transport chain (ETC) mutants examined increased sensitivity to CCCP.

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Chapter 1. Introduction

1.1 Introduction

Bacteria are able to colonise almost any niche available in the natural environment, including terrestrial, aquatic and host environments. Some bacteria have adapted to survive in highly specific conditions while others have evolved so they can survive in a wide variety of environments (Aujoulat et al., 2012). This is a testament to their adaptability because a new environment can come with a different set of stresses which the bacterium needs to overcome in order to survive. Even if the bacterium is limited to one particular niche, the stresses it will encounter will vary due to the fluctuations in conditions that occur in nature. A limiting factor of almost all habitats is the abundance of nutrients. Bacteria have developed several strategies in order to survive, including antibiotic production (Procopio et al., 2012). Antibiotics were commandeered by man-kind in order to treat infections, however, misuse has led to the development of drug resistant pathogens which plague the healthcare system (Ventola, 2015a). Due to the low discovery rate of new antibiotics, and the growing number of antibiotic resistant bacteria, research is essential to elucidate the exact mechanisms by which antibiotics cause cell death.

The first part of this introduction will focus on the life cycle of bacteria and how they are able to survive stress within the environment, eventually focusing upon nutrient starvation as this is a stress common to the majority of bacteria. The survival capacity of bacteria within host environments has proved to be an issue for human-kind, with the development of “super bugs” which are multi-drug resistant. In addition to this there are phenotypes which can tolerate otherwise lethal concentrations of antibiotics, after removal of the antibiotic stress cells can undergo proliferation. Therefore, the second part of this introduction will focus on the antibiotic resistance issue, and some of the strategies taken to combat this growing problem.

1.2 The bacterial life cycle

The bacterial growth cycle is divided into five phases, lag, exponential, stationary, death and long-term stationary, when bacteria are initially provided with nutrients and optimum conditions (temperature, aeration etc.). Upon inoculation of fresh media there is an initial period in which there is no observable growth (Figure 1.1), the so-called lag phase. It is believed that during this time the bacteria are

acclimatising to their new environment, and generating the appropriate enzymes for growth and utilisation of the existing nutrients (Rolfe et al., 2012). Once acclimatised, the bacterium begins to grow and divide using cellular machinery known as the divisome. The assembly of the divisome is brought about by FtsZ, which is a tubulin homologue and forms a ring like structure at the division site (Adams and Errington, 2009; Gamba et al., 2009). For rod shaped cells such as *Bacillus subtilis* and *Escherichia coli* this occurs usually at the midpoint of the cell. In addition, the Z-ring recruits downstream components of the divisome in order to form the cell division machinery (Adams and Errington, 2009). The development of two daughter cells is achieved through constriction of the "Z-ring". The division time varies between bacterial species, ranging from 20 minutes to days/weeks. Growth and division becomes exponential (exponential or logarithmic phase), but eventually nutrients become limited, at which time they will enter the stationary growth phase (Kolter et al., 1993) (Figure 1.1). In the stationary phase, bacteria produce secondary metabolites which are compounds not essential for growth such as antibiotics, however, they can be beneficial to the bacteria (Ruiz et al., 2010).

After prolonged incubation in stationary phase, if there is no change to the environmental conditions such as an influx of nutrients, the bacterial culture will enter the death phase (Figure 1.1). During this phase, the majority of the population will lyse. The death phase is reproducible across different species, however, the primary mechanisms that cause cells to die still remain unclear. Two main hypotheses have been postulated (Finkel, 2006). The first is that the environment can no longer sustain a high population level (Nystrom, 2003). As a result, cells begin to accumulate damage which they are no longer able to repair and, consequently, start to lyse. The second is that the bacteria sense they are at a high population density and nutrients have become limited. As a result, they initiate a mechanism known as programmed cell death (PCD), resulting in a portion of the population "committing suicide" and thus providing nutrients for the remaining cells (Finkel, 2006). It is still unclear which of these mechanisms are the cause of death and it is also conceivable that both are responsible. It has been shown for *E. coli* that not all cells lyse during death phase. Rather, a portion of the population survives for an extended period of time (months and years) without the addition of nutrients. This phenomenon has been referred to as long-term stationary phase (Finkel, 2006).

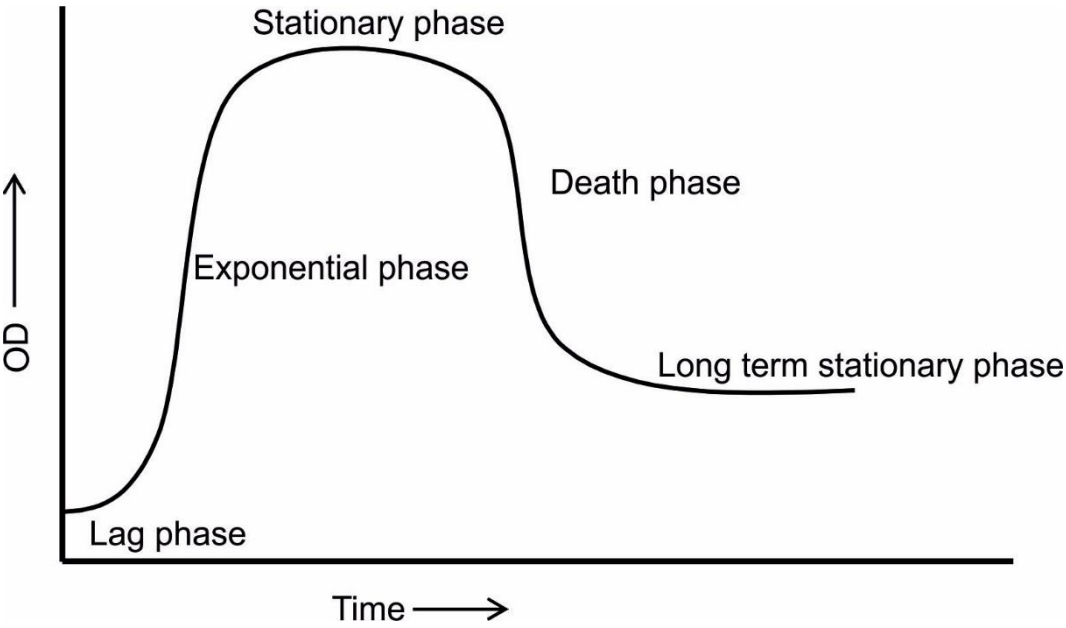

Figure 1.1 Bacterial growth phases.

Upon inoculation of fresh media, the bacteria will first exhibit no increase in population count (lag phase). It is believed that this period is when they are acclimatising to the new conditions. Eventually the bacterial population will increase at a logarithmic rate (exponential phase) with bacterial growth and division occur at a maximum rate consuming available nutrients until these become exhausted. After this phase, cells enter stationary phase. Continuous incubation inevitability leads to death phase, during which the cells begin to lyse resulting in a decrease to the population. Not all cells will undergo lysis. A portion of the population can enter a long term stationary phase, which can persist for years. Figure modified from (Finkel, 2006).

1.3 Bacterial stress survival

The ever changing conditions to which bacteria are exposed constantly cause them to switch from phases of active growth to periods of survival, and eventually to death. The way bacteria cope or fail to survive hostile conditions is crucial for understanding the full life cycle of microbial life. Within the bacterial kingdom there is an assortment of strategies that allow bacteria to survive unfavourable conditions and to endure until the hazards are no longer present (McDougald et al., 2002; Roszak and Colwell, 1987). The strategy utilised depends upon the species and the external stimuli. Fluctuations in conditions such as pH, temperature, salinity, oxygen saturation, water availability and nutrient content can induce stress responses which lead to changes in gene expression aimed at protecting essential functions and processes (Fawcett et al., 2000). Due to the numerous stresses a bacterium can encounter, many of the stress responses have overlapping pathways. These have been well studied in *E. coli* and *B. subtilis*, the main Gram-negative and Gram-positive model organisms, respectively (Asadishad et al., 2014; Murata et al., 2011), along with numerous other bacterial species (Roszak and Colwell, 1987). How bacteria cope during unfavourable conditions, and the survival strategies they employ, are important to understand as it will allow us to utilise bacteria more efficiently in industry and give us insight into how to combat them.

Stresses which bacteria encounter can be divided into five broad categories: (a) physiochemical and chemical parameters, including temperature, oxygen, redox-state, pressure and pH, (b) toxic compounds, including antibiotics and toxins, (c) cellular interactions, be it host cells during infection or coexisting bacteria during high cell densities, (d) nutritional deprivation or starvation (amino acid, carbon, phosphate etc.) and (e) water availability. Equally, the methods used to overcome these stresses can be broadly categorised: (a) elimination of the stress factor, (b) repair of damage incurred, and (c) escape from the stress. More than one of these methods can be used at a given time in order to increase survival chances. There are many specific stress responses, including heat shock, cold shock, oxidative stress, acid stress, envelope stress, to name a few. However, here I will focus on responses to nutrient starvation, and the effect observed on the cells. Oxidative stress will be discussed later.

1.3.1 *Viable but non-culturable*

One of the simplest methods to determine cell viability within a culture is by plating on a nutrient rich medium and counting the colony forming units (CFU). As a result of this, culturability became the standard for viability and the growth observed provided proof of life. This method relies upon the cells within the culture to generate a colony when supplied with nutrients. However, the inability to grow does not necessarily indicate cell death (Ramamurthy et al., 2014). The development of new techniques, such as microscopy and flow cytometry provided new insight into the discrepancies between life and death. The culturing technique has also proven limited when trying to select bacteria from the environment. It has been estimated that we are able to culture less than 2 % of all the bacteria present (Wade, 2002). In addition to this, when readily culturable bacteria such as *E. coli* are exposed to nutrient exhaustion, they can enter a non-culturable state. This is seen as a reduction in the CFU even though the cells remain intact microscopically (Xu et al., 1982). The discrepancy between the two detection methods has been attributed to the so-called "viable but non-culturable" (VBNC) phenomenon. This refers to a state in which bacteria do not produce colonies on media that would usually be sufficient for growth, but are still nevertheless alive (Oliver, 2005). The VBNC state has been investigated in a variety of bacteria, but was first documented in *E. coli* and *Vibrio cholerae* (Xu et al., 1982). Since the initial discovery, many bacteria including human pathogens have been shown to enter a VBNC state. However, it has been observed these bacteria do not express virulence factors while in a VBNC state, yet after resuscitation their virulence returns (Oliver, 2010).

Not only have changes to virulence been observed for the VBNC state, but changes to cell wall and membrane composition, physical and chemical resistance, cell morphology and gene expression have also been documented (Li et al., 2014). Changes to cell morphology include cell rounding, reduction in cell size and additional cell shape changes such as spiralling. Morphological changes cannot be used to identify the VBNC as similar changes have been observed in bacteria that are not in a VBNC state. Cell shrinking has been observed for *Staphylococcus aureus* (Watson et al., 1998), while for the fish pathogen *Flavobacterium columnare* a coiling phenotype was observed after 7 days incubation in water. After 14 days the majority of the population underwent coiling (Arias et al., 2012). It was also shown that the addition of nutrients could return cells to a normal state. It is believed these

changes in cell structure either enhances nutrient uptake, or lowers the nutrient cost of cellular maintenance.

Compared to exponentially growing cells, VBNC cells have a lower metabolic rate (Li et al., 2014). This could explain the increased chemical and physical resistance. In addition, they have increased cross-linking of their peptidoglycan (Signoretto et al., 2000). For *Vibrio vulnificus* VBNC cells have been shown to be more resistant to sonication, and *Mycobacterium smegmatis* VBNC cells are resistant to higher temperatures (Anuchin et al., 2009; Weichart and Kjelleberg, 1996). Recently, a study has shown that VBNC cells of *V. vulnificus* have a high resistance to ethanol, oxidative and osmotic stress, low pH, and antibiotics in comparison to exponentially growing cells (Nowakowska and Oliver, 2013). VBNC cells have also been shown to be tolerant to antibiotics, but they are also tolerant to additional stresses such as extreme pH and temperature (Nowakowska and Oliver, 2013). A phenotype similar to VBNC is persistence. Persister cells are a subpopulation of cells in a culture that are tolerant to lethal concentrations of antibiotics (Lewis, 2010), this will be discussed later.

Although changes to environmental conditions are known to induce the VBNC state, little is known about the genetic control mechanisms, although it has been shown that OxyR and RpoS play an important role (Li et al., 2014). A loss in culturability during cold temperatures was shown for *V. vulnificus.* This effect could be duplicated at ambient temperatures with mutations to *oxyR* (Kong et al., 2004). The analysis of major stress regulators identified RpoS as a factor involved in the generation of VBNC cells (Li et al., 2014). *E. coli* and *Salmonella* species that were depleted for RpoS entered a VBNC state faster. In the case of *E. coli,* the *rpoS* mutant became unculturable after 21 days when incubated at 4 ºC, while it took 33 days for the parental *E. coli* strain to reach that state (Boaretti et al., 2003). However, it was reported that *rpoS* was expressed continuously during the entry into VBNC for *V. vulnificus* (Smith and Oliver, 2006).

1.3.2 *Stringent response*

A broadly conserved bacterial stress response system is the stringent response. This mechanism allows for adaptation to nutrient exhaustion, and can be activated by different stress and starvation signals. The alarmones guanosine 5’-diphosphate 3’-diphosphate (ppGpp) and guanosine 5’-triphosphate 3’-diphosphate

((p)ppGpp), denoted together as (p)ppGpp, are induced during amino acid starvation (Spira et al., 1995). Two enzymes control (p)ppGpp levels, RelA (PSI, or ppGpp synthetase I)) and SpoT (PSII). RelA synthesises (p)ppGpp as a result of amino acid starvation which causes an increase in uncharged tRNA. In *E. coli,* (p)ppGpp has been shown to bind to RNA polymerase (RNAP) along with DksA, which provides resistance to starvation through altering transcription of certain genes (Paul et al., 2004). Genes involved in amino acid biosynthesis and transport are induced while rRNA and tRNA synthesis is repressed resulting in a reduction in overall translation (Srivatsan and Wang, 2008). For *E. coli*, (p)ppGpp has been shown to have an effect on the formation of persisters cells which are a dormant phenotype. Strains lacking *relA* and *spoT* produce less persister cells upon glucose exhaustion when compared to wild type *E. coli* cells (Amato et al., 2013). Similarly to this, deletions of *relA* and *spoT* have been shown to decrease the virulence of *Burkholderia pseudomallei* (Muller et al., 2012). The mode of action of (p)ppGpp can vary between bacteria as it does not always bind to RNAP. For *B. subtilis* it was elucidated that (p)ppGpp inhibits Gmk and HprT, two enzymes which affect GTP synthesis, resulting in a decrease of GTP levels (Kriel et al., 2012). Research has indicated there are a wide variety of signals and conditions that result in (p)ppGpp synthesis.

1.3.3 *Long term survival during nutrient exhaustion*

Long term survival of bacteria under nutrient poor conditions has been investigated in many different bacterial species (Arias et al., 2012; Britos et al., 2011; Byrd et al., 1991; Fung et al., 2010; Mandel and Silhavy, 2005; Watson et al., 1998). Endospores, a dormant cell phenotype, can survive under unfavourable conditions for many years until favourable conditions return allowing for growth. Nevertheless, this phenotype is limited to only a few genera of bacteria including *Bacillus* and *Clostridium* (de Hoon et al., 2010). However, bacteria that cannot produce a spore are also able to survive under nutrient poor conditions.

Several studies have investigated the length of time bacteria can survive without nutrients. The pathogenic *Salmonella typhimurium* was investigated for survival in prolonged batch culture and produced viable cells even after 110 days (Turner et al., 2000). Other studies have investigated the survival of fish pathogens, organisms that can survive in aquatic environments which have very limited nutrients. *Flavobacterium psychrophilum*, which is responsible for rainbow trout fry syndrome, was shown to produce culturable cells when incubated in stream water for 19 weeks,

however, it was only 0.07 % of the population (Vatsos et al., 2003). The study also performed a live/dead stain revealing that after 10 weeks 94.5 % of the population were “live” cells, yet only 2.2 % of these cells were culturable, suggesting a VBNC phenotype (Vatsos et al., 2003). In the context of live/dead stains, life and death is determined by the integrity of the membrane. If the membrane has been compromised the bacterium is considered to be dead or dying. For a live/dead stain cells with a compromised membrane will stain one colour while cells with an intact membrane will stain a second colour.

A second fish pathogen *F. columnare,* which causes columnaris disease, was shown to produce colonies for 14 days when incubated in ultra-pure water (Arias et al., 2012). The starved cells underwent morphological changes (coiling), which could be reversed with the addition of nutrients. Additional morphological changes have been seen in other starvation studies. For instance, cell shrinking was seen for *Micrococcus luteus* during the 70-plus days CFUs were detected. The cell diameter decreased from 1.3 µm to 0.4 µm over the first 10 days and then remained relatively constant (Kaprelyants and Kell, 1993). A decrease in cell size was also observed for *S. aureus* during a 20 day starvation incubation (Watson et al., 1998). In this study cells remained sensitive to penicillin G while under starvation conditions suggesting growth is still occurring as penicillin targets the cell wall. In contrast to the sensitivity observed with *S. aureus*, 6 week old *Mycobacterium tuberculosis* cultures showed tolerance to a 7 day treatment with rifampicin or isoniazid (Betts et al., 2002). *Brucella suis,* the causative agent of swine brucellosis, was shown to produce CFUs for 6 weeks when incubated in a salt solution based upon the minimal medium of *Brucella*. This study also highlighted that *B. suis* was not surviving off of nutrients generated from lysed cells (Al Dahouk et al., 2013). This was shown by washing the *B. suis* cultures after 3 weeks of starvation, and resuspending in “fresh” salt solution. No difference in the CFU was observed between the washed and unwashed cultures. This would suggest *B. suis* is not surviving through cryptic growth, which is believed to have an important role during nutrient deprivation. Cryptic growth is defined as the utilisation of nutrients released from dead cells.

1.3.4 *Growth advantage in stationary phase*

It has been observed that during the death phase not all cells lyse in an *E. coli* culture. These cultures have been maintained for 5 years with only the addition of water (Finkel, 2006). This growth phase has been termed long-term stationary phase

and, unlike stationary phase cells, the cells in this phase are more dynamic and showed cell division. This survival has been attributed to the growth advantage in stationary phase (GASP) phenotype which is defined as "*the ability of cells aged in long-term batch cultures to outcompete cells from younger cultures*" (Finkel, 2006).

The GASP phenotype can be observed through the mixing of different aged cultures. For instance, by mixing a 10 day old culture with a 1 day old culture, it was shown that the older cells out compete the younger cells, eventually forcing them to extinction (Finkel and Kolter, 1999). These types of competition experiments utilise antibiotic resistance markers in order to distinguish between the different cells. The GASP phenotype can be observed in the majority of *E. coli* batch cultures after 10 days of incubation (Finkel, 2006). The advantage of the GASP phenotype is not due to cells adapting to their new environment but it is genetically determined. This was revealed by two methods. Firstly cells that developed GASP maintained the phenotype after repeated exponential growth incubations (Zambrano et al., 1993a). Secondly, mutations associated with GASP can be cloned into strains which have not been aged, giving raise to the GASP phenotype (Zambrano et al., 1993a). Several GASP mutations have been identified in *E. coli*. The first identified was in *rpoS* which encodes RpoS, an alternate sigma factor (Zambrano et al., 1993b). Additional mutations identified include the *ybeJ-gltJKL* cluster, that encodes a high-affinity aspartate and glutamate transporter, and *lrp,* that encodes leucine-responsive protein respectively (Zinser and Kolter, 2000; Zinser et al., 2003). Continued incubation of these cultures beyond the 10 days resulted in the development of novel GASP mutations, indicating that long-term stationary phase cultures are highly dynamic. GASP has also been observed in *Salmonella* species, indicating that the phenotype is not specific to *E. coli* long term survival (Bacun-Druzina et al., 2007).

1.4 *B. subtilis* differentiation

Another method for increasing survival of bacterial populations is differention, this results in distinct subpopulations of genetically identical yet specialised cell types (Lopez et al., 2009). During times of stress, such heterogeneity provides the greatest probability that a portion of population will survive until favourable conditions return. This is known as a bet-hedging strategy and has been thoroughly studied in *B. subtilis* (Figure 1.2) (Lopez et al., 2009). The different cell types *B. subtilis* can differentiate into include competent cells, a state by which bacteria can take up exogenous DNA and incorporate it into their genome. *B. subtilis* can also differentiate

into a motile cell allowing the cell to move through a concentration gradient (Kearns and Losick, 2005). Cells may also become cannibals, whereby *B. subtilis* cells kill their brethren in order to use the released nutrients (Gonzalez-Pastor, 2011). Another cell type available within the differentiation options is a miner. These cells utilise extracellular enzymes to generate nutrients (Lopez et al., 2009). It is also possible for a cell to become a matrix producer; these cells construct a biofilm (Vlamakis et al., 2013). *B. subtilis* can also produce an endospore, which has DNA encased within a highly protective coat (Nicholson et al., 2000). The regulation pathways of these cellular differentiation processes are overlapping (Figure 1.3).

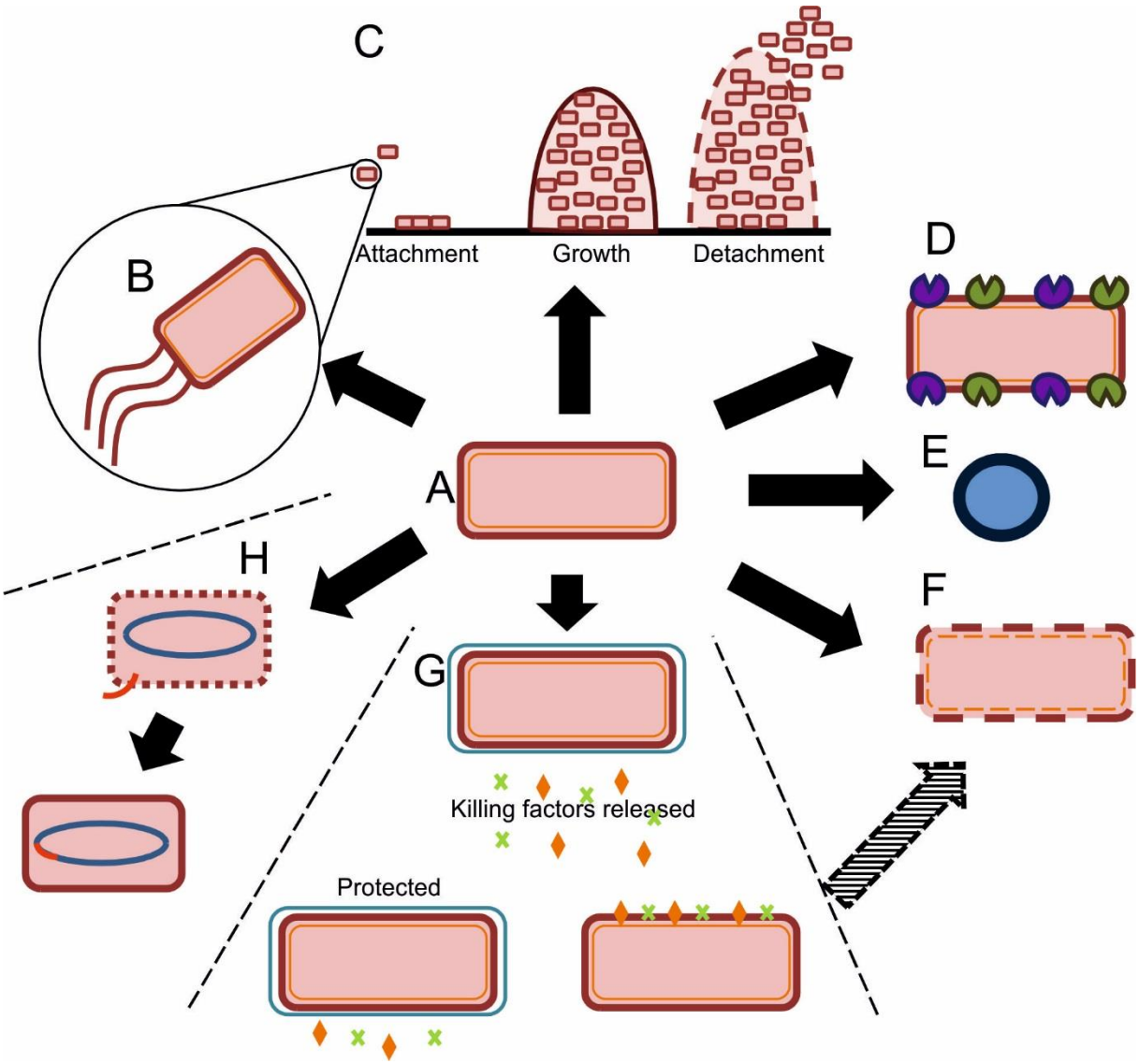

Figure 1.2 ***B. subtilis*** **differentiation phenotypes.**

A graphical representation of the phenotypes *B. subtilis* can differentiate into. A) Vegetative undifferentiated cell. B) Motile cell. C) Formation of a biofilm. D) Secretion of extracellular enzymes. E) Endospore. F) Dead cell undergoing lysis. G) Cannibalistic cell. H) Competent cell incorporating exogenous DNA. Figure modified from (Lopez and Kolter, 2010).

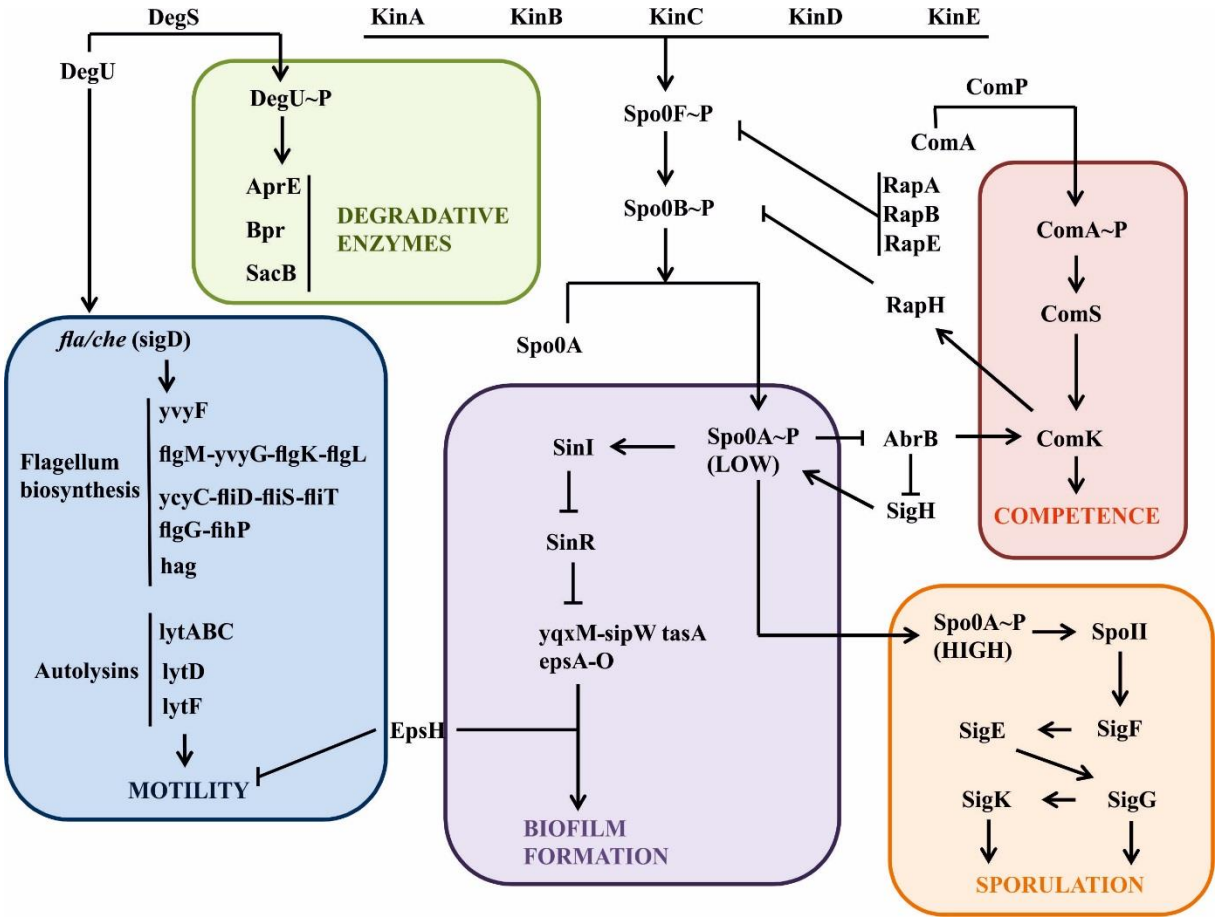

Figure 1.3 Regulatory network of differentiation pathways in *B. subtilis*.

A network representation of the major regulators involved in the pathways related to cellular differentiation of *B. subtilis*. Genes related to specific differentiation processes are grouped together by coloured boxes. Green represents the production of extracellular enzymes, red represents competence, orange represents sporulation, purple represents biofilm formation and blue represents motility. Figure modified from (Lopez et al., 2009).

1.4.1 *Motility*

In response to positive or negative stimuli, *B. subtilis* has the ability to move towards or away from the stimulus, respectively. This is achieved by the use of a flagellum, which is an external whip like structure and through rotation propels the bacterium forward (Guttenplan et al., 2013). There are two forms of motility *B. subtilis* can adopt, swarming and swimming. Swarming is a process by which groups of cells move across a solid surface, whereas swimming is the process of an individual cell moving in liquid medium (Calvio et al., 2005). The movement of a single cell is either in a straight direction or the cell is moving in place, “tumbling”. In order to move in a straight-line bacteria will use chemotaxis. This is achieved through the presence of a chemical gradient. Bacteria will move towards the highest concentration of food (e.g. glucose) or away from toxic chemicals (e.g. phenol).

The flagellum is divided into 3 sections, the basal body which anchors the flagellum and traverses the peptidoglycan layer (Mukherjee and Kearns, 2014). The second section is called the hook and is a curved hollow cylinder which acts as a universal joint (Courtney et al., 2012). The last component of the flagellum is the filament, which is a helical structure constructed from repeating protein monomers of flagellin (Mukherjee and Kearns, 2014). The propulsion of the flagellum allowing for movement comes from a rotating protein complex located in the anchor, the “fuel” for the complex is the flow of protons driven by the proton motive force (PMF) (Blair, 2003).

There are many genes required for a cell to become motile. The foremost are located in the *fla-che* operon. A key gene apart of this operon is *sigD*-encoding sigma factor SigD (Kearns and Losick, 2005), which is required for expression of the *hag* locus. Together these genes encode proteins which make up the basal body, flagellin, motor proteins and flagellar filament proteins (Lopez et al., 2009). Additionally, SigD induces the production of autolytic enzymes which ensure the cell is separated after cell division, allowing the bacterium to swim free. The *fla-che* operon is regulated by the unphosphorylated form of DegU (Tsukahara and Ogura, 2008). Therefore upon phosphorylation of DegU the induction of motility genes would decrease.

1.4.2 *Extracellular enzymes*

Another cellular differentiation process in *B. subtilis* is a cellular state that can produce large amounts of extracellular enzymes such as proteases (such as AprE) and polysaccharases (such as levansucrase) (Marvasi et al., 2010). Extracellular proteases and polysaccharases are made to provide extra nutrients through the degradation of exogenous proteins into small peptides, and polysaccharides into mono- and disaccharides that can be readily be utilised by the bacterium. At the end of exponential growth, *B. subtilis* can secrete at least 8 extracellular proteases (Park et al., 2004). These include the 2 major ones, alkaline serine protease subtilisin (AprE) and a neutral protease (NprE). There are 6 minor proteases; metalloprotease (Mpr), wall-associated extracellular protease (WprA), bacillopeptidase F (Bpr), Vpr, Epr and neutral protease B (NprB) (Park et al., 2004). Proteases are utilised in industry as components of detergent products, the majority of which are generated by *Bacillus* species.

Some of these extracellular enzymes are under the control of the DegS-DegU two-component system (Kunst et al., 1994). The master regulator DegU is phosphorylated by the kinase DegS (Dahl et al., 1991) (Figure 1.3). While DegU is a positive regulator of *aprE,* this gene is also controlled by negative regulators, including AbrB, SinR and ScoC (Abe et al., 2009). These proteins are regulated by Spo0A, indicating that Spo0A~P contributes to the production of extracellular proteases by the repression of the negative *aprE* regulators. It has been shown that Spo0A~P and phosphorylated DegU levels must reach a certain threshold before *aprE* expression occurs (Veening et al., 2008a). The high level of phosphorylated DegU that induces extracellular enzymes represses motility. This is understandable since it is not beneficially to move away while secreting enzymes.

1.4.3 *Competence*

Another example of cellular heterogeneity that occurs when *B. subtilis* cells enter stationary phase is the development of competence. A small portion of the population, approximately 10 – 20 %, halt growth and become genetically competent in response to high cell density (Sadaie and Kada, 1983). Competence is a state in which bacteria are able to uptake exogenous DNA. This phenotype allows *B. subtilis* to be very amenable to genetic modifications permitting rapid strain construction.

The development of competence depends on the activation of the transcription factor ComK (van Sinderen et al., 1995b). ComK activates its own expression as well as the transcription of all genes required for transformation (van Sinderen et al., 1995b). The positive feedback loop driving *comK* expression determines the bistable expression pattern of competence. The cellular ComK levels are normally kept too low to trigger this auto-stimulation. However, stochastic fluctuations can cause ComK concentration to exceed the threshold level for activation in certain cells upon which the positive feedback loop is activated (Smits et al., 2005). This causes ComK to be produced at very high levels, and to induce the expression of the downstream genes required for competence (Vansinderen and Venema, 1994). ComK is repressed by AbrB, however, anti-repression of AbrB is achieved by Spo0A~P, underlining the important role of Spo0A in the development of different phenotypes in *B. subtilis* (Figure 1.3).

1.4.4 *Cannibalism*

Although endospores are robust and allow for survival in adverse times, sporulation is energy expensive, time consuming, and a committed process. Therefore, if nutrients were to become available sporulating cells would be at a disadvantage since the sporulation process will continue. The cannibalism phenotype allows for the delay of sporulation through the release of nutrients (Gonzalez-Pastor et al., 2003). These nutrients come from the lysing of sister cells, which are susceptible to the toxins released from the cannibalistic cells. The cannibalism operons *skf* (sporulation killing factor) and *sdp* (sporulation delaying protein) are repressed by AbrB, therefore, the repression of AbrB by Spo0A~P allows for the activation of these genes (Greene and Spiegelman, 1996) (Figure 1.3). Furthermore, the promoter for the *skf* operon has a high affinity binding site for Spo0A~P. As a result of this, the *skf* operon is activated through two methods: the direct activation by Spo0A, and the indirect repression by AbrB (Fujita et al., 2005).

The *skf* operon contains 8 genes (*skfA-H*) that are responsible for the production and release of the killing factor which in turn results in the lysis of surrounding *B. subtilis* cells (Gonzalez-Pastor, 2011). In addition, the operon is also responsible for the immunity to the killing factor. The *sdp* cluster is made up of two operons, *sdpABC* and *sdpRI* (Hofler et al., 2016). The toxin SpdC, and its secretion, is encoded for by the operon *spdABC*. Initially SpdC was thought to be a signalling peptide involved in delaying sporulation, but later it was shown to be a toxin (Perez

Morales et al., 2013). The immunity to SpdC comes from *sdpRI* which is induced in the presence of the toxin. The combination of these toxins is enough to lyse sister cells that have not started to undergo sporulation.

1.4.5 *Biofilm*

Nearly all bacterial species produce biofilms. Biofilms are communities of bacteria attached to surfaces encased in an extracellular matrix (Vlamakis et al., 2013), providing them with protection to external stresses. Biofilms can form on almost all surfaces, and have been shown to form on clinical devices as well as pipes and tubes in industrial structures (Vlamakis et al., 2013). Biofilms are utilised in waste water treatment indicating they are therefore not only a hindrance but also have beneficial uses to man-kind (Vlamakis et al., 2013). In the case of *B. subtilis*, the biofilm is formed through the secretion of an extracellular matrix which is primarily composed of exopolysaccharide (EPS) and proteins. This structure provides protection from external stresses including lethal concentrations of antibiotics.

For biofilm synthesis to begin, low levels of Spo0A~P are required, similar to that of cannibalism. The anti-repression of two regulators (AbrB and SinR) by Spo0A~P allows for the activation of genes required for the secretion of the biofilm matrix (Banse et al., 2008). AbrB and SinR have overlapping repression targets (*eps* operon) indicating a high degree of control (Chu et al., 2006). AbrB is repressed directly by Spo0A~P while SinR is inhibited by SinI through the formation of protein-protein complexes which prevents SinR from binding to DNA (Colledge et al., 2011). Within a population only a subset of cells will inactivate SinR. Therefore, only a subpopulation will express genes required for biofilm formation (Ogura, 2016). Even within a biofilm population there will still be subpopulations of motile and spore forming cells which, upon disassembly of the biofilm, are dispersed into the environment (Stewart and Franklin, 2008).

The high levels of Spo0A~P required for sporulation can also act as a repression for pathways that require low levels of Spo0A~P. The *sinI* promoter has both high and low affinity activators for Spo0A~P, at low levels of Spo0A~P the high affinity activator site is bound and SinI is produced (Chai et al., 2008). However *sinI* expression is reduced when Spo0A~P levels rise, resulting in the low affinity activator sites being bound by Spo0A~P. As a result of this, once sporulation is activated, the cell halts biofilm formation (Vlamakis et al., 2013). Therefore, not only do levels of

Spo0A~P dictate which pathways are active but also the length of time they remain active. Due to both biofilm formation and cannibalism requiring only low levels of Spo0A~P, this allows for the development of a population with biofilm producers and cannibalism toxin producers. The toxin would kill sister cells which do not have immunity resulting in an amplified number of biofilm producers within the population, and thus an increase in biofilm construction.

1.4.6 *Sporulation*

Sporulation is the process by which an endospore is formed (Errington, 1993). The spore is a specialised cell type that is capable of surviving harsh environmental conditions including extreme temperatures, ultraviolet radiation, desiccation, freezing, detergents, chemical reagents and hydrolytic enzymes (Setlow, 2006). Survival under such conditions is likely because they are metabolically dormant and partially dehydrated. Not surprisingly, they can survive for extended periods of time with reports of spores germinating after decades (Vaishampayan et al., 2012). The ability to generate an endospore is restricted to only a couple of bacterial genera, including *Clostridium* and *Bacillus*; these include the pathogenic bacteria *Bacillus anthracis* and *Clostridium difficile* (Paredes-Sabja et al., 2014; Spencer, 2003). The commitment for a cell to sporulate is the result of a complex regulatory cascade known as phosphorelay that involves multiple kinases (KinA, B, C, D and E), and culminates in the phosphorylation of the transcription factor Spo0A (Figure 1.3). KinA has been shown to be the major kinase responsible for sporulation initiation as overexpression during exponential growth induces entry into sporulation (Stephenson and Hoch, 2002). The kinases A and B phosphorylate Spo0F which, in turn phosphorylates Spo0B, which finally phosphorylates Spo0A (Tojo et al., 2013).

Once the commitment to sporulation has been made, the first morphological change occurs with the formation of the asymmetric septum (Errington, 1991). During normal cell division the septum forms at mid-cell. However, during sporulation the septum forms closer to one of the cell poles (Figure 1.4). This divides the cell into two compartments known as the mother cell (larger compartment) and the forespore (smaller compartment). SpoIIE is required for the correct placement of the asymmetric septum and deletion of *spoIIE* has been shown to affect septum placement (Barak et al., 1996). The DNA is transported into the forespore by SpoIIIE (Bath et al., 2000), and the forespore is engulfed by the mother cell, a process similar to phagocytosis. The membrane of the mother cell expands around the forespore

culminating at the tip of the cell (Broder and Pogliano, 2006). SpoIIIE has been shown to be important for the membrane fusion (Liu et al., 2006).

Once the forespore is fully engulfed, the forespore is matured into the spore. This is achieved by the formation of multiply layers, including the cortex a modified cell wall that is synthesised outside the spore membrane (Driks, 1999). The cortex is then surrounded with a multi-layered proteinaceous coat (McKenney et al., 2013). After the production of the endospore it is released into the environment through lysis of the mother cell which is achieved by autolysins (Figure 1.4). The process that awakes these dormant spores is known as germination and occurs in response to environmental signals (Black et al., 2005).

Although the long term survival of *B. subtilis* has been observed this is usually attributed to the resistance of the spore. A study investigating VBNC *B. subtilis* in drinking water saw culturable cells for over 30 days (Byrd et al., 1991), however, this study never determined the number of spores within the culture. Therefore it is unclear how *B. subtilis* survived these conditions. Additionally it has been observed that during the death phase a subpopulation of cells survive while others lyse or sporulate (Veening et al., 2008b). In this thesis, the survival of these non-sporulating cells under nutrient exhaustion was investigated, in order to elucidate their survival strategy, and how long they can survive under nutrient deprivation.

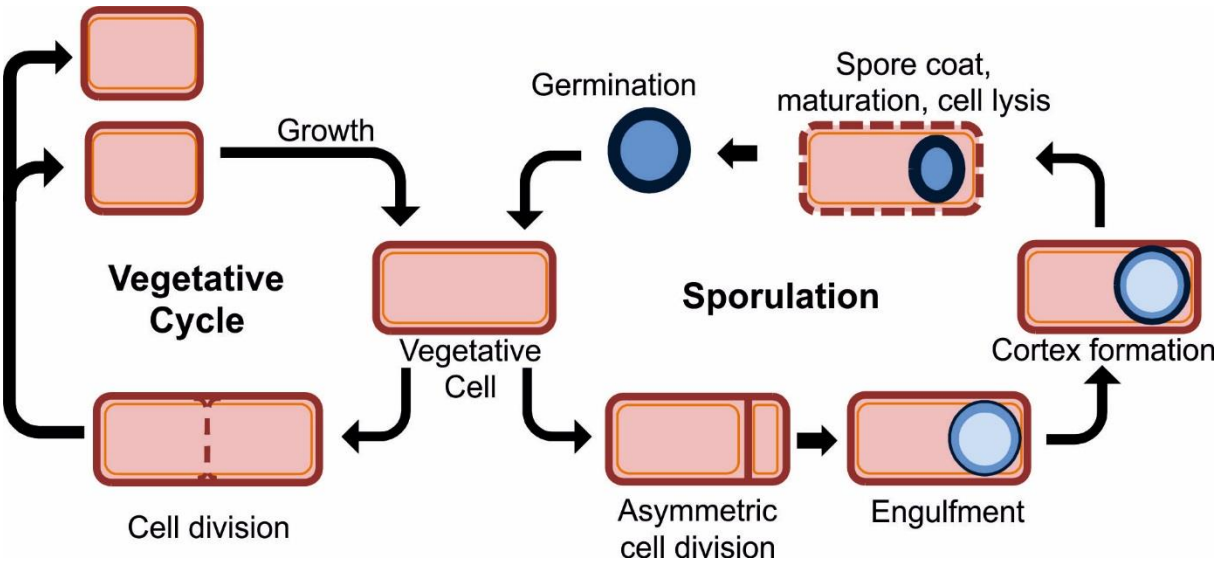

Figure 1.4 The sporulation cycle of *B. subtilis*.

The sporulation pathway of *B. subtilis* is initiated after nutrient exhaustion. Instead of a vegetative division site developing at mid-cell, an asymmetric septum develops closer to one of the cell poles, thereby dividing the cell into a larger compartment (mother cell) and a smaller compartment (forespore). After the DNA has been transported into the forespore it is engulfed by the mother cell. After engulfment, the spores protective layers are produced in order to protect the DNA. Once the spore has been produced, the mother cell undergoes lysis in order to release the spore. Upon environmental stimulation, the spore will undergo germination producing again a vegetative cell. Figure adapted from (McKenney et al., 2013).

1.5 Persister cells

Survival strategies that allow bacteria to survive times of nutrient exhaustion can also provide protection to additional stresses. One such phenotype is the so-called persister cell type which was first discovered in the 1940s by Joseph Bigger. He observed the failure of penicillin to sterilise exponentially growing cultures of *S. aureus* (Bigger, 1944). Bacterial persistence differs from antibiotic resistance because persister cells do not proliferate while the antibiotic is present, however, they can switch back to a growing state. As a result of removing the antibiotic stress, the cells generate a new culture which remains sensitive to antibiotics and again produces a small portion of persisters (Keren et al., 2004). The drug tolerance of persister has been attributed to their non-growing / slow growing state. This is achieved due to antibiotics primarily targeting active processes such as cell wall or protein synthesis (Figure 1.5). Despite the fact that investigating persisters is difficult due to their low numbers within a population, significant progress has been made over the last few decades. During the 1980s mutants of *E. coli* were identified that generated high frequencies of persisters (Moyed and Bertrand, 1983). The mutation was discovered to be within the “high persister” *hipA* gene, which increased persistence 1000 fold (Moyed and Bertrand, 1983). It was later discovered that the *hipA* gene encoded the toxin of a toxin-antitoxin system (Korch et al., 2003), the counter part of which is *hipB*, which encodes a repressor of *hipBA* transcription (Korch and Hill, 2006).

Bacterial population heterogeneity is an effective strategy employed by bacteria for population survival. However, it was unclear whether persisters were formed due to the addition of the antibiotic, or were already present within the population. Through utilising the *hipA E. coli* mutant which generates high quantities of persisters, microscopy revealed that normally growing bacterial populations have subpopulations of slow growing cells (Balaban et al., 2004). Many pathogenic bacteria, including *E. coli*, *S. aureus*, *M. tuberculosis* and *Pseudomonas aeruginosa*, generate persisters and this phenomenon has been associated with reoccurring reinfections.

1.6 Antibiotic resistance

The discovery of antibiotics over 80 years ago revolutionised the healthcare system, and allowed for the successful treatment of microbial infections. Antibiotics

are naturally produced by microorganisms as a method to kill competitive organisms within the environment (Mlot, 2009). The introduction of antibiotics has extended the human life span, and allowed for advancement in medicine and surgery. Penicillin was first prescribed in the 1940s and successfully treated serious infections. During World War II the use of penicillin saved the lives of soldiers with bacterial infections. However, not long after the introduction penicillin resistance became a clinical problem. In response to this threat new antibiotics were discovered. The successful application of antibiotics since their discovery can be attributed to the vast numbers that were discovered during the golden era of antibiotic discovery (1950 – 1960) (Davies, 2006). Antibiotics can be classically divided into two different groups, bactericidal and bacteriostatic (Mulligan and Cobbs, 1989). The latter only halt the growth of bacteria, while the former kill bacteria. The antibiotics in common practice target major but relatively few bacterial processes including cell wall synthesis (e.g. penicillin, vancomycin), transcription and translation (e.g. rifampicin and tetracycline respectively), DNA replication (e.g. quinolones), the membrane (e.g. polymyxin B and daptomycin) and other essential metabolic processes (Figure 1.5) (Kohanski et al., 2010).

Unfortunately, antibiotic resistance has been observed for virtually all antibiotics that have been developed. In addition to this, human pathogens are developing multiple resistance giving rise to “super bugs”, including methicillin resistant *S. aureus* (MRSA) (Kock et al., 2010), vancomycin resistant *Enterococci* (VRE) (Cetinkaya et al., 2000), *Streptococcus pneumoniae* (Nuermberger and Bishai, 2004), *Neisseria gonorrhoeae* (Unemo and Shafer, 2014), multi-drug resistant tuberculosis (MDR TB), and extensively drug resistant tuberculosis (XDR TB) (Seung et al., 2015). Several factors have brought about this wide spread resistance including overuse, extensive agriculture use, inappropriate prescribing, and the low discovery rate of new antibiotics. In addition to this, bacteria are able to transfer genes between species by horizontal gene transfer (Barlow, 2009), and resistance can be brought about by spontaneous mutants. Bacterial antibiotic resistance is becoming a serious health issue (Andersson and Hughes, 2011).

Additionally, it is now well known that bacterial populations can develop small fractions of persistent cells capable of tolerating antibiotic treatment (Dawson et al., 2011). In order to try to increase antibiotic effectiveness, and possibly to limit the onset of resistance mechanisms, it is increasingly important to not only try and

develop new antibiotics, but also to understand the mode of action of known antimicrobial compounds, this includes the secondary effects they may have after affecting their primary target. Recently, it has been proposed that antibiotics like beta-lactams, aminoglycosides and fluoroquinolones cause the production of reactive oxygen species (ROS) and therefore it was proposed that ROS contributes to the lethality of these antibiotics (Kohanski et al., 2007), this has remained controversial.

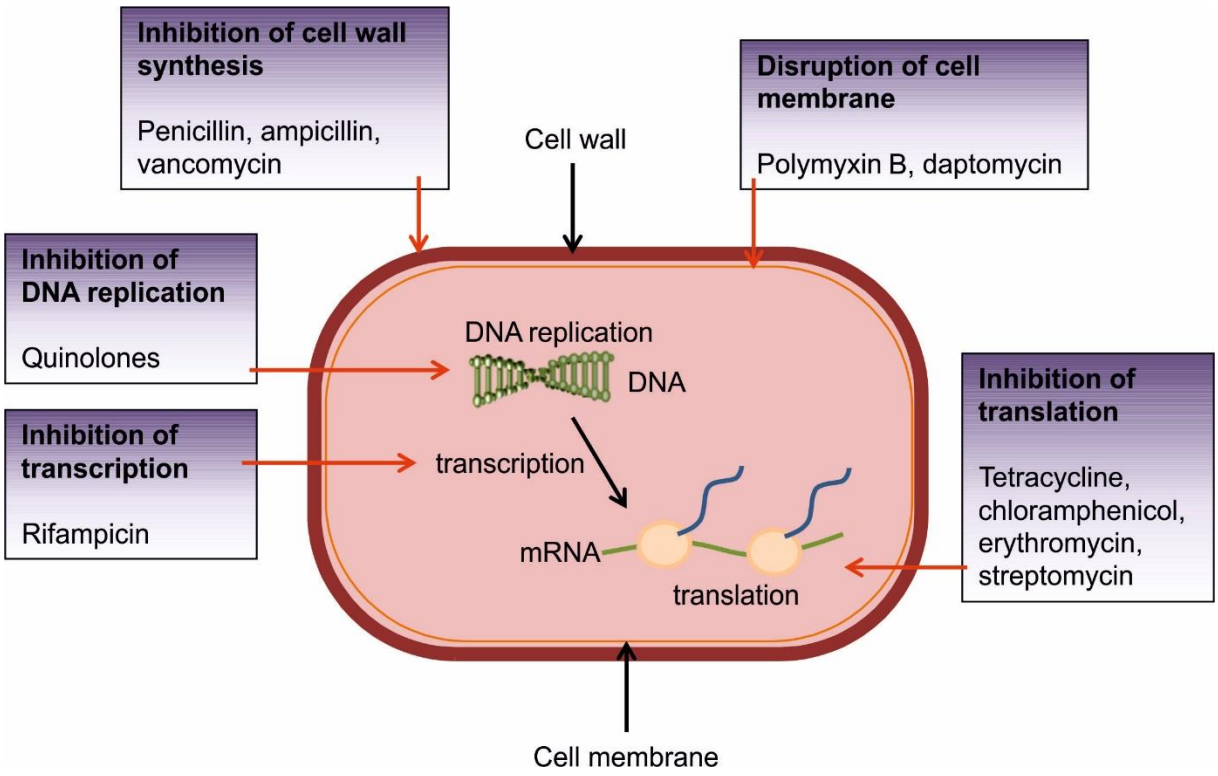

Figure 1.5 Common antibiotic targets.

Antibiotics are either bacteriostatic or bactericidal through targeting essential bacterial processes. The major targets of antibiotics are cell wall synthesis, the cell membrane, DNA replication, transcription, and translation. These cellular processes have differences between prokaryotes and eukaryotes allowing for specific targeting of bacterial cells within a human host. Figure adapted from (Lewis, 2013).

1.7 Antibiotics induce oxidative stress

The first study showing that antibiotics cause increased ROS production was published in 2007 (Kohanski et al., 2007). By use of fluorescent dyes which are sensitive to ROS production, it was shown in *E. coli* that ampicillin, kanamycin and norfloxacin cause the generation of hydroxyl radicals (HO•). Additionally it was shown that in the presence of an iron chelator or a hydroxyl radical scavenger, the killing efficacy of the three antibiotics was reduced (Kohanski et al., 2007; Wang et al., 2010). Further evidence supporting the generation of ROS by antibiotics was revealed through the increased susceptibility of DNA repair mutants (Δ*recA*) to all three of the antibiotics examined (Kohanski et al., 2007). Furthermore, an increase in sensitivity to antibiotic treatment was observed for *E. coli* oxidative stress mutants (Δ*sodA* and Δ*sodB*) (Feld et al., 2012).

In 2008, based on the work performed by Konanski, a model was proposed for the generation of ROS by aminoglycosides (kanamycin and gentamycin) in *E. coli* (Kohanski et al., 2008). These bactericidal antibiotics target the 30S ribosome subunit (Davis, 1987) causing mistranslation, resulting in immature membrane proteins being translocated across the membrane. The resulting misfolded proteins trigger phosphorylation of CpxA, which in turn phosphorylates CpxR, one of the key regulators of the envelope stress response (Merdanovic et al., 2011). However, CpxA also activates ArcA which regulates respiratory and metabolic genes (Iuchi et al., 1989; Ronson et al., 1987). The activation of respiration may bring about the formation of more free radicals (Kohanski et al., 2008). A more recent study has provided further evidence for ROS generation by antibiotics (Dwyer et al., 2014) through the use of a broad spectrum of ROS sensitive fluorescent dyes. Moreover, it has also been shown that the efficacy of antibiotic treatment was decreased under anaerobic conditions, and could be increased through the exposure to oxygen. Additionally, the overexpression of catalase or MutS, a DNA mismatch repair enzyme, reduced the lethality of antibiotics. Therefore, strong evidence exists for antibiotics inducing ROS, thus causing cellular damage as a secondary function.

However, the notion that oxidative stress is the cause of lethality of antibiotics has been disputed. First, oxidative stress triggered by antibiotic treatment did not appear universal among bacteria as *Listeria monocytogenes* oxidative stress mutants (Δ*sod* and Δ*fri*) were not more susceptible to ampicillin, gentamycin, or norfloxacin (Feld et al., 2012). Moreover, in another study it was shown that the killing efficacy of

these antibiotics was the same with and without the presence of oxygen, and *E. coli* mutants lacking catalase and peroxidase (Hpx-) were not more susceptible (Liu and Imlay, 2013). Furthermore, it was proposed that the redox fluorescent probes used in the original studies are not actually detecting ROS, but showed increased fluorescence due to increased cell size related to stress induced cell division blockage (Paulander et al., 2014). In addition, it has been shown that the fluorescent probes can also be oxidised by cytochrome *c* and other cellular components.

1.8 Oxidative Stress

1.8.1 *ROS and membrane potential generation*

During bacterial aerobic respiration toxic forms of oxygen are generated in the form of ROS. These include hydrogen peroxide (H_2O_2), the superoxide anion radical (O_2-) and HO• (Figure 1.6) (Imlay, 2002). The majority of these products are generated through sequential reduction of oxygen via respiratory enzymes. Respiration in bacteria consists of three major components, glycolysis, tricarboxylic acid (TCA) cycle, and the electron transport chain (ETC). Oxidative stress can also be induced by environmental agents such as near-UV radiation, and several compounds including paraquat, a widely available herbicide (Cabiscol et al., 2000). In addition, there are compounds that target the ETC, such as rotenone, antimycin A and cyanide, resulting in oxidative stress. Cells are deemed to be under oxidative stress when they are unable to cope with the level of ROS being generated.

The electron carriers NADH and FADH produced by glycolysis and the TCA cycle are oxidised at ETC complex I and complex II, respectively (Figure 1.7). Complex II is also a part of the TCA cycle carrying out the oxidation of succinate to fumarate (Shimizu, 2013). These electrons then flow through redox reactions while moving down the ETC until terminating at the terminal electron acceptor oxygen. From complex I and II the electrons are transferred to a quinone, either ubiquinone or menaquinone depending upon the organism, which carry the electrons to complex III (Kracke et al., 2015). The quinone molecules are reduced by the Q-cycle, a series of oxidation and reduction reactions, passing the electrons one at a time to cytochrome *c* which is highly conserved across species (Cramer et al., 2011). Complex IV is the final protein in the flow of electrons. Here the electrons reduce oxygen to form water (Figure 1.7). For *B. subtilis* there are alternative terminal oxidases, cytochrome *aa3* or cytochrome *bd*, which accept electrons directly from the menaqunione pool. This is

known as the quinol oxidase branch while the electron flow through complex III and IV is known as the cytochrome oxidase branch (Winstedt and von Wachenfeldt, 2000). During the flow of electrons down the ETC protons are transported across the membrane by complex I, II and IV. The movement of protons results in a pH difference known as ΔpH, along with membrane potential these make up the PMF. The membrane potential has been shown to be important for several cellular processes such as ion homeostasis, ATP synthesis, transport, protein secretion and protein localisation (Dimroth et al., 2000; Strahl and Hamoen, 2010).

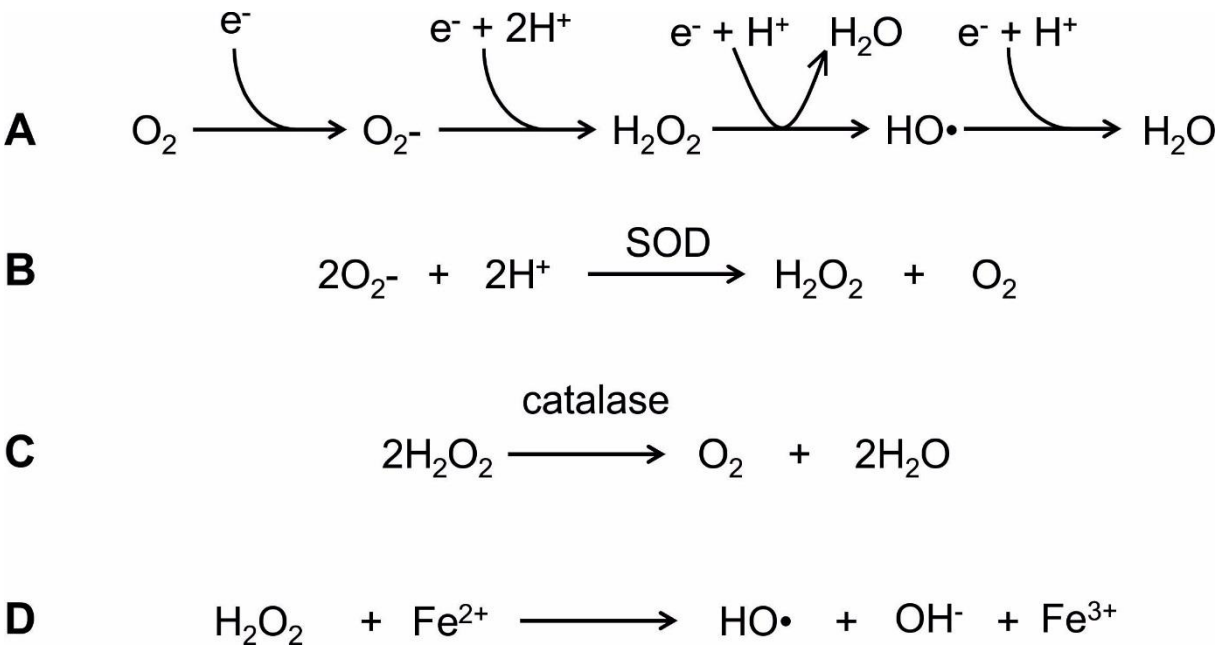

Figure 1.6 Oxidative stress.

A) Reduction series of oxygen to water by way of superoxide, hydrogen peroxide and hydroxyl radical. B) Conversion of superoxide to hydrogen peroxide by superoxide dismutase (SOD). C) Conversion of hydrogen peroxide to water by catalase. D) Fenton reaction; the reaction between hydrogen peroxide and iron to form hydroxyl radical.

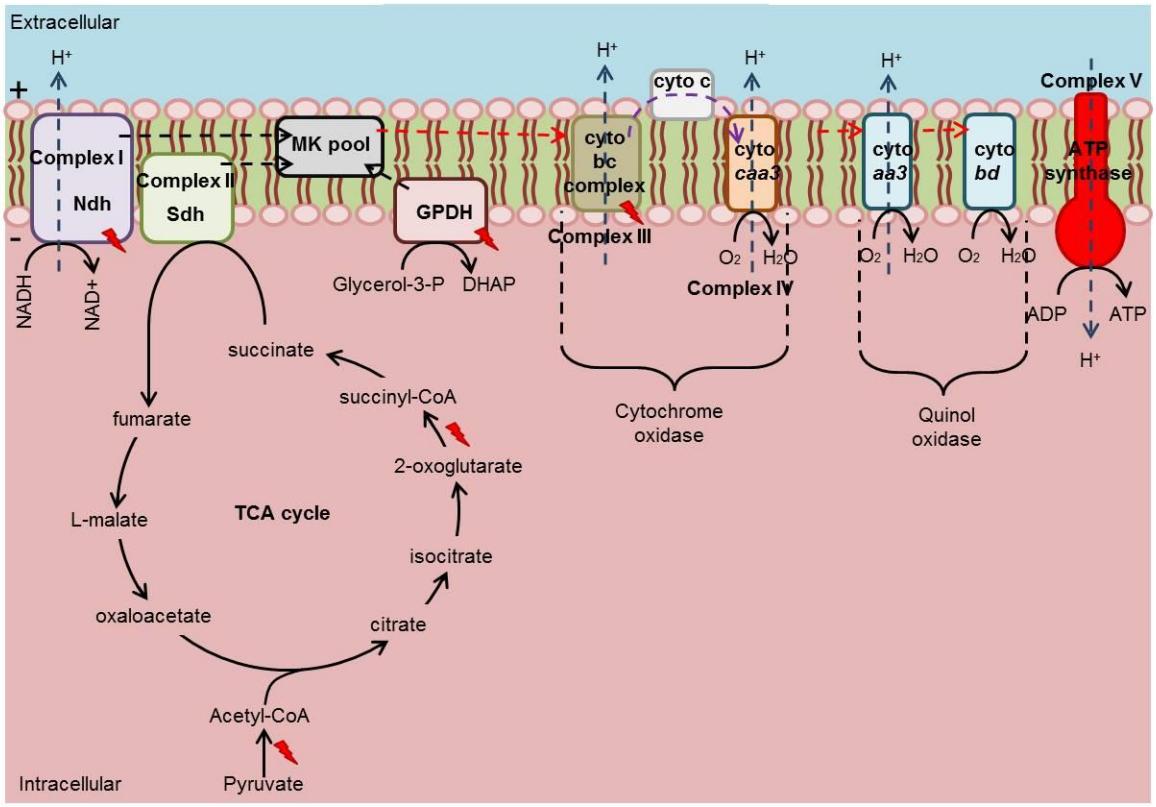

Figure 1.7 Cartoon representation of the TCA cycle and electron transport chain. Pyruvate, generated from glycolysis, is converted into acetyl-coenzyme A (CoA) by decarboxylation. Acetyl-CoA can then be utilised by the tricarboxylic acid (TCA) cycle, which consists of 8 enzymatic reactions to generate energy by producing NADH and FADH, which transport electrons to the electron transport chain (ETC). NADH dehydrogenase (Complex I - Ndh), succinate dehydrogenase (Complex II - Sdh) and glycerol-3-phosphate dehydrogenase (GPDH) transfer electrons to menaquinone (MK) generating a MK pool. From this electrons travel either the cyctochrome oxidase (Complex III and IV), or quinol oxidase route both terminating with oxygen reduced to water. During the movement of electrons from complex to complex, the reactions are coupled to the translocation of protons across the membrane. These protons are used to produce ATP from ADP by ATP synthase (Complex V). Black dashed arrows show the flow of electrons to the MK pool while red dashed arrows show the movement from the MK pool. Purple dashed arrows indicate the flow of electrons through cytochrome oxidase. Dark blue dashed arrows indicate the movement of protons across the membrane. Red lightning bolts represent known potential sources of ROS. Abbreviations: cyto, cytochrome; glycerol-3-P, glycerol-3-phosphate; DHAP, dihydroxyacetone phosphate. Figure adapted from (Shimizu, 2013).

1.8.2 *ROS damage and defence*

The ROS generated by either aerobic respiration or from an environmental stress can damage DNA, RNA, proteins and lipids. The base and sugar components of DNA are open to attack from ROS resulting in single or double strand breaks and can cause cross-links (Zgur-Bertok, 2013). The damage incurred by ROS has been attributed to HO•. Hydroxyl radicals are formed through the Fenton reaction, by which H_2O_2 reacts with Fe^{2+} producing HO• (Figure 1.6) (Winterbourn, 1995). The HO• are the most reactive species, however, they are also short lived and, as a result, they primarily cause damage close to the site of their formation. During oxidative stress lipid peroxidation can occur as a result of ROS, this is where electrons are removed from the lipids. This primarily affects polyunsaturated fatty acids in the membrane (Cabiscol et al., 2000). Reactions with polyunsaturated fatty acids can result in the formation of reactive aldehydes (Cabiscol et al., 2000). Proteins can be damaged by ROS through oxidation of amino acids, or by the end products of lipid peroxidation. The superoxide anion can attack the iron-sulphur cluster of proteins which affect the functionality of the protein, and releases free Fe^{2+} that can be used in Fenton reactions (Imlay, 2006). Additionally, protein oxidation can cause conformational changes to the protein structure, thereby decreasing or blocking enzyme activity. Highly susceptible amino acids are cysteine, methionine and histidine. Additional amino acids which are susceptible include, lysine, proline and tryptophan.

Defence mechanisms to oxidative stress involve the expression of enzymes that protect against ROS and that repair the resulting damage. These include detoxifying enzymes such as superoxide dismutases (SODs), peroxidases, catalases, alkyl hydroperoxide reductases and the organic hydroperoxide resistance proteins (Imlay, 2008; Mostertz et al., 2004; Seaver and Imlay, 2001). The defence by SODs is achieved through the dismutation of O_2- to H_2O_2 and O_2 (Figure 1.6). There are three main classes of SODs which differ in metal cofactor, (Mn, Fe or Cu/Zn). The main intracellular SOD in *B. subtilis* is SodA, which has a manganese cofactor (Inaoka et al., 1999). There are two other SOD enzymes, SodF and SodC, that have an iron-sulphur cluster, and a zinc metal cofactor, respectively. The H_2O_2 produced by SODs can also cause oxidative stress through Fenton reactions (Lloyd and Phillips, 1999). H_2O_2 can be removed by catalases forming H_2O and O_2 (Figure 1.6) (Hassett and Cohen, 1989). KatA the major catalase of *B. subtilis,* is encoded by *katA* that is part of the PerR regulon (Herbig and Helmann, 2001). In the absence of

H_2O_2 PerR binds to DNA repressing genes in the PerR regulon. Upon H_2O_2 exposure, PerR becomes oxidised and can no longer bind to the DNA allowing for induction of the PerR regulon. PerR regulates several genes including *katA* (catalase), *mrgA*, *ahpCF* (alkyl hydroperoxide reductase) and *hemAXCDBL* (haem biosynthesis enzymes) (Chen and Helmann, 1995a; Haikarainen and Papageorgiou, 2010; Zuber, 2009). In addition to removing H_2O_2 the PerR regulon is also involved in sequestering intracellular iron by up-regulation of *mrgA* and fur, attempting to remove any Fenton reactions.

Spx is an oxidative stress regulator, induced specifically by thiol-specific oxidative stress. Under the regulation of Spx is the synthesis of molecules involved in maintaining the redox balance within the cell such as bacillithiol. It is believed this low-molecular-weight thiol is the “glutathione” of *B. subtilis*, although it has been shown that bacillithiol and glutathione have different biophysical characteristics (Gaballa et al., 2010). Also under the regulation of Spx are *trxA* and *trxB*, encoding thioredoxin and thioredoxin reductase respectively. Thioredoxins are present in all domains of life, and are involved in protecting proteins from oxidative damage. Thioredoxin is an antioxidant that catalyses the reduction of disulphide bonds that can arise from ROS interactions with proteins (Zeller and Klug, 2006). Upon DNA damage the SOS response is activated by RecA which binds to ssDNA stimulating cleavage of LexA, allowing for transcription of genes required for DNA repair (Zgur-Bertok, 2013).

1.9 Membrane potential targeting compounds

Since non-growing bacteria, including persisters, are tolerant to most current antibiotic treatments, it is important to find methods that effectively kill these cell types. The major targets of antibiotics are mostly active processes for instance cell wall synthesis or transcription and translation (Figure 1.5). In dormant phenotypes these processes are not active and as a result antibiotics are ineffective. Nevertheless, the membrane is essential whether the cells grow or not. The dissipation of the membrane potential through ionophores such as valinomycin has been shown to be affective against both actively growing and dormant *M. tuberculosis* cell types (Rao et al., 2008). Therefore dissipation of the membrane potential could be an effective method for treating persister cells.

Besides antibiotics, there are compounds such as carbonyl cyanide m-chlorophenylhydrazone (CCCP) that are capable of killing bacteria by also disrupting membrane potential. CCCP in anionic form becomes protonated in the periplasm forming a neutral compound which can diffuse across the membrane releasing the proton into the cytoplasm. With the anion form of CCCP re-established it is again attracted to the positive side of the membrane, the periplasm, to obtain a new proton thus continuing the cycle, transferring protons from the periplasm to the cytoplasm disrupting membrane potential and ΔpH (Kasianowicz et al., 1984). Valinomycin is a naturally occurring compound produced by *Streptomyces* species, which is able to transport potassium ions (K^+) across the membrane due to its cyclic dodecadepsipeptide structure. The ring structure generates a hydrophilic centre, which accommodates the K^+ ion, and a hydrophobic exterior, which allows for diffusion within the cell membrane. The function of valinomycin has been shown to be dependent upon a high external concentration of K^+ ions (Te Winkel et al., 2016). Moreover, both CCCP and valinomycin depend upon the pH of the medium. Valinomycin has been shown to be ineffective below pH 7.5 (Tempelaars et al., 2011), conversely, CCCP has reduced effectiveness above pH 7.5 (Wada et al., 1992). In addition to these compounds there are many others which result in depolarisation of the membrane, including gramicidin produced by *Bacillus brevis*, and cereulide produced by *Bacillus cereus*, to name a couple (Nakai et al., 2005; Tempelaars et al., 2011).

Although dissipation of membrane potential causes cell death, the exact mechanism by which this occurs is not fully understood. Studies have identified processes that are dependent upon the membrane potential. It was shown for *B. subtilis* and *E. coli* that the membrane potential is also important for protein localisation (Strahl and Hamoen, 2010). By the utilisation of GFP tagged proteins, it was shown that upon dissipation of membrane potential the localisation of proteins, such as MinD, FtsA and MreB were disrupted. Additionally, membrane potential has been observed to be critical for the generation of ATP (Dimroth et al., 2000). Further to this, for *B. subtilis* dissipation of membrane potential has been shown to affect respiration (Azarkina and Konstantinov, 2002; Schirawski and Unden, 1998). It was suggested that electron flow through the ETC was disrupted at the dehydrogenase-menaquinone due to the inhibition of 2,6-dichlorophenolindophenol (DCPIP) and N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (TMPD) becoming

reduced (Azarkina and Konstantinov, 2002). However the exact location of disruption was never identified.

1.10 Aims of this thesis

B. subtilis is known to survive for long periods of time. However this has been largely attributed to the formation of an endospore. Very few studies have examined the long-term survival of *B. subtilis* cells that do not produce endospores. The survival of bacteria incapable of sporulation is well documented, suggesting *B. subtilis* cells might be able to survive without sporulation. Therefore, one of the aims of this thesis was to examine the survival of these non-sporulating cells under nutrient deficient conditions. A further aim is to characterise these cells during long term incubation during nutrient deprivation, for instance are these cells in a dormant state? Additionally, the factors important for survival were also investigated.

Due to the development of antibiotic resistance and the presence of antibiotic tolerant phenotypes, it is important to develop new antibiotics and understand the mode of action and any secondary effects antibiotics may cause. As a consequence, one of the aims of this thesis is to investigate the effect of membrane-dissipating compounds on dormant (stationary phase) *B. subtilis* cells. The modes of action of the two dissipating compounds under investigation are already known, however, the exact reason for cell death after membrane potential dissipation is unknown; therefore, another aim is to determine what, after loss of membrane potential, contributes to cell death.

Chapter 2. Material and methods

2.1 Maintenance and growth of strains

Nutrient agar (NA) (Oxoid) was used for routine selection and maintenance of both *B. subtilis* and *E. coli* strains. Supplements were added as required: chloramphenicol (5 µg/ml), erythromycin (1 µg/ml), kanamycin (2 µg/ml), spectinomycin (50 µg/ml), tetracycline (10 µg/ml), ampicillin (100 µg/ml) and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). For liquid cultures cells were cultured in either a Spizizen minimum medium (SMM) derivative ($(NH_4)_2SO_4$ 15 mM, K_2HPO_4 80 mM, KH_2PO_4 44 mM, $Na_3C_6H_5O_7$ 3 mM, 0.5 % glucose, $MgSO_4$ 6 mM, 0.2 mg/ml tryptophan, 0.02 % casamino acids and 0.00011 % Fe-NH_4-citrate), Luria-Bertani (LB), valinomycin media (tryptone 10 mg/ml, yeast extract 5 mg/ml, HEPES 50 mM and KCl 300 mM) or Penassay broth (PAB).

2.2 Strain construction

Strains used in this study are listed in table 2.1. All strains constructed during this investigation were done so by transformation (Anagnostopoulos and Spizizen, 1961; Young and Spizizen, 1961). For DG037, the erythromycin resistance cassette was removed using pDR244, this was performed according to the protocol provided by BGSC. In order to remove the plasmid along with the resistance cassette transformed cells are incubated at 42 ºC overnight. All strains containing a gene with a clean deletion were checked by polymerase chain reaction (PCR) using the oligonucleotides listed in table 2.2. In order to change the erythromycin marker in PG344, plasmid pErm::Spec was used. This plasmid inserts a spectinomycin marker into erythromycin thus removing the antibiotic resistance of erythromycin.

2.2.1 *Genome extraction*

Single colonies were inoculated in 4 ml PAB with 0.5 % glucose and incubated overnight at 37 ºC. Cultures were centrifuged for 10 minutes at 6000 rpm (Hettich Universal 320) and the supernatant discarded. The pellet was resuspended in 100 µl 50 mM ethylenediaminetetraacetic acid (EDTA), 300 µg/ml lysozyme and 300 µg/ml RNase, and incubated at 37 ºC for 60 minutes. 500 µl of nuclei lysis solution was added and the samples were incubated at 80 ºC for 5 minutes, and then cooled to room temperature. 200 µl of protein precipitation solution was added and the sample vortexed at high speed for 20 seconds, then kept on ice for 10 minutes. The samples

were then centrifuged at 13000 rpm (Thermo Heraeus Pico 17) for 10 minutes and the supernatant containing the DNA was transferred to an Eppendorf containing 600 µl isopropanol (room temperature). The samples were mixed by gently inverting the tubes until a visible mass of DNA was detected. Tubes were centrifuged for 10 minutes at 13000 rpm (Thermo Heraeus Pico 17), and supernatant discarded. The pellet was washed with 600 µl 70 % ethanol and centrifuged for 5 minutes at 13000 rpm (Thermo Heraeus Pico 17). The supernatant was removed and tubes were air dried for 15 minutes. The pellet was resuspended by incubating with 200 µl elution buffer at 65 ºC for 15 minutes and stored at -20 ºC until required.

2.2.2 *Transformation*

Competent *B. subtilis* cells were generated by starter cultures of strains grown in 10 ml of SMM overnight at 37 ºC. Overnight cultures underwent a 25 fold dilution into 10 ml of fresh SMM and were allowed to grow for 3 hours at 37 ºC. 10 ml of pre-warmed starvation media ($(NH_4)_2SO_4$ 15 mM, K_2HPO_4 80 mM, KH_2PO_4 44 mM, $Na_3C_6H_5O_7$ 3 mM, glucose (0.5 %) and $MgSO_4$ (6 mM)) was added to the cultures which underwent a further 2 hours incubation. A range of DNA/plasmid concentrations (100 – 600 ng) were then incubated together with the competent cells for 1 hour at 37 ºC, allowing for the uptake of DNA/plasmid. Transformed cells were then subsequently plated onto NA plates supplemented with the appropriate antibiotics.

2.2.3 *PCR amplification*

PCR amplification was used to amplify DNA fragments from plasmids or chromosomal DNA templates. The PCR reaction was carried out in a reaction made up of 0.02 U/µl of Q5 DNA polymerase (NEB), 1x of Q5 buffer, 200 µM of dNTPs (Promega), 0.5 µM of each primer, < 1000 ng of DNA template made up to a final volume of 50 µl with sterile distilled water. PCR was carried out in a PCR machine (Techne) with a typical reaction cycle of: 98 ºC for 30 seconds (initial denaturation) followed by 25 cycles of: 98 ºC for 10 seconds (denaturation), 55 ºC for 20 seconds (annealing) and 72 ºC for 1 minute per kb (extension) and finished with a final extension of 72 ºC for 2 minutes.

Oligonucleotides used in this study were designed using clone manager and purchased from Eurogentec and are listed in table 2.2. All oligonucleotides were dissolved in water to give a 10 µM stock solution and stored at -20 ºC. PCR

purification was performed using QiAquick PCR purification kit (Qiagen) and was used according to the manufacturer's protocol.

2.2.4 *Agarose gel electrophoresis*

DNA samples were mixed with DNA loading dye (Qiagen) in a 5:1 ratio and loaded onto 1 % agarose (Sigma-Aldrich) gel made using 1x TBE buffer (90 mM Tris-borate and 2 mM EDTA). Gels were stained with ethidium bromide (Invitrogen) and visualized with a UV transilluminator (Syngene transluminator) and molecular sizes were estimated using either a 1 kb or 100 bp ladder (NEB).

2.3 Starvation assay

Strains were cultured in the SMM derivative at 37 ºC with shaking for 48 hours. Following incubation, 10 ml of cells were collected by vacuum filtration using a 47 mm filter membrane with 0.45 µm pores (ThermoFisher). Cells were resuspended into 10 ml starvation buffer ($(NH_4)_2SO_4$ 15 mM, K_2HPO_4 80 mM, KH_2PO_4 44 mM, NaCl 50 mM and $MgSO_4$ 811 µM) by vortexing. Filtered cultures were incubated at 37 ºC with shaking for 14 days. Periodic sampling was performed to determine the CFU through serial dilutions and plating on NA. Plates were incubated overnight at 37 ºC and colonies counted the following morning.

2.4 Starvation stress survival assay

Samples from cultures undergoing starvation were taken on days 0, 7 and 14 and the following compounds were added as required;100 or 1000 µg/ml ampicillin, 5 or 50 µg/ml chloramphenicol, 10 or 100 µM valinomycin and 100 or 1000 µM paraquat. Cultures were incubated for 8 hours at 37 ºC with aeration and samples were taken every 4 hours in order to determine the CFU through serial dilutions and plating on NA. Plates were incubated overnight at 37 ºC and colonies counted the following morning.

2.5 Spore assay

The number of spores present within a population was determined by heating samples from starved cultures. Samples were heated for 25 minutes at 80 ºC after which samples were serially diluted and plated onto NA and incubated overnight. The following morning colonies were counted. Additionally, the CFU for samples that

were not heat-treated were determined. This allowed for the proportion of the population that were spores to be calculated.

2.6 Membrane potential dissipation assay

Strains were cultured in valinomycin media overnight at 37 ºC with aeration. Following incubation, cells were mixed with either 100 µM CCCP, 100 µM valinomycin or 1 % DMSO. Supplements were added as required; 10 mM tiron, 150 mM thiourea, 500 µM FerroZine, 500 µM 2,2'-bipyridyl, antimycin A (50 µM, 100 µM, 200 µM and 400 µM) and potassium cyanide (KCN) 5 mM. Cultures were incubated for 10 hours at 37 ºC with aeration, and samples were taken every 2 hours in order to determine the CFU through serial dilutions and plating on NA. Plates were incubated overnight and colonies counted the following morning.

For optical density (OD_{600}) measurements, from the overnight cultures 200 µl of *B. subtilis* cells were incubated with 100 µM CCCP, 100 µM valinomycin or 1 % DMSO in 96 well plates. The plates were incubated for 10 hours at 37 ºC in a microtitre plate reader (FLUOstar OPTIMA) and the ODs were monitored at 600 nm over time. Readings were taken every 5 minutes with 247s of double orbital shaking at 200 rpm, after each time point.

2.7 ATP assay

ATP levels were determined using the ATP bioluminescence assay kit HS II following manufacturer's instructions. This assay was performed in black walled 96 well plates. Briefly, samples (100 µl), taken every 2 hours from cells treated with or without CCCP or valinomycin, of which a sample of 25 µl was lysed using cell lysis reagent provided in the kit with a 5 minute incubation at 25 ºC. The luciferase reagent was added through automation of a FLUOstar OPTIMA plate reader, 50 µl was pumped in at a speed of 420 µl/s. After which the luminescence was measured. A standard curve was generated using known concentrations of ATP provided in the kit.

2.8 Anaerobic conditions

Strains were cultured in valinomycin media overnight at 37 ºC with aeration. Following incubation, cells were mixed with either 100 µM CCCP, 100 µM valinomycin or 1 % DMSO. 10 mM tiron or 150 mM thiourea were added as required. Cultures were then added to an anaerobic chamber and oxygen was removed using

AnaeroGen 2.5L sachets. Cultures were incubated for 10 hours under anaerobic conditions. The CFU was determined prior to incubation and after the 10 hour period through serial dilutions and plating on NA.

2.9 Microscopy

In general, microscopic experiments were performed using the Nikon Eclipse Ti microscope. Images were acquired using metamorph 6 (Molecular Devices, Inc) and analysed with ImageJ (National Institutes of Health). For visualisation, 0.3 µl samples were mounted on a thin layer of 1.2 % agarose and a 0.13 – 0.17 mm glass coverslip (VWR) was placed on top.

2.9.1 *Determination of cell length*

Cell length was determined through microscopic visualisation of cells using 0.4 µg/ml FM595 membrane dye (Molecular Probes). FM595 dye was added to samples of starved cultures and cell length calculated using ImageJ for approximately 100 cells. To determine change in cell length during starvation, samples were treated with and without 10 µM 3-methoxybenzamide (3-MBA) for 48 hours at 37 ºC with shaking. Microscopy was then performed and the change in cell length was determined using ImageJ for approximately 100 cells. The cell length was determined after 0 and 48 hours and the difference was calculated for cells treated with and without 3-MBA.

2.9.2 *Induction of gfp*

Microscopy determination of GFP induction levels was achieved using an IPTG inducible GFP *B. subtilis* strain (*phyper-spank-sfGFP* - DG017). Samples from cultures in starvation conditions were incubated for 4 hours with and without 1 mM IPTG at 37 ºC. Cells were visualised microscopically and GFP levels were calculated using ImageJ for approximately 100 cells by measuring the average level of fluorescence within whole cells.

2.9.3 *Membrane potential*

Membrane potential determination was achieved using the voltage-sensitive dye 3,3'-Dipropylthiadicarbocyanine iodide ($DiSC_3(5)$), which accumulates on hyperpolarised membranes. The membrane potential was assessed for starved cells by incubating cells with 2 µM $DiSC_3(5)$ for 5 minutes at 37 ºC (Eppendorf Thermomixer Compact) followed immediately by microscopy using commonly

available Cy5-filter sets. As a control, logarithmic growth phase cells were also treated with $DiSC_3(5)$ and also with or without 10 µg/ml gramicidin, a depolarising agent.

2.9.4 *Superoxide detection*

Superoxide detection was achieved using a superoxide sensitive probe, OxyBURST Green H_2DCFDA succinimidyl ester (2',7'-dichlorodihydrofluorescein diacetate, SE) (ThermoFisher). The superoxide level was assessed in cells treated with 100 µM CCCP and 100 µM valinomycin. As a control, cells were treated with 100 µM paraquat a superoxide inducing compound. Samples were taken at 0 and 120 minutes after CCCP, valinomycin or paraquat was added, mixed with 50 µM OxyBURST Green and incubated for 15 minutes. Excess probe was washed away through centrifugation at 10000 rpm (Thermo Heraeus Pico 17) for 1 minute and cells were resuspended in SMM salts after which the cells were visualised by microscopy.

2.10 Growth curve

B. subtilis cells were grown in LB at 37 ºC overnight, the following morning cells underwent a 25 fold dilution into prewarmed LB and grown to exponential phase (OD_{600} 0.2 – 0.3), the cultures were then diluted to and OD of 0.02 in prewarmed LB. The OD of these cultures was then monitored, at OD_{600} using a Beckman DU 650 spectrophotometer, for 5 hours.

2.11 Competition assay

Competition assays were performed for Δ*spoIIE* vs. Δ*spoIIE divIVA-gfp rpoB** and Δ*spoIIE* vs. Δ*spoIIE divIVA-gfp*. *B. subtilis* cultures were set up in accordance with the starvation assay, on day 4 of starvation the cultures were mixed and monitored for an additional 10 days. The fraction of the population for each strain was determined through two methods: (a) antibiotic resistance on NA plates, and (b) GFP fluorescence. On days 0, 3, 7 and 10, after culture combination, samples were taken serially diluted and plated on to NA plates which were incubated overnight at 37 ºC. The following morning 30 – 50 colonies were patched onto chloramphenicol containing NA plates and NA only plates, and were incubated overnight at 37 ºC. The next morning colonies were counted for chloramphenicol resistance.

To determine the fraction of the population by GFP fluorescence samples were taken on days 0, 3, 7 and 10, after culture combination. For visualisation, 0.3 µl

samples were mounted on a thin layer of 1.2 % agarose and a 0.13 – 0.17 mm glass coverslip (VWR) was placed on top. Phase contrast and GFP fluorescent images were acquired using a Nikon Eclipse Ti microscope. Images were processed using ImageJ. To avoid bias, phase contrast images were used to count approximately 100 cells after which the number of fluorescent cells was determined using the corresponding fluorescent images.

2.12 Marker frequency analysis

Chromosomal DNA was extracted from cultures, set up in accordance with the starvation assay, using a DNeasy Blood and Tissue Kit (Qiagen). For logarithmic and stationary controls cells were cultured in SMM to OD_{600} 0.2 – 0.3 or cultured overnight respectively. PCRs were carried out with GoTaq (Promega) qPCR mix, and the Q-PCR was performed in a Rotor-Gene Q Instrument (Qiagen). Primers 5'-GATCAATCGGGGAAAGTGTG-3' and 5'-GTAGGGCCTGTGGATTTGTG-3', were used for quantification of the origin, amplifying the region between *dnaA* and *dnaN*. Primers 5'-TCCATATCCTCGCTCCTACG-3' and 5'-ATTCTGCTGATGTGCAATGG-3', were used for quantification of the terminus, amplifying the region downstream of *yocG*. The *oriC* to terminus ratio was calculated using the crossing points (CT) and PCR efficiency a relative quantification analysis (ΔΔCT) using Rotor-Gene Software version 2.0.2 (Qiagen). The results were normalised to the *oriC* to terminus ratio of *B. subtilis* spore DNA which has an *oriC* to terminus ratio of 1.

2.13 Genome sequencing

All sequencing was performed by the University of Amsterdam and the data analysis was carried out by Dr. Henrik Strahl.

2.14 Microarray

B. subtilis (Δ*spoIIE*) were set up in accordance with the starvation assay, however, a 1 L volume of SMM was used for culturing and 1 L of starvation buffer. On the 14th day of starvation 1 L cultures underwent suction filtration using 90 mm filter membrane with 0.45 µm pores (Millipore). Cells were collected into a pellet and instantly frozen in liquid nitrogen and stored at -80 ºC. All microarray experimental procedures and data analysis was performed by the University of Amsterdam and Prof. Leendert Hamoen respectively.

2.15 Determining protein concentration

The protein concentration of the buffer during starvation was determined using a Bradford assay (Bio-Rad) according to manufacturer's instructions. Samples from starvation cultures were taken through the incubation period (days 0, 4, 7, 11 and 14), cells were removed by centrifugation (5 minutes at 8000 rpm (Thermo Heraeus Pico 17)). The supernatant was transferred to a clean tube and used for the Bradford assay.

2.16 Strains and plasmids

Table 2.1 List of strains and plasmids used in this study

Strain	Genotype	Source or reference
BSB1	*trp+*	BaSysBio consortium
PG1	*trpC2, cmR, divIVA-gfp*	P. Gamba
PG344	*spoIIE::erm*	P. Gamba
PG344_2	*spoIIE::er(spec)m*	This work
DG001	*spoIIE::erm, divIVA-gfp*	This work
bSS47	*spo0A::kan*	S. Syvertsson
DG004	*spoIIE::erm, spo0A::kan*	This work
JK8	*trpC2 clpP::spec*	J. Kirstein
DG005	*spoIIE::erm, clpP::spec*	This work
JK42	*trpC2 clpX::kan*	J. Kirstein
DG006	*spoIIE::erm, clpX::kan*	This work
WB800	*nprE aprE epr bpr mpr::ble nprB::bsr vpr wprA::hyg*	(Wu et al., 2002)
ΔWB800	*nprE aprE epr bpr mpr::ble nprB::bsr vpr wprA::hyg spoIIE::erm,*	This work
2682	*recA::tet*	L. Hamoen
DG010	*spoIIE::erm, recA::tet*	This work
BRB01	*trpC2, nprB*	(Pohl et al., 2013)
BRB02	*trpC2, nprB, aprE*	(Pohl et al., 2013)
BRB03	*trpC2, nprB, aprE, epr*	(Pohl et al., 2013)
BRB04	*trpC2, nprB, aprE, epr, bpr*	(Pohl et al., 2013)
BRB05	*trpC2, nprB, aprE, epr, bpr, nprE,*	(Pohl et al., 2013)
BRB06	*trpC2, nprB, aprE, epr, bpr, nprE, mpr*	(Pohl et al., 2013)
BRB07	*trpC2, nprB, aprE, epr, bpr, nprE, mpr, vpr*	(Pohl et al., 2013)
BRB08	*trpC2, nprB, aprE, epr, bpr, nprE, mpr, vpr, wprA*	(Pohl et al., 2013)
Δ1 protease	*trpC2, nprB, spoIIE::erm*	This work
Δ2 protease	*trpC2, nprB, aprE, spoIIE::erm*	This work
Δ3 protease	*trpC2, nprB, aprE, epr, spoIIE::erm*	This work
Δ4 protease	*trpC2, nprB, aprE, epr, bpr, spoIIE::erm*	This work

Δ5 protease	*trpC2, nprB, aprE, epr, bpr, nprE, spoIIE::erm*	This work
Δ6 protease	*trpC2, nprB, aprE, epr, bpr, nprE, mpr, spoIIE::erm*	This work
Δ7 protease	*trpC2, nprB, aprE, epr, bpr, nprE, mpr, vpr, spoIIE::erm*	This work
Δ8 protease	*trpC2, nprB, aprE, epr, bpr, nprE, mpr, vpr, wprA, spoIIE::erm*	This work
ΔwprA	*wprA::erm*	BGSC
DG034	*wprA, spoIIE::erm*	This work
rpoB*	*spoIIE::erm, divIVA-gfp, rpoB**	This work
ΔmmgB	*mmgB::erm*	BGSC
DG097	*spoIIE::er(spec)m, mmgB::erm*	This work
ΔmanA	*manA::erm*	BGSC
DG100	*spoIIE::er(spec)m, manA::erm*	This work
ΔacoA	*acoA::erm*	BGSC
DG091	*spoIIE::er(spec)m, acoA::erm*	This work
ΔyobH	*yobH::erm*	BGSC
DG098	*spoIIE::er(spec)m, yobH::erm*	This work
ΔyisJ	*yisJ::erm*	BGSC
DG095	*spoIIE::er(spec)m, yisJ::erm*	This work
ΔytcP	*ytcP::erm*	BGSC
DG101	*spoIIE::er(spec)m, ytcP::erm*	This work
ΔyezD	*yezD::erm*	BGSC
DG092	*spoIIE::er(spec)m, yezD::erm*	This work
ΔcwlH	*cwlH::erm*	BGSC
DG096	*spoIIE::er(spec)m, cwlH::erm*	This work
ΔyesQ	*yesQ::erm*	BGSC
DG094	*spoIIE::er(spec)m, yesQ::erm*	This work
ΔyrdD	*yrdD::erm*	BGSC
DG093	*spoIIE::er(spec)m, yrdD::erm*	This work
ΔyteP	*yteP::erm*	BGSC
DG104	*spoIIE::er(spec)m, yteP::erm*	This work
ΔyxlE	*yxlE::erm*	BGSC

DG090	*spoIIE::er(spec)m, yxlE::erm*	This work
ΔyvnK	*yvnK::erm*	BGSC
DG109	*spoIIE::er(spec)m, yvnK::erm*	This work
OC003	*abrB::spec*	(Chumsakul et al., 2011)
DG024	*spoIIE::erm, abrB::spec*	This work
SinI	*sinI::spec*	L. Hamoen
DG019	*spoIIE::erm, sinI::spec*	This work
codY	*codY::spec*	S. Syvertsson
DG051	*spoIIE::erm, divIV-gfp::chl, codY::spec*	This work
comK	*comK::kan*	H. Strahl
DG021	*spoIIE::erm, divIV-gfp::chl,comK::kan*	This work
sigD	*sigD::kan*	S. Syvertsson
DG049	*spoIIE:erm, divIV-gfp::chl,sigD::kan*	This work
sigB	*sigB::chl*	J. Stülke
DG057	*spoIIE::erm, sigB::chl*	This work
pnpA	*pnpA::kan*	L. Hamoen
DG048	*spoIIE:erm, divIV-gfp::chl, pnpA::kan*	This work
ΔnucB	*nucB::erm*	BGSC
DG067	*spoIIE::er(spec)m, nucB::erm*	This work
ΔnucA	*nucA::spec*	L. Hamoen
DG047	*spoIIE::erm, nucA::spec*	This work
ΔperR	*perR::erm*	BGSC
DG055	*spoIIE::er(spec)m, perR::erm*	This work
Spx	*spx::kan*	S. Syvertsson
DG025	*spoIIE::erm, spx::kan*	This work
ΔsodA	*sodA::erm*	BGSC
DG037	*spoIIE::erm, sodA*	This work
ΔkatA	*katA::erm*	BGSC
DG051	*spoIIE::erm, katA*	This work
ΔmrgA	*mrgA::erm*	BGSC
DG043	*spoIIE::erm, mrgA*	This work
ΔmsrA	*msrA::erm*	BGSC
DG045	*spoIIE::erm, msrA*	This work
ΔmsrB	*msrB::erm*	BGSC

DG044	*spoIIE::erm, msrB*	This work
ΔsodF	*sodF::erm*	BGSC
DG042	*spoIIE::erm, sodF*	This work
ΔsodC	*sodC::erm*	BGSC
DG038	*spoIIE::erm, sodC*	This work
YK1461	*yjlD(ndh)::tn-kan*	(Kawai et al., 2015)
DGYK1461	*spoIIE::erm, divIVA-gfp::chl, yjlD(ndh)::tn-kan*	This work
YK1462	*qoxB::tn-kan*	(Kawai et al., 2015)
DGYK1462	*spoIIE::erm, divIVA-gfp::chl, qoxB::tn-kan*	This work
ΔbshA	*bshA::erm*	BGSC
DG078	*spoIIE::er(spec)m, bshA::erm*	This work
ΔbshB1	*bshB1::erm*	BGSC
DG079	*spoIIE::er(spec)m, bshB1::erm*	This work
ΔbshB2	*bshB2::erm*	BGSC
DG080	*spoIIE::er(spec)m, bshB2::erm*	This work
ΔbshC	*bshC::erm*	BGSC
DG081	*spoIIE::er(spec)m, bshC::erm*	This work
ΔsdhC	*sdhC::erm*	BGSC
DG103	*spoIIE::er(spec)m, sdhC::erm*	This work
ΔpdhB	*pdhB::erm*	BGSC
DG102	*spoIIE::er(spec)m, pdhB::erm*	This work
ΔglpD	*glpD::erm*	BGSC
DG082	*spoIIE::er(spec)m, glpD::erm*	This work
ΔqcrA	*qcrA::erm*	BGSC
DG106	*spoIIE::er(spec)m, qcrA::erm*	This work
lytABCDEF	*lytABC::neo, lytD::tet, lytE::chl, lytF::spc*	R. Daniel
DG022	*lytABC::neo, lytD::tet, lytE::chl, lytF::spc, spoIIE::erm*	This work
ΔrelA	*relA::erm*	BGSC
DG077	*spoIIE::er(spec)m, relA::erm*	This work
Hspank	*p_hyper-spank-sfGFP::spec*	T. Ewen
DG017	*p_hyper-spank-sfGFP::spec, spoIIE::erm*	This work
FB113	*cwlJ::tet, sleB::spec*	(Paidhungat et al., 2001)

Plasmid	**Genotype**	**Source or reference**
pDR244	*cre, spec, amp*	BGSC
pErm::Spec	*erm::spec*	(Steinmetz and Richter, 1994)

2.17 Primers

Table 2.2 Primers used to confirm clean deletions

Primer	Sequence	Gene
DG1	5’-GATCCTCCGGTGCTTGTG-3’	*Forward aprE*
DG2	5’-GGCCGCATCTGATGTCTTTG-3’	*Reverse aprE*
DG3	5’-GATACGCTTGACATCCCGAC-3’	*Forward bpr*
DG4	5’-GAACGCTCCGCCTACCAG-3’	*Reverse bpr*
DG5	5’-GCGCGATCCTTCACATAGCC-3’	*Forward nprE*
DG6	5’-GCCTCATTCGGTTAGACAGCG-3’	*Reverse nprE*
DG7	5’-CACCCGAGTGAATGTGC-3’	*Forward epr*
DG8	5’-CCTGCGAGCAGCAGTAATTC-3’	*Reverse epr*
DG9	5’-GCGGATTACACTGTTGAAGG-3’	*Forward mpr*
DG10	5’-CTCTGTACTCGGCTCCTCATC-3’	*Reverse mpr*
DG11	5’-GCTTATACTGGCATATGGAGC-3’	*Forward nprB*
DG12	5’-CATCGAGCTTATGAAAGAGCG-3’	*Reverse nprB*
DG13	5’-CTTAATCACAAGAGATATCCAC-3’	*Forward vpr*
DG14	5’-CTTATGAACAGAGACGAATTGC-3’	*Reverse vpr*
DG15	5’-GGAGGCCTGTGGGTCGGCTTC-3’	*Forward wprA*
DG16	5’-CGGCTTATCGGTATTCGATTGC-3’	*Reverse wprA*
DG19	5’-GAGCACCGCGCCGATTAGCACG-3’	*Forward sodA*
DG20	5’-CTTATCGTCCATCAGAAGG-3’	*Reverse sodA*

Chapter 3. Development of long duration starvation assay

3.1 Introduction

The generation of the endospore by *B. subtilis* allows the bacterium to survive for extended periods of time under harsh conditions. Nevertheless, sporulation is not adopted by 100 % of the population, a phenomenon which was shown clearly in a time-lapse microscopy experiment (Veening et al., 2008b). During the death phase of the population, when spores are released and others cells lyse, it was observed that a small subpopulation of *B. subtilis* cells did neither sporulate nor lyse. In order to study these cells an assay was developed. The experiments involved in the development of said assay are discussed in this chapter.

Within the literature there is no standard technique for investigating how bacteria respond to nutrient exhaustion, due to the diversity of bacterial species. There has been an array of assays used to study the survival of different bacteria under nutrient limitation (Arias et al., 2012; Britos et al., 2011; Byrd et al., 1991; Finkel, 2006; Fung et al., 2010; Mandel and Silhavy, 2005; Watson et al., 1998). In most of these studies bacteria are cultured to stationary phase and either resuspended into a new solution, or incubated further in their original solution. The latter does not remove any excess nutrients or secondary metabolites, and nutrients released from lysed cell. The resuspension of cells into a fresh solution upon reaching stationary phase is commonly used for aquatic bacteria (Arias et al., 2012; Vatsos et al., 2003). It is important to ensure that changes, e.g. pH, during the resuspension procedure are minimal as this may affect the survival of the bacteria.

3.2 Results

3.2.1 *Long term incubation of sporulation deficient B. subtilis cells*

B. subtilis spores can survive extreme conditions, and are able to survive for very long periods of time (Setlow, 1994, 2006). Since *B. subtilis* spores would hamper our starvation survival study, we removed this ability through utilising a *spoIIE* deletion mutant strain of *B. subtilis* that is unable to sporulate. SpoIIE is essential for the correct formation of the asymmetric septum during the early stages of sporulation (Barak et al., 1996). *B. subtilis spoIIE* deletion mutant cells were grown to stationary phase (overnight incubation) in either LB or SMM, after which the culture was left at 37 ºC with or without shaking (Figure 3.1A and B). During 100 days of incubation for shaking cultures, a decrease in the CFU by 6 and 5 orders of magnitude for SMM and LB respectively was observed (Figure 3.1A).

Since *B. subtilis* survives in a soil environment, aeration might be an issue. Therefore static, non-shaking, cultures were tested for their ability to survive long-term incubation for 100 days. For LB cultures a similar drop in CFU was observed for static and aerated cultures (Figure 3.1B). This time, the non-shaking SMM culture showed a similar CFU to that of the LB culture (Figure 3.1B). These experiments highlighted the capacity of non-sporulating cells to survive extended periods of time under nutrient deprivation. In the following experiments shaking was utilised because of biofilm-like formation on the tube wall and water-air interface that might interfere with the assay. These formations were not observed in the shaking cultures.

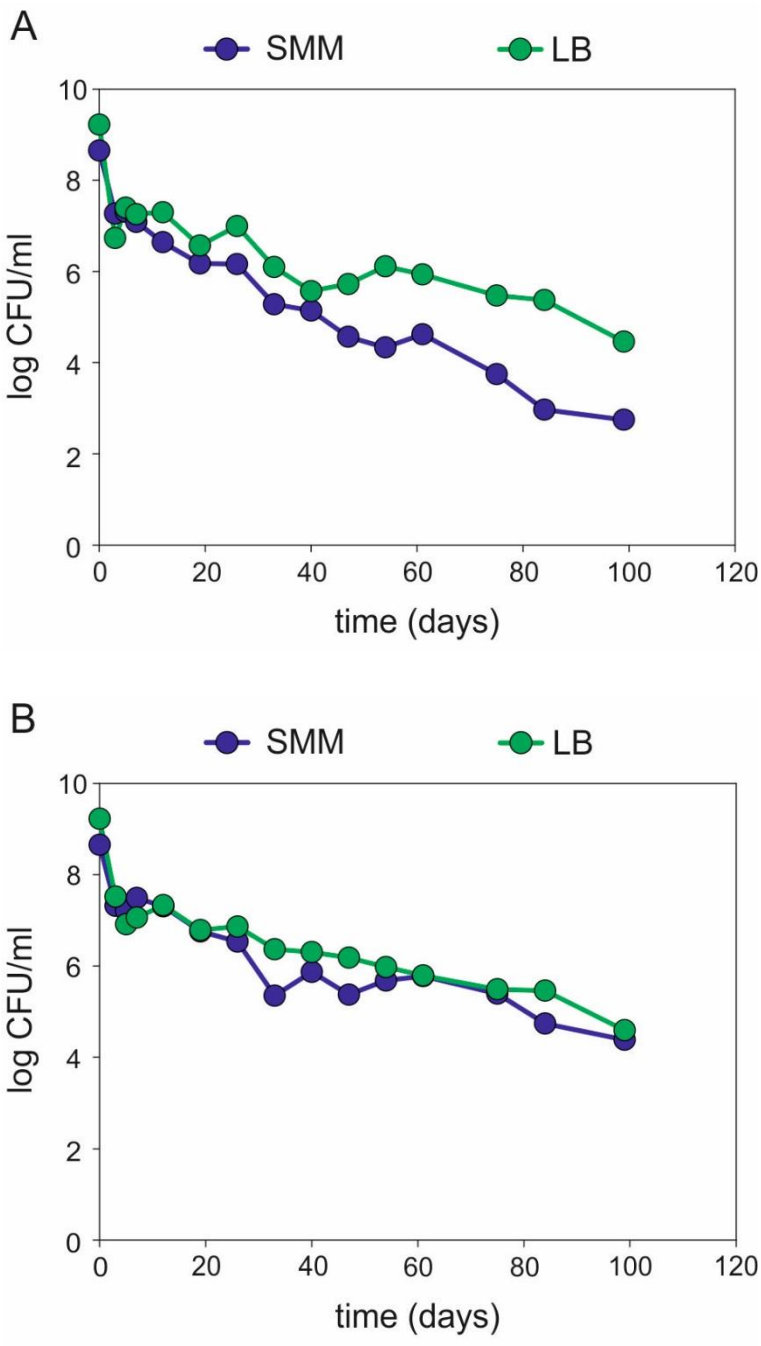

Figure 3.1 Long term survival of non-sporulation *B. subtilis* cells in different media. Cultures of Δ*spoIIE* were incubated in either LB or SMM media overnight at 37 °C. A) Cultures were left in the shaker at 37 °C. B) Cultures were taken out of the shaker after overnight growth and left at 37 °C on the bench. CFUs were determined by serial dilution and plating on NA. The graph represents the data of a single experiment.

3.2.2 *Improving starvation conditions*

In the previous experiment over flow metabolites were still present in the media broth. Therefore, cells from an overnight culture were washed, via centrifugation, and resuspended into three different nutrient limited solutions: SMM salts, starvation buffer and D.I. water. SMM salts are the base for SMM medium. However, this buffer still contains sodium citrate that might be a potential carbon source. Therefore, sodium citrate was replaced with sodium chloride (called starvation buffer). Cells were cultured overnight in either SMM or LB medium, centrifuged, and resuspended into one of the three solutions. CFUs were monitored for approximately 60 – 80 days. Interestingly, the CFU for all 3 wash solutions were very similar during the incubation period (Figure 3.2A, B and C), and showed in all cases a decrease of approximately 5 orders of magnitude. The majority of the decrease occurred within the first 7 – 10 days of incubation, followed by a plateau for the remainder of incubation. The initial growth medium showed also no effect on the CFUs.

Since the growth media had no effect on survival, and SMM medium is more defined and results in the cells becoming carbon starved, therefore, SMM medium was utilised for the initial growth of the bacteria. The ‘starvation buffer’ was chosen because it provided pH buffering conditions. This was achieved through the presence of 44 mM potassium dihydrogen phosphate and 80 mM dipotassium hydrogen phosphate, in addition the starvation buffer contained 15 mM ammonium sulphate, 50 mM sodium chloride and 811 µM magnesium sulphate. The experiments were limited to 14 days since the main reduction in CFU occurs during the first 7 days after which the CFU plateaued.

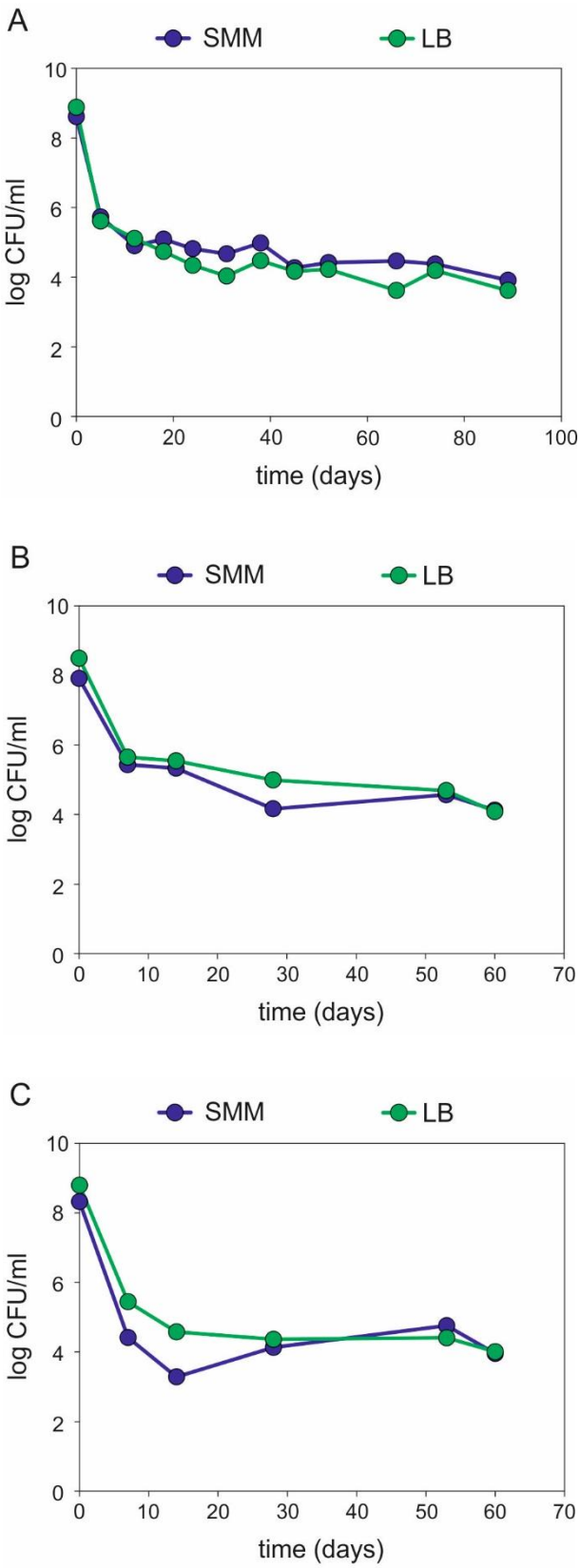

Figure 3.2 Resuspension of cultures in nutrient deficient solutions.

Cultures of Δ*spoIIE* were incubated in either LB or SMM overnight at 37 °C. Cultures were washed through centrifugation and resuspended in (A) SMM salts, (B) starvation buffer, and (C) water. CFUs were determined by serial dilution and plating on NA. These graphs represent the data of a single experiment.

The method initially used to wash the cells was centrifugation; however, centrifugal forces can stress the cells, therefore, dialysis and filtration were tested. Dialysis is the mildest method to remove nutrients; however, it turned out to be extremely difficult to sample the dialysing culture for CFU measurement without contaminating the culture. CFUs were only achieved at the start (day 0) and the end (day 14) of the experiment, and in fact the CFU was lower compared to cells washed by centrifugation (Figure 3.3). For this reason, we decided to test filtration as a means to remove excess nutrients. Cultures were filtered through a 0.45 µm membrane, and detached from the membrane by vortexing for 20 seconds. The CFU before and after filtration revealed there was no loss in CFU (not shown). As shown in figure 3.3, washing by filtration gave the same result compared to a culture that was washed by centrifugation. Filtration was taken forward as the cell collection method as it was the faster method.

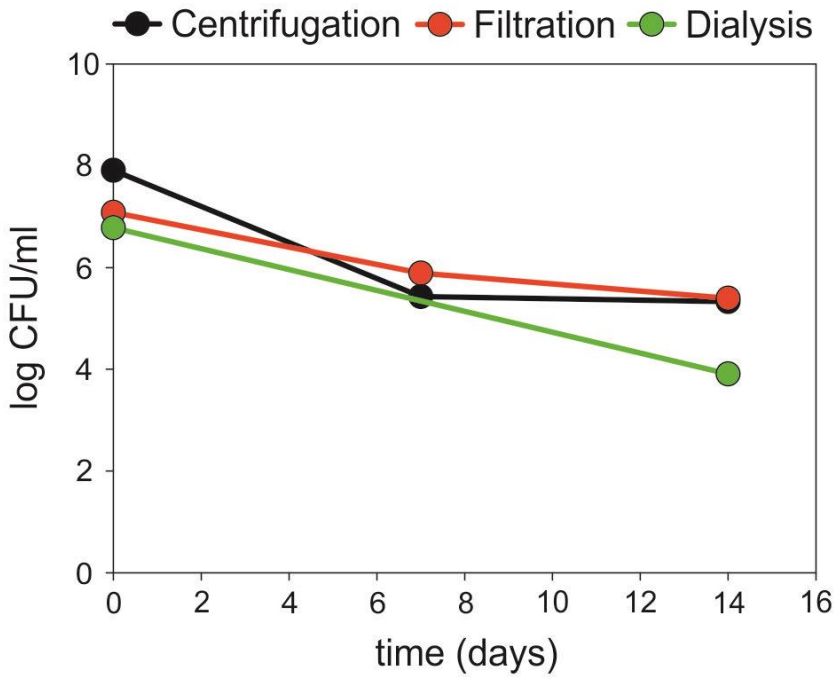

Figure 3.3 The effect of different wash techniques on starvation survival.
Cultures of Δ*spoIIE* were incubated in SMM medium overnight. The following morning cells were washed through centrifugation, filtration or dialysis. For centrifugation and filtration, cells were resuspended into starvation buffer and incubate with shaking at 37 °C. The dialysis cassette was incubated in 2 L of starvation buffer at 37 °C. CFUs were determined by serial dilution and plating on NA. This graph represents the data of a single experiment.

The initial reduction in CFU is also reflected in a strong reduction in optical density that can be observed by eye (not shown). A large number of cells that lyse will provide nutrients, which could be potentially used by the remaining cells. Most lysis of the overnight culture appeared to occur after 2 days incubation (without washing). Therefore, the day of filtering after inoculation was examined. Figure 3.4 shows the starvation survival of these washed cultures, where day 0 represents the time after cells were washed and resuspended into starvation buffer. Clearly, the day of washing had no effect on the overall CFU trend, and in all cases there was a decrease for roughly 7 days before a plateau was established. Since most of the culture lysed in 2 days (48 hour) incubation, we used this time point to filter cells in further experiments. The final experimental setup is schematically presented in figure 3.5.

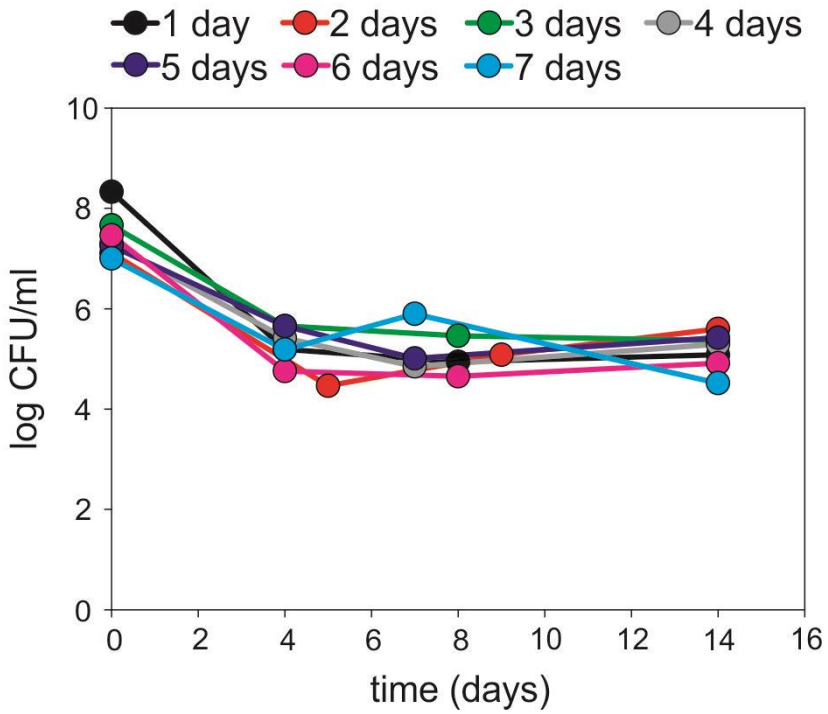

Figure 3.4 The effect of different incubation lengths before washing.

Cultures of Δ*spoIIE* cells were incubated for 1, 2, 3, 4, 5, 6 or 7 days in SMM at 37 ºC before filtration and resuspension into starvation buffer. Resuspended cells were incubated at 37 ºC for 14 days. CFUs were monitored throughout incubation. This graph represents the data of a single experiment.

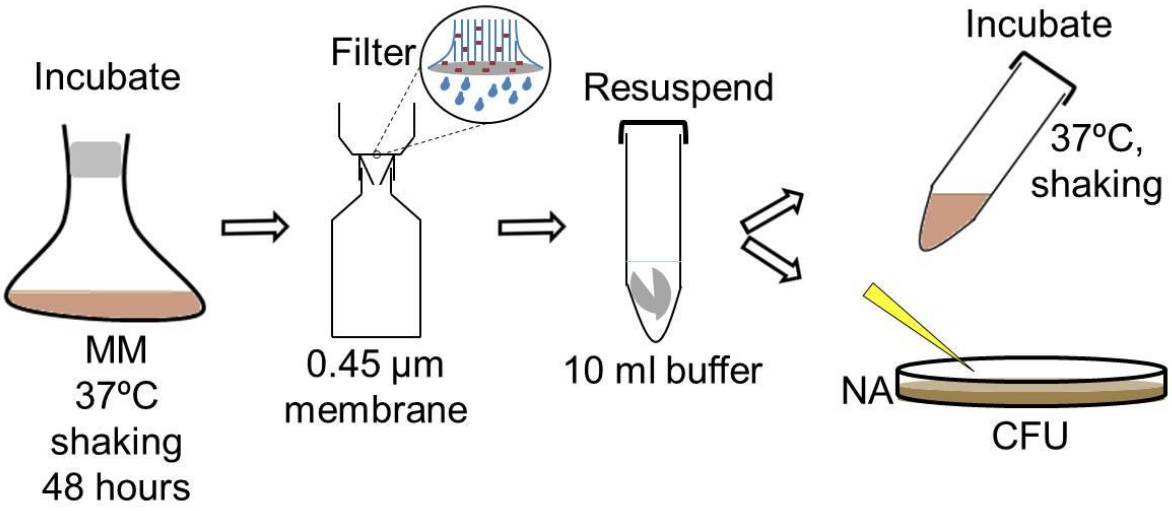

Figure 3.5 Starvation assay protocol.

SMM was inoculated with a single *B. subtilis* colony and incubated for 48 hours at 37 ºC under shaking. Cells were subsequently washed through filtration using a 0.45 µm membrane, and resuspended in 'starvation buffer'. Cultures were incubated at 37 ºC with aeration. The CFU was monitored for the duration of incubation by serial dilution and plating on NA.

3.2.3 *The survival of different non-sporulating strains*

Sporulation is regulated by an intricate stepwise signal transduction cascade that is initiated by the phosphorylation of the response regulator Spo0A. One of the first genes that are activated by Spo0A~P is ***spoIIE***. Although Spo0A is the key sporulation regulator, it is involved, directly and indirectly, in many starvation induced processes, including competence and biofilm formation (Hamon and Lazazzera, 2001; Mirouze et al., 2012). The blockage location of the sporulation cascade might affect long-term starvation, to assess this, a *spo0A* and a ***spoIIIE*** mutant were tested. The latter is involved in translocation of DNA into the forespore from the mother cell (Burton et al., 2007). The deletion of *spo0A* showed the same trend as a *spoIIE* mutant, a slight decrease in CFU was observed for the *spoIIIE* mutant after 14 days (Figure 3.6). During sporulation the formation of the asymmetric septum is known as the “point of no return”. Up until this point cells can leave the sporulation process. However, after its formation the cells are committed to sporulation. As a result *spoIIIE* deletion strains neither complete sporulation nor leave the process, causing them to be “stuck” and eventually lyse. For that reason a *spoIIE* deletion was used as the background phenotype for sporulation deficient *B. subtilis* cells. The *spo0A* deletion was not used because it is a regulator of over 300 genes (Molle et al., 2003).

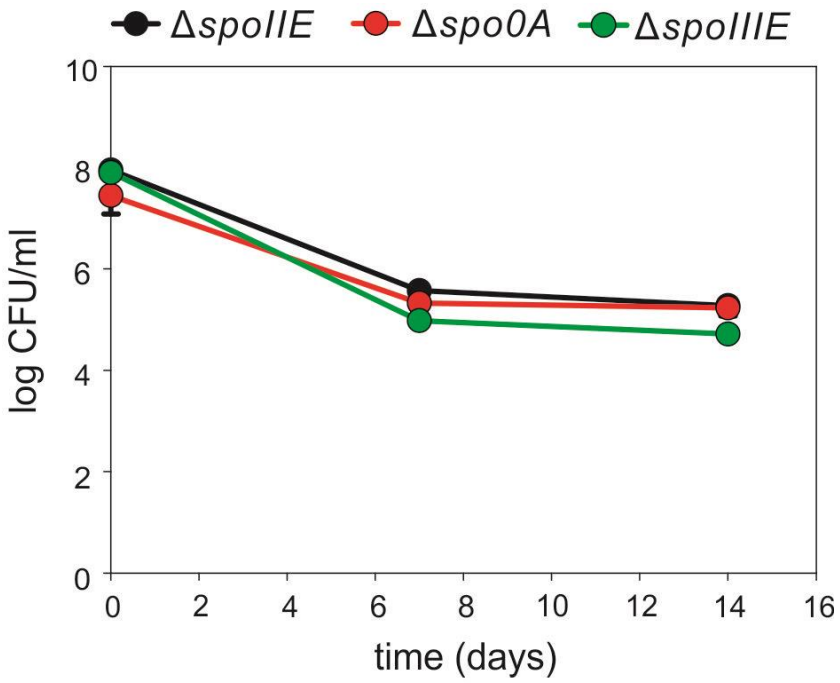

Figure 3.6 Survival of different sporulation deficient strains.

Cultures of Δ*spoIIE*, Δ*spo0A* or Δ*spoIIIE* were set up in accordance with the starvation assay. CFUs were monitored throughout incubation. This graph represents the data of a single experiment.

3.3 Conclusion

Throughout the literature many long term starvation survival experiments have been performed (Arias et al., 2012; Mandel and Silhavy, 2005; Watson et al., 1998). However, the methods vary substantially and no method has been described for *B. subtilis*. The reason for that is obvious since sporulation is considered an end-point for *B. subtilis* differentiation.

The final starvation assay is depicted in figure 3.5, and consists of a 48-hour incubation in a SMM derivative, this medium was chosen because it is chemically defined, and additionally the phosphate buffer base was used for the starvation buffer. The 48 hour incubation period was determined to be the minimum amount of time for the majority of the population to lyse allowing for the removal of over flow metabolites and any energy sources derived from the lysed cells. After incubation cells are collected through vacuum filtration. This collection method was chosen because it proved to be more efficient than centrifugation and dialysis. The starvation buffer was developed using the SMM phosphate buffer as a base, this meant when cells were resuspended there should be less of an environmental shock. The CFUs were determined through serial dilutions in LB and plating on NA plates. A variety of solutions were tested for the serial dilution ranging from water to LB however there was no difference in the number of colonies generated (data not shown). Additionally, different concentrations of NA plates were tested, ranging from 100 % to 50 % (data not shown), again no difference in the number of colonies produced was observed.

The preliminary experiments of this thesis have highlighted the capacity of non-sporulating *B. subtilis* cells to survive long term incubation. Additionally, they allowed for the development of an assay in which *B. subtilis* can be incubated under nutrient deprivation in a reproducible and controllable manner.

Chapter 4. Investigating the survival of non-sporulating *B. subtilis* under starvation conditions

4.1 Introduction

B. subtilis has a well-known capacity to survive unfavourable conditions for an extended period of time. This has been largely contributed to its ability to produce an endospore (Lopez et al., 2009). The spore is resistant to many of the environmental stresses a bacterium can encounter (Nicholson et al., 2000); however, sporulation is not adopted by the whole population due to the bet-hedging strategy employed by *B. subtilis* (Lopez et al., 2009). Additional cell types *B. subtilis* can differentiate into during times of stress include competence, a state by which bacteria become naturally transformable, and are able to uptake exogenous DNA and assimilate it into their genome. *B. subtilis* can also differentiate into a motile cell allowing for movement to and from certain stimuli (Kearns and Losick, 2005). Cells may also become cannibals, allowing them to kill their brethren in order to use the nutrients released (Gonzalez-Pastor, 2011). Another cell type available within the differentiation options is a so-called miner. These cells utilise extracellular enzymes to release otherwise unavailable nutrients (Lopez et al., 2009). It is also possible for a cell to become a matrix producer capable of biofilm formation (Vlamakis et al., 2013).

The mechanisms mentioned above are not limited to *B. subtilis*. For instance other species including *Streptococcus pneumoniae* (Steinmoen et al., 2002), *Haemophilus influenzae* (Macfadyen et al., 2001), and *Neisseria gonorrhoeae* (Aas et al., 2002) are able to become naturally competent. However, the ability to produce an endospore is restricted to specific genera including *Bacillus* and *Clostridium* (de Hoon et al., 2010). Despite the spore being an extremely effective method in providing protection from numerous stresses, it is not wide spread among the bacterial kingdom.

Since non-sporulating bacteria are able to survive for extended periods of time and since sporulation is limited to a subpopulation of cells in a *B. subtilis* population, the question arises whether *B. subtilis* that do not sporulate would still be able to survive in nutrient poor conditions. This question was also triggered by a time-lapse study of a *B. subtilis* micro-colony revealing that a small portion of the population did neither sporulate nor underwent lysis during death phase of the life cycle (Veening et al., 2008b). From studies of bacteria unable to sporulate it is conceivable these cells

have the capacity to survive for an extended period of time, even when they do not sporulate.

Aims of this chapter

The aim of this chapter was to investigate how well and by what mechanism *B. subtilis* cells that do not sporulate survive under nutrient poor conditions. Due to the heterogenic development of sporulation and fluctuating nutrient conditions, it is plausible this cell type occurs frequently.

4.2 Results

4.2.1 *Survival of sporulating and non-sporulating B. subtilis cultures*

Previous work have shown that *B. subtilis* cells can survive for over 3 weeks in water (Byrd et al., 1991). However, these experiments were performed with a *B. subtilis* strain that can undergo sporulation, moreover, in these experiments it was unclear which percentage of CFU originated from germinating spores. To examine this, the survival efficiency of our wild type strain (BSB1) was measured in our salt (starvation) buffer (Chapter 3) and in water, as this is a likely environment *B. subtilis* can encounter. Wild type cultures were set up in accordance with the starvation assay, consisting of a 48 hour incubation in SMM at 37 ºC after which cells were washed through filtration and resuspended in starvation buffer or water. CFUs were monitored for over 100 days through plating on NA (Figure 4.1A). The percentage of spores present within the population was determined through heating of samples of the culture at 80 ºC for 25 minutes followed by CFU determination on NA plates. This temperature shock is sufficient to kill vegetative cells while leaving spores unharmed and thus the CFU is a result of spore germination.

Under the conditions examined here, wild type *B. subtilis* cultures generated colonies for over 100 days (Figure 4.1A). An initial decrease in the CFU of approximately two orders of a magnitude was observed for the first 7 days of incubation (Figure 4.1A). After this a plateau in the CFU was established and no further decrease was seen for the duration of monitoring. No detectable difference between the CFUs, or number of spores was observed between cells incubated in buffer or water (Figure 4.1A and B). An initial increase from approximately 0 % to 70 – 80 % of spores was observed over the first 14 days. Over the remaining time the spore percentage remained relatively stable (Figure 4.1B). This indicates that there are a percentage of cells that does not sporulate and seem to be able to survive nutrient deprived conditions.

After establishing the rate of survival for cells able to sporulate, the corresponding ability was tested for non-sporulating cells. For this aim, a Δ*spoIIE* strain was tested under the same conditions as the wild type (starvation buffer and water). Surprisingly, a trend remarkably similar to the wild type was observed for Δ*spoIIE* cultures. In contrast to wild type cultures which maintained their CFU-values, a continuing although remarkably slow and gradual reduction of CFU was observed

for Δ*spoIIE*-culture (Figure 4.1C). In this case, the CFU decreased an additional order of a magnitude within 63 days, followed by a further slowing trend. Here a difference in CFU was detected between cultures incubated in starvation buffer, or in water. For water cultures a ten-fold lower CFU was observed after 14 days. This difference was maintained for the remainder of monitoring with only small fluctuations. Despite the lower CFU counts, our experiments show that non-sporulating *B. subtilis* cells are able to survive very long starvation periods.

It could be argued that the non-sporulating cells in the wild type culture originate from spores that germinate and that the Δ*spoIIE* mutant is therefore not a relevant model system. To investigate this, the survival of a strain unable to germinate after the spore was produced (FB113) was tested. If non-sporulating cells in our starvation assay originated from germinating cells then the CFU should decrease over time. However, the trend observed for this strain was similar to that of wild type cells for at least 11 days. On day 14 there was some decrease in the CFU (Figure 4.2). This suggests that non-sporulating *B. subtilis* can survive for weeks without sporulating.

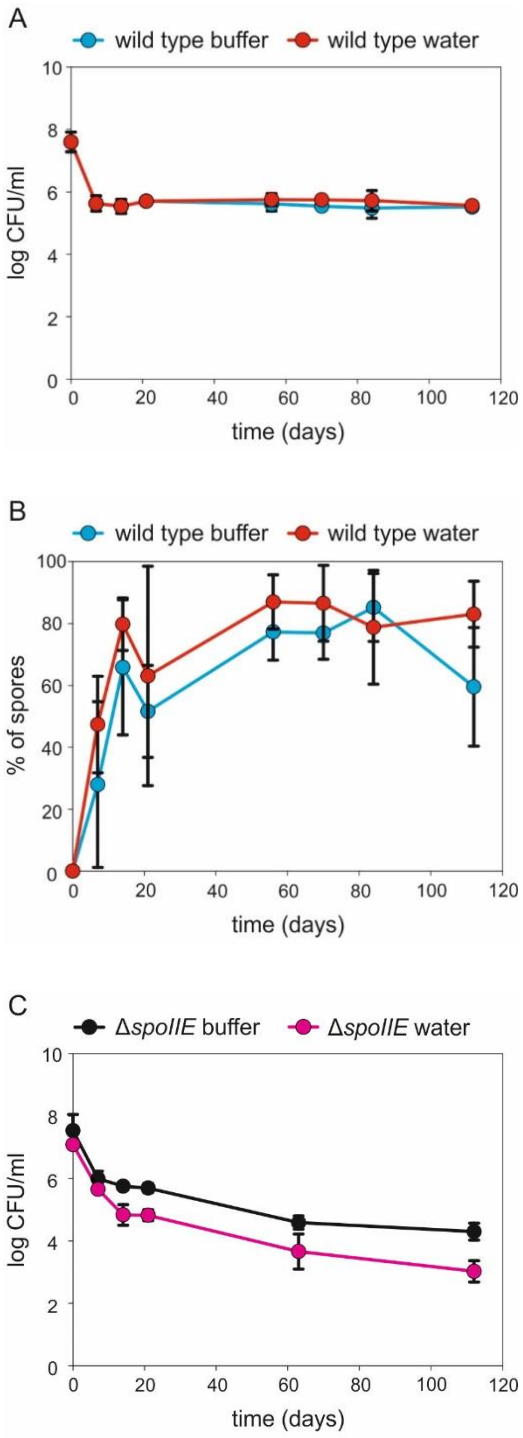

Figure 4.1 Long term survival under nutrient poor conditions.

Strains were inoculated in SMM and incubated for 48 hours at 37 ºC, after which cultures were washed through filtration and resuspended into either starvation buffer or D.I. water. Cultures were then incubated at 37 ºC and the CFU was monitored periodically. A) Total CFU for wild type cultures incubated in either buffer or water. B) Percent of spores present in wild type cultures. Spores were determined by incubating samples from cultures at 80 ºC for 25 minutes, then determining the CFU. C) CFU for Δ*spoIIE* cultures incubated in either buffer or water. The graphs represent the average and standard deviation of 3 independent experiments.

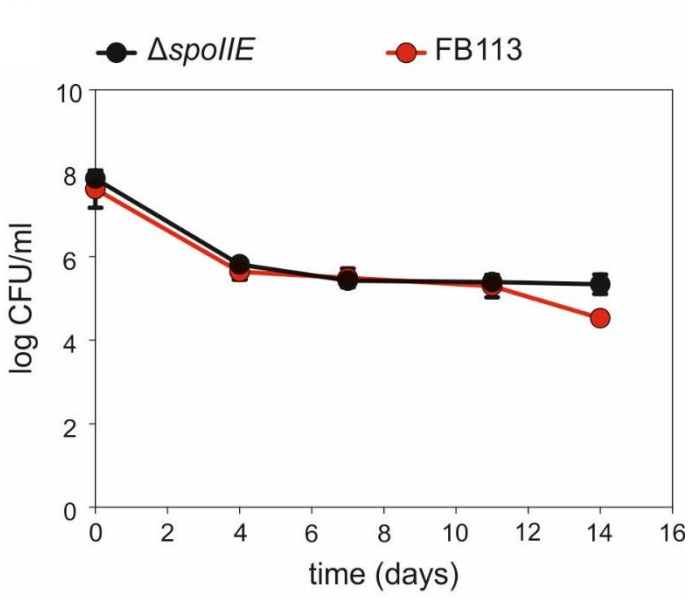

Figure 4.2 The effect of germination deficiency during starvation incubation.
Strains were inoculated in SMM and incubated for 48 hours at 37 ºC, after which cultures were washed through filtration and resuspended into starvation buffer. Cultures were then incubated at 37 ºC and the CFU was monitored periodically. Graph represents the average of 3 individual experiments with standard deviations.

4.2.2 *Metabolic activity*

The ability of non-sporulating *B. subtilis* cultures to survive nutrient limitation for long periods of time raises the question about their level of metabolically active. For this aim, the cell membrane potential was assessed as a proxy for active metabolism using an assay based on the voltage-sensitive dye $DiSC_3(5)$ (Te Winkel et al., 2016). In order to determine the membrane potential, samples were taken from starved cultures on days 7, 11 and 14 and the membrane-potential-dependent cellular accumulation of $DiSC_3(5)$ was analysed using a Nikon Ti fluorescence microscope. As a control, the membrane potential of logarithmic phase cells was determined in the presence and absence of membrane potential dissipating ionophore gramicidin (Figure 4.3A and B). For days 7, 11 and 14 the membrane potential was more comparable to the membrane potential of exponentially growing cells.

The membrane potential assay suggested that these cells were, after an initial adaptation period, indeed metabolically active. To verify this crucial finding with an independent method, the ability of the cells to actively transcribe and translate was assessed through induction of GFP from an IPTG inducible promoter. For this aim, samples from days 0, 7 and 14 were incubated in the presence and absence of IPTG for 4 hours (Figure 4.4), followed by fluorescence microscopy. Consistent with the low energy state of the cells, samples from day 0 showed only a low level of IPTG-induced fluorescence and over 90 % of the population had fluorescence values below 500 a.u. In contrast, samples from day 7 and 14 showed a higher level of induced fluorescence. For day 7 samples, 90 % of the population showed fluorescence between 1000 and 4000 a.u. The highest GFP fluorescence levels were seen on day 14 with over 80 % of the population displayed cellular fluorescence of over 5000 a.u. (Figure 4.4, Figure 7.1).

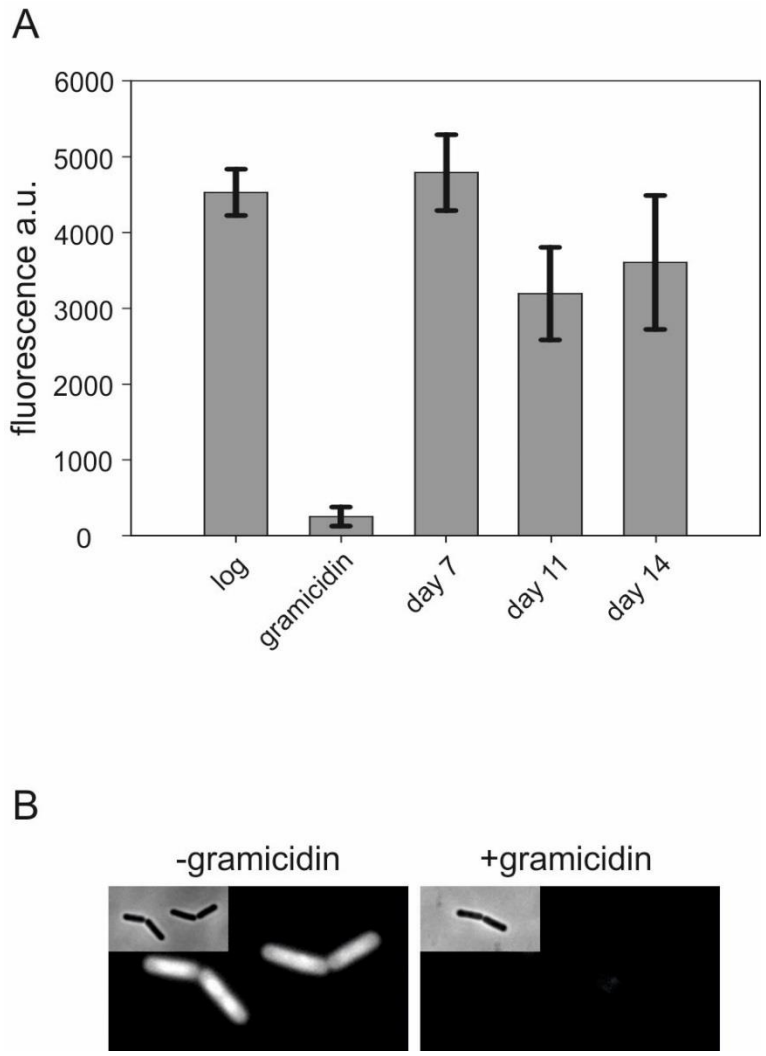

Figure 4.3 Membrane potential levels during starvation incubation.
Cultures of Δ*spoIIE* were set up in accordance with the starvation assay. On days 7, 11 and 14 samples were taken and incubated with 2 µM $DiSC_3(5)$ for 5 minutes followed by fluorescence microscopy using Nikon Ti. A) The average cellular fluorescence intensity was measured using ImageJ. As a control, exponentially growing cells (OD_{600} 0.2) were incubated with $DiSC_3(5)$ for 5 minutes in the presence and absence of 10 µg/ml gramicidin. The fluorescence intensity of approximately 100 cells was measured. The graph depicts the average and standard deviation of 3 independent experiments. B) Fluorescent microscopy of *B. subtilis* cells stained with $DiSC_3(5)$ in the absence and presence of gramicidin (left and right image respectively). Scale bar represents 2 µm.

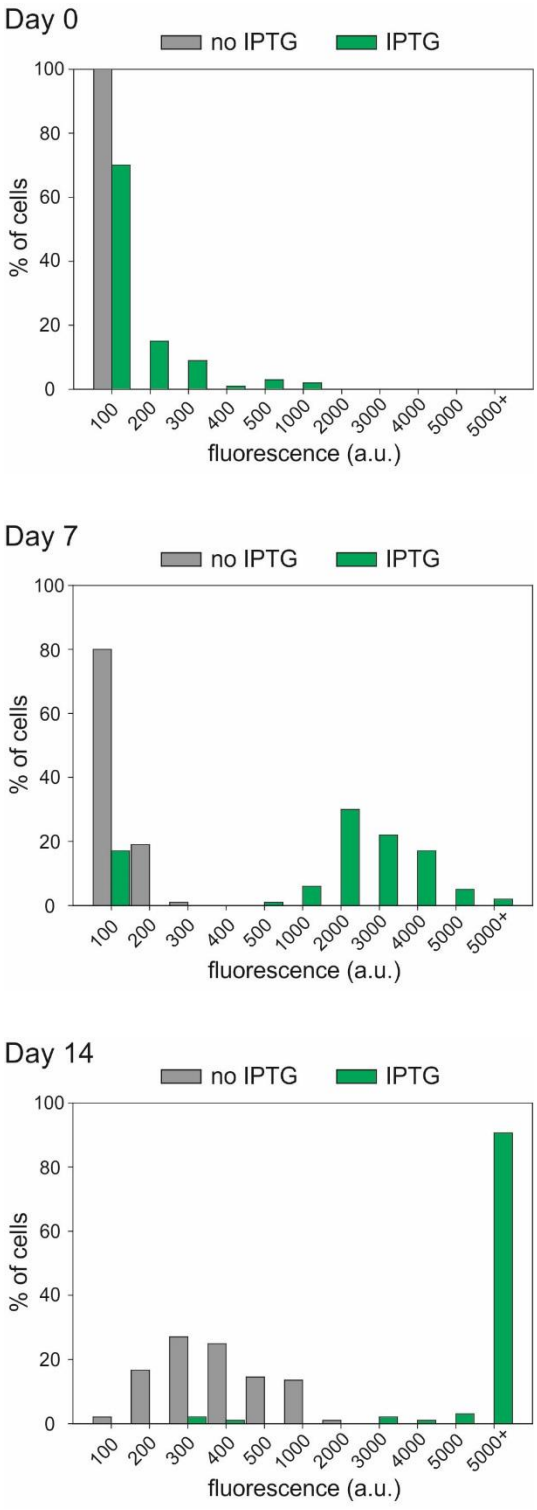

Figure 4.4 Inducing *gfp* during starvation incubation.

Cultures of *amyE::hyper-spank-sfGFP in a* Δ*spoIIE* background were set up in accordance with the starvation assay. On days 0, 7 and 14 samples were taken and incubated with and without IPTG for 4 hours, after which fluorescent images were acquired using a Ti Nikon microscope, and the GFP fluorescence levels measured using ImageJ. Approximately 100 cells were counted to determine the average GFP levels within a population. Graphs depict a representative of three independent experiments (Experimental replicates figure 7.1).

4.2.3 *Cell growth and length*

Morphological changes, including cell shrinkage and coiling, have been observed during times of nutrient exhaustion in several species of bacteria including, *S. aureus* and *F. columnare* (Arias et al., 2012; Watson et al., 1998). Therefore, the size and morphology of *B. subtilis* cells was assessed. Over the first 2 days of incubation in nutrient poor conditions, a decrease in the average cell length from approximately 2.5 µm to approximately 1.6 µm was observed (Figure 4.5). Over the remaining 12 days of incubation the average cell length remained stable.

Now the key question that arises is whether non-sporulating starved cells are actually dormant and non-dividing. The reduction in cell length and very low nutrient levels suggests that cells are dormant, however, the membrane potential and GFP expression measurements suggests that these starved cells are quite active. If the starved cells started to grow again, it is likely that they become sensitive for antibiotics. Therefore the sensitivity of these cells to different antibiotics was investigated. Culture samples were treated with either ampicillin, which targets the cell wall, chloramphenicol, which targets protein synthesis (Kohanski et al., 2010), valinomycin, which targets membrane potential (Ahmed and Booth, 1983), and paraquat, which induces the formation of ROS (Cabiscol et al., 2000). Two concentrations were tested for each compound. Untreated starved samples showed a plateau in the CFU over the 8 hour treatment period, except for the samples withdrawn from day 0 cultures which exhibited a small decrease in CFU (Figure 4.6A).

Exponential cells treated with 100 µg/ml ampicillin (Amp100) showed a decline of approximately 4 orders of magnitude over the 8 hours indicating potent bactericidal action. In contrast, only a comparably minor killing was observed for any of the starvation condition samples (Figure 4.6B). The observed decrease in CFU was enhanced when the ampicillin concentration was increased to 1000 µg/ml (Amp1000) (Figure 4.6C). Chloramphenicol did not exhibit a substantial killing in any of the tested conditions, consistent with the bacteriostatic mode of action (Rahal and Simberkoff, 1979) (Figure 4.6D and E).

The two concentrations of valinomycin tested, 10 µM and 100 µM (Val10 and Val100 respectively) triggered identical effects on the CFU (Figure 4.6F and G). The CFU for exponentially growing cells was reduced by approximately 2 orders of

magnitude, while day 0 cultures showed a reduction of 4 orders of magnitude. Cells from days 7 and 14 were slightly less sensitive than day 0 cells and exhibited a reduction of viability of approximately 2 and 3 orders of magnitude, respectively (Figure 4.6G).

The final compound tested was the oxidative stress inducer paraquat, a herbicide known to cause ROS (Cabiscol et al., 2000). The first tested paraquat concentration of 100 µM (Para100) had no effect on exponential cells, nor on day 7 or 14 cells. However, cells from day 0 showed a decrease of approximately 1.5 orders of magnitude. The small sensitivity of day 0 cells was again observed when treated with the higher concentration of 1000 µM paraquat (Para1000) whereas day 7 and 14 cultures remained unaffected (Figure 4.6H and I). However, exponential cells were sensitive against the elevated concentrations of paraquat, resulting in a decrease in CFU of approximately 3.5 orders of magnitude (Figure 4.6I). Thus, the slowly growing nutrient-starved cells exhibit a tolerance towards ampicillin and paraquat which is superior to logarithmically growing cells.

The antibiotic treatments were inconclusive and, as a consequence, a new experiment was designed in order to elucidate if the starved cells were undergoing growth. For this aim, the activity of the key cell division protein FtsZ was blocked by the inhibitor 3-methoxybenzamide (3-MBA) (Ohashi et al., 1999). Samples were taken periodically during the 14 day incubation (day 0, 2, 4, 7, 9, 11 and 14) and were incubated with and without 3-MBA for 48 hours. A change in cell length was determined by taking microscopic images at time 0 hours and 48 hours. As shown in figure 4.7A and B, for day 0 samples a reduction in cell length was observed irrespective of 3-MBA treatment that matches the overall cell length reduction shown in figure 4.5. Interestingly, during later starvation time points, a clear and constant increase in cell length was observed, ranging between 0.6 – 1.0 µm when cells were incubated with 3-MBA. This experiment unequivocally proves that non-sporulating starved cells start to grow again.

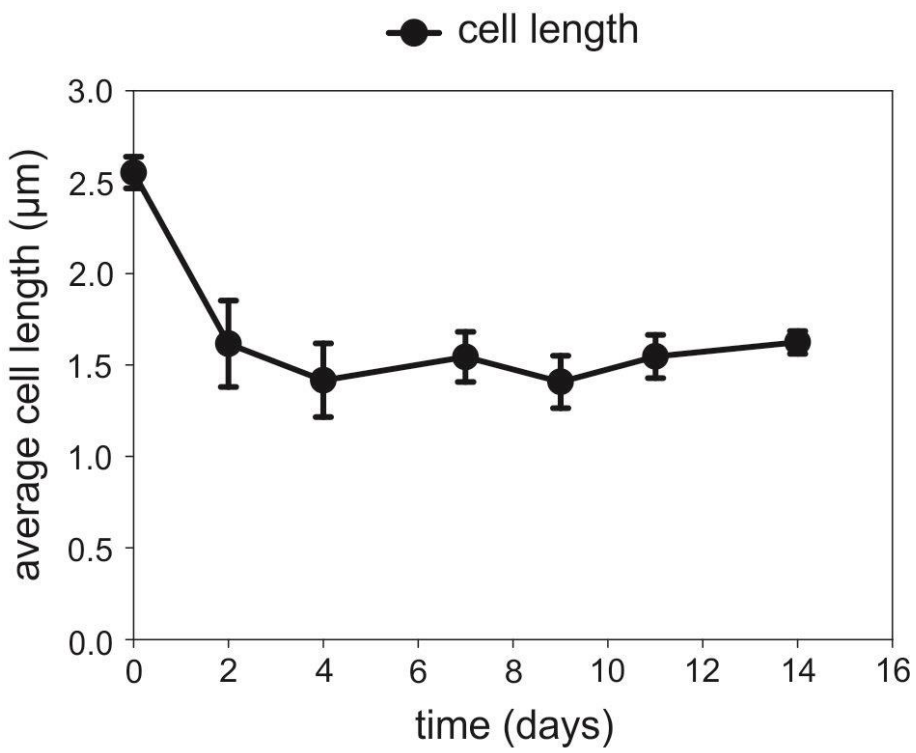

Figure 4.5 Average cell length during starvation incubation.

Cultures of Δ*spoIIE* were set up in accordance with the starvation assay. On days 0, 2, 4, 7, 9, 11 and 14 the average cell length was determined by visualisation of cells through microscopy and image analysis by ImageJ. The lengths of approximately 100 cells, for each time point were counted for 3 independent experiments. The graph represents the average and standard deviation of the 3 independent experiments.

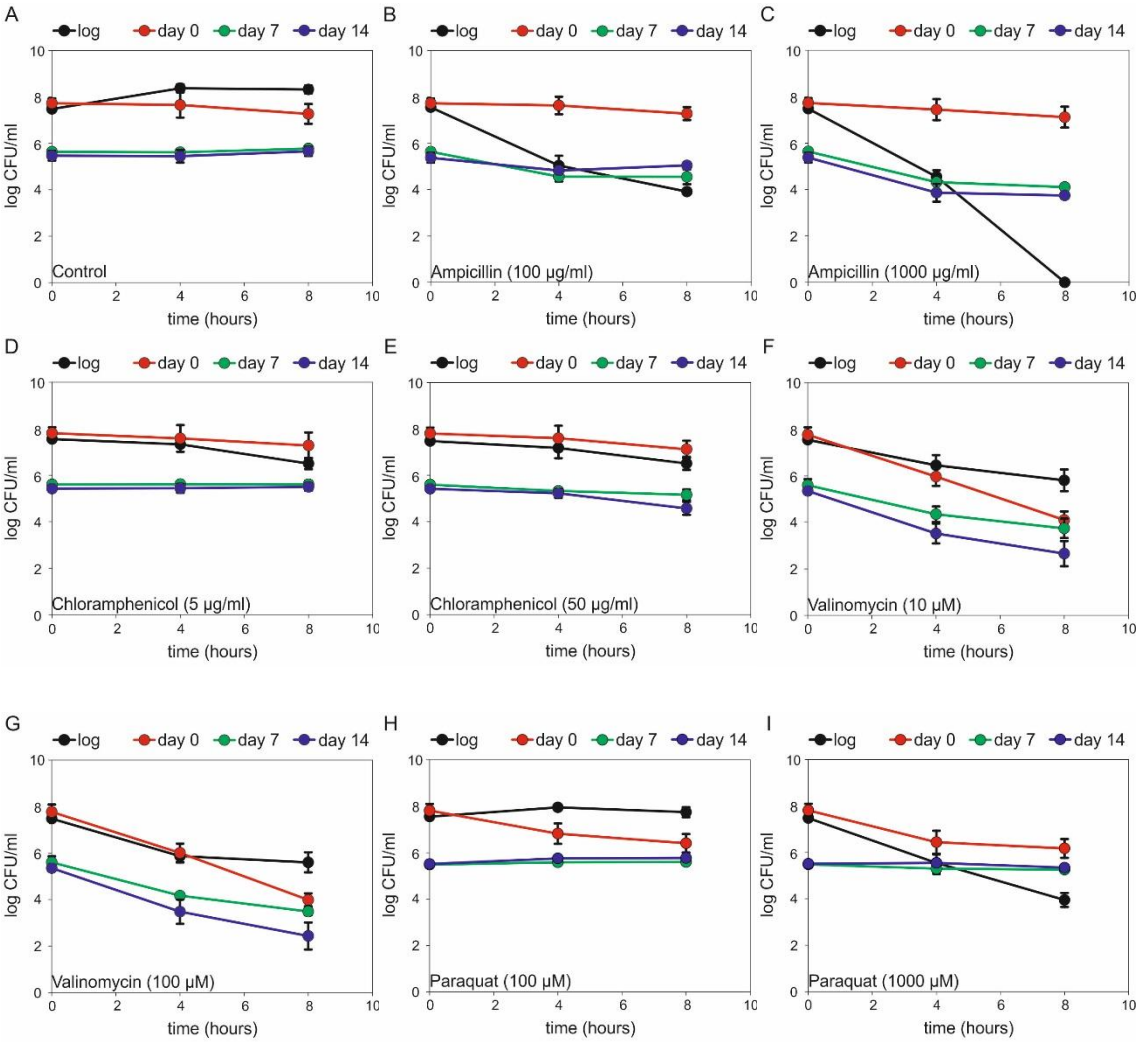

Figure 4.6 Antibiotic sensitivity under starvation conditions.

Cultures of Δ*spoIIE* were set up in accordance with the starvation assay. Samples were taken from cultures on days 0, 7 and 14, and treated with either: A) untreated, B) ampicillin 100 µg/ml, C) ampicillin 1000 µg/ml, D) chloramphenicol 5 µg/ml, E) chloramphenicol 50 µg/ml, F) valinomycin 10 µM, G) valinomycin 100 µM, H) paraquat 100 µM and I) paraquat 1000 µM, for 8 hours. As a control, exponential phase cells were treated in the same manner as the starvation samples with all the same compounds and concentrations. All graphs represent the average of 3 independent experiments with standard deviation.

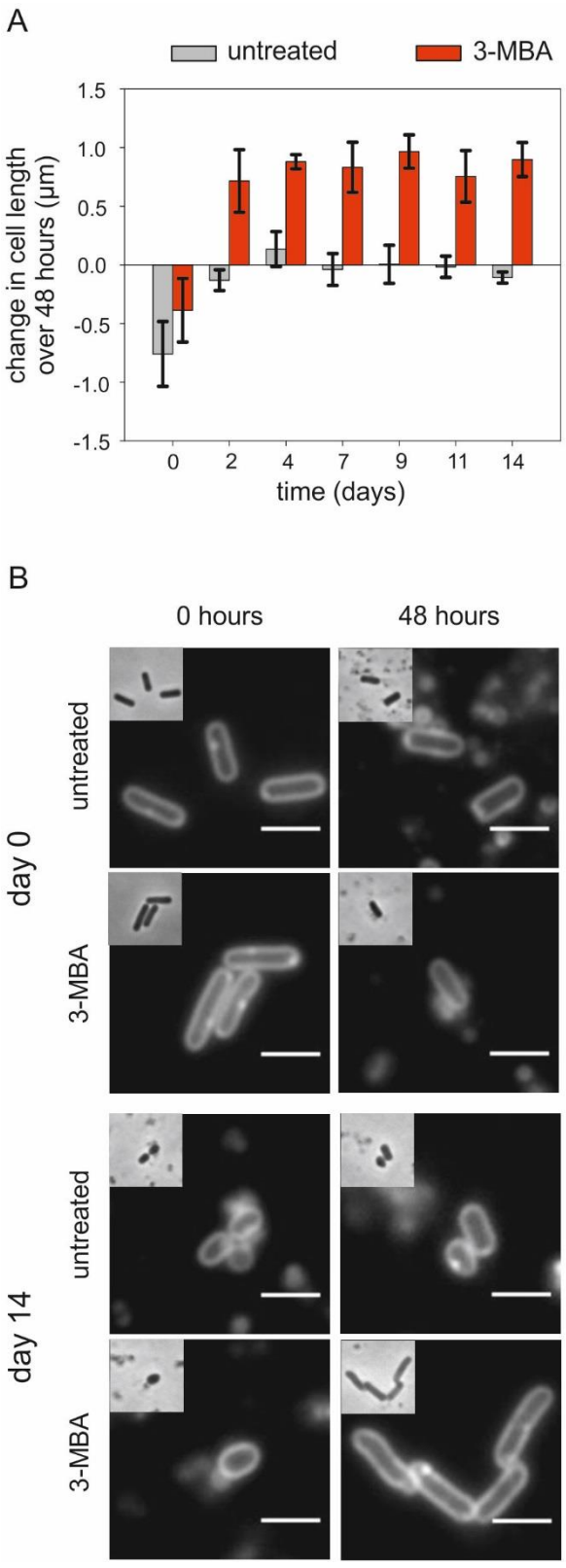

Figure 4.7 Change in cell length over a 48 hour period in the presence of FtsZ inhibitor 3-methoxybenzamide.

Cultures of Δ*spoIIE* were set up in accordance with the starvation assay. On days 0, 2, 4, 7, 9, 11 and 14 samples were taken and incubated with (red bars) and without (grey bars) 10

mM 3-methoxybenzamide (3-MBA) for 48 hours. Images were taken using a Nikon Ti microscope at hours 0 and 48, cells were stained with FM595 membrane dye (0.4 µg/ml). Cell length was determined using ImageJ. The average change in cell length was calculated from measuring approximately 100 individual cells for each time point. A) Represents the change in cell length over the 48 hour treatment period. The graph represents the average and standard deviation of the 3 independent experiments. B) Shows example images visualising the observed change in cell length for day 0 and day 14. Scale bar represents 2 µm.

The FtsZ inhibitor-experiment provided an indication that cells were undergoing slow growth during the starvation period. To verify that these slow-growing cells were undergoing cell division, active cell division sites were visualized using a strain encoding DivIVA-GFP. DivIVA is a cell division site regulator which recruits proteins to the division septum (Edwards and Errington, 1997). As a consequence, localization of DivIVA can be used to identify cell division sites. Throughout starvation incubation, samples were analysed for the presence of division sites by fluorescent microscopy. Over the course of incubation there was a low frequency of division sites (Figure 4.8). On day 0 only approximately 5 % of the population had a division site, the number of division sites gradually increased during starvation, the greatest increase occurring between days 0 and 4. On day 11 approximately 25 % of the cell population showed a division site, day 14 showed a similar level (Figure 4.8A and B). This was a greater value than observed for stationary phase cells, which showed division sites in less than 20 % of the population. Conversely these were all much lower when compared to exponentially growing cells, for this growth phase approximately 50 % of the population had a division site.

In order for a cell to divide and produce two daughter cells the DNA must be replicated, therefore, with an indication that cell division was occurring the frequency of DNA replication was analysed by marker frequency analysis, determining the *oriC* to terminus ratio. A noticeable difference was detected in the initiation of DNA replication for logarithmically growing cells and cells which are under starvation incubation, beyond day 4. Logarithmic cells showed an unsurprising higher *oriC* to terminus ratio of approximately 2.2 (Figure 4.9), while marker frequency analysis performed on cells during starvation all showed a ratio of approximately 1.1 (Figure 4.9). These data are consistent with the observed slow increase in cell length upon inhibition of cell division, thus supporting the conclusion that *B. subtilis* is undergoing slow growth in the nutrient-starvation conditions.

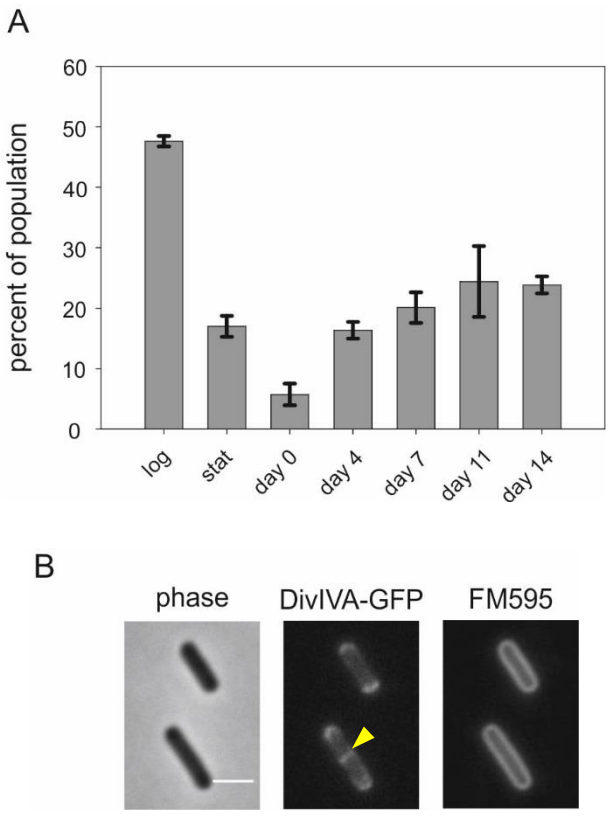

Figure 4.8 Division sites present during starvation incubation.

Cultures of Δ*spoIIE divIVA-gfp* were set up in accordance with the starvation assay. A) On days 0, 4, 7, 11 and 14 samples were taken and observed using fluorescent microscopy. Images were processed using ImageJ. To avoid bias, phase contrast images were used to select approximately 100 cells after which the number of divisions sites present within the selected cells were counted using the fluorescent images, DivIVA-GFP and membrane dye FM595. The graph represents the average and standard deviation of 3 independent experiments. B) Fluorescent microscopy of DivIVA-GFP *B. subtilis* cells highlighting a division site, indicated by the yellow arrow. Scale bar represents 2 µm.

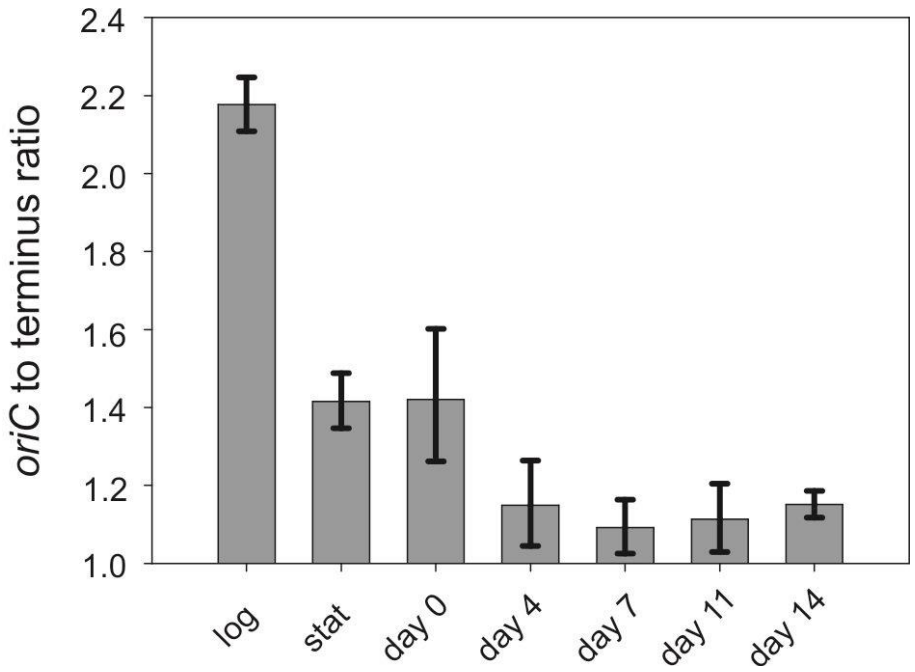

Figure 4.9 DNA replication initiation.

Genomic DNA was extracted from Δ*spoIIE* cultures either growing logarithmically (0.2 – 0.3 OD_{600}) or in stationary phase (overnight incubation) or under starvation incubation (day 0, 4, 7, 11 and 14). Cells were either cultured in SMM at 37 ºC or set up in accordance with the starvation assay. Extracted DNA underwent marker frequency analysis to determine the *oriC* to terminus ratio.

4.2.4 *Nutrient availability*

The presence of metabolic activity, protein synthesis, and growth indicates that the cells still have an available energy source although they were washed and resuspended in a nutrient free solution. Figure 4.1C clearly shows a decrease in CFU, suggesting that the majority of the cell population are undergoing lysis during the first 7 days of incubation. This cell lysis would result in the release of nutrients, and thereby explain the sustained metabolism and growth. To test the availability of lysis-derived proteins in the cell culture, a Bradford assay was utilised. Indeed, an increase in the protein concentration between day 0 and 4 was observed (Figure 4.10).

In addition to monitoring protein levels, the removal of nutrients released by cell lysis was achieved by resuspension of cells into fresh starvation buffer. For this aim, cells incubated for 7 days were washed for a second time, and resuspended into fresh starvation buffer. A clear additional decline of the CFU was observed as a result of resuspension into fresh buffer (Figure 4.11A). As a control, parallel cultures were resuspended back into their own filtrate instead of fresh starvation buffer. In this case no decrease in CFU was observed, indicating the wash procedure was not causing the additional reduction in CFU. In addition to resuspension in their own filtrate, the addition of low concentrations of casamino acids (0.02 %), peptone or tryptone (0.5 µg/ml) to the fresh starvation buffer after the 7 day wash was enough to maintain the CFU, no decrease was seen (Figure 4.11B).

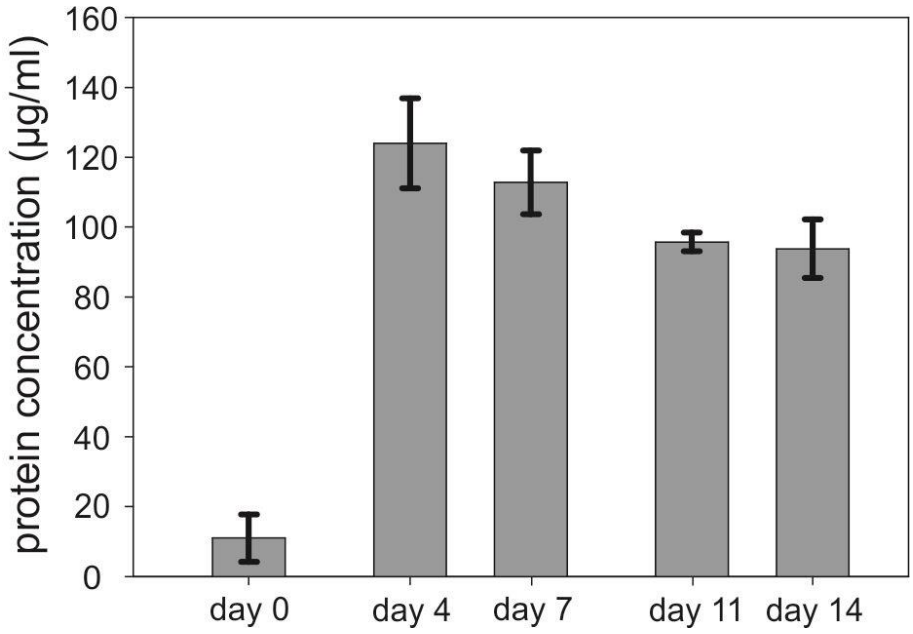

Figure 4.10 Protein concentration of the starvation buffer throughout incubation. Cultures of Δ*spoIIE* were set up in accordance with the starvation assay. On days 0, 4, 7, 11 and 14 samples were taken, cells were removed by centrifugation, and the supernatant underwent a Bradford assay to determine the protein concentration. The graph represents the average of 3 experiments with standard deviation.

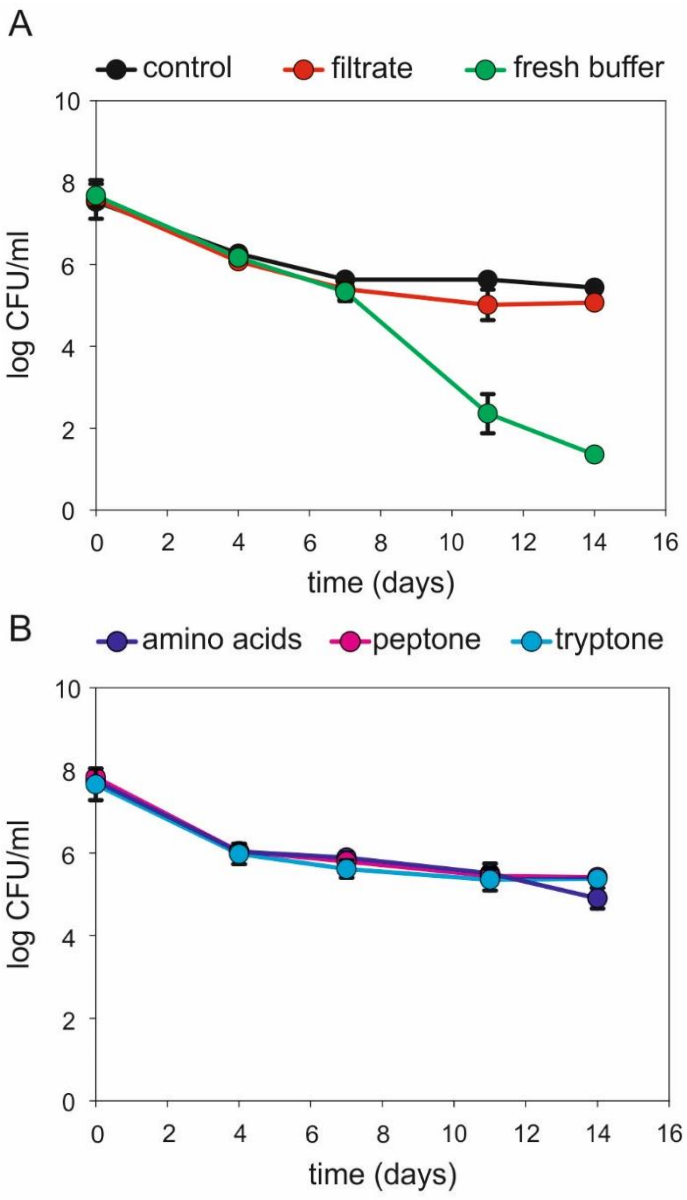

Figure 4.11 Additional washing and supplementation during starvation incubation. Cultures of Δ*spoIIE* were set up in accordance with the starvation assay. A) The effect of an additional wash step was examined for its consequences on survival. After 7 days the "fresh buffer" culture was washed by filtration and resuspended into fresh starvation buffer, while the "filtrate" culture was resuspended back into the filtrate. B) The consequence of a resuspension into fresh starvation buffer supplemented with casamino acids (0.02 %), peptone (0.5 µg/ml) or tryptone (0.5 µg/ml) was assessed. The graphs represent the average and standard deviation of 3 independent experiments.

4.2.5 *Starvation survival of different B. subtilis strains*

In the previous sections it was established that a subpopulation of *B. subtilis* resuspended in a nutrient free medium remains metabolically active, and undergoes slow growth using nutrients provided by lysis of the remaining cell population. To gain a deeper understanding into the involved differentiation and adaptation pathways, a candidate-based approach was chosen to identify genetic components associated with survival under nutrient limitation. For this aim, mutants lacking genes involved in cell differentiation pathways (*spo0A*, *comK*, *abrB*, *sinI*, *sigD* and *codY*) (Bai et al., 1993; Chen and Helmann, 1995b; Molle et al., 2003; Ratnayake-Lecamwasam et al., 2001; Robertson et al., 1989; Serror and Sonenshein, 1996), and in stress response pathways (*sigB*, *pnpA*, *relA*, *recA* and *clpP*) (Benson and Haldenwang, 1993; Kruger et al., 2000; Lovett et al., 1989; Wang and Bechhofer, 1996; Wendrich and Marahiel, 1997) were analysed. All the tested strains also contained the *spoIIE* deletion. No increase or decrease in survival was observed for all the above mentioned strains (Figure 4.12A and B).

Based on results indicting that *B. subtilis* remains metabolically active and undergoes slow growth using nutrients released by their lysed brethren, a strain carrying a deletion of all 8 extracellular proteases produced by *B. subtilis* (ΔWB800) was investigated (Wu et al., 2002). In addition to the release of proteins, DNA would also be present and provide a nutrient source. Therefore, strains carrying single deletions of extracellular nucleases (*nucA* and *nucB*) were also tested (van Sinderen et al., 1995a). No difference was observed between the nuclease deletion strains and the *spoIIE* mutant. However, a 100 fold decrease in survival was observed for the 8 extracellular protease deletion strain (Figure 4.12C). To test if this phenotype was due to a specific protease, strains with sequential protease deletions (*nprB*, *aprE*, *epr*, *bpr*, *nprE*, *mpr*, *vpr* and *wprA*) were examined. Again, the only strain to show a decrease in survival was a strain lacking all 8 extracellular proteases (Figure 4.12D). A single deletion of *wprA* was tested to confirm this deletion was not the cause of the reduced CFU. The reduced viability is thus a consequence of a missing extracellular protease activity as such, rather than one specific protein.

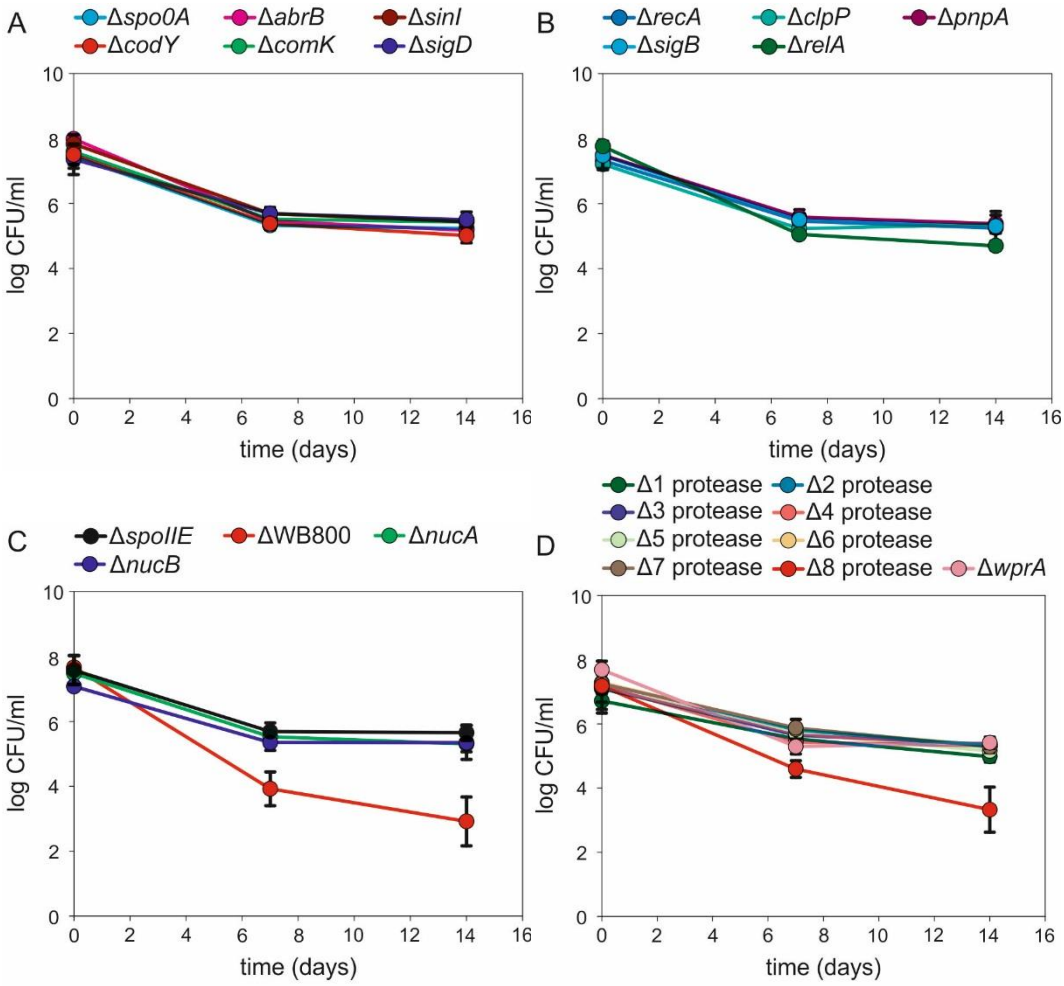

Figure 4.12 Survival of different mutants under starvation conditions.

All cultures were set up in accordance with the starvation assay. A) Starvation survival of strains carrying deletion of cell differentiation pathway regulators. B) Starvation survival of strains carrying deletion of stress response genes. C) Starvation survival of strains deficient for extracellular proteases and nucleases. D) Starvation survival strains carrying sequential deletions of protease-encoding genes. Graphs represent the average and standard deviation of 3 independent experiments.

4.2.6 *Transcriptome profiling*

The candidate-approach to identify genes involved in the starvation survival thus far have only revealed the importance of the extracellular proteases, most likely responsible for releasing nutrients from lysis-derived proteins. No relevant regulatory mechanism and differentiation pathways were identified for the slow growth state observed during starvation incubation using this approach. In order to identify potentially crucial adaptation processes important for the long term starvation survival, the transcriptome of 14 day old, exponential, and stationary phase cultures were compared using RNA-seq. Culture pellets generated for microarray analysis were isolated from all three growth phases by filtration, this was achieved through using 1 L cultures in order to collect enough cell material for analysis, and collected cells were immediately frozen with liquid nitrogen and stored at -80 ºC. Up-regulated genes are listed in table 7.1, the top 20 are shown in bold.

Out of the up-regulated genes (Table 7.1), 13 genes were chosen because of their level of up-regulation, and the specificity for the starvation-induced slow growth conditions (Table 4.1). The capacity of strains carrying individual deletions of the candidate genes to survive starvation conditions was assessed under identical conditions as the previous mutants. Knockouts of these genes were obtained from the Bacillus Genetic Stock Center (BGSC) and were transformed into the Δ*spoIIE* background to remove any sporulation-specific effects. Unfortunately, the 13 deletion strains showed no difference in survival compared to the Δ*spoIIE* strain (Figure 4.13).

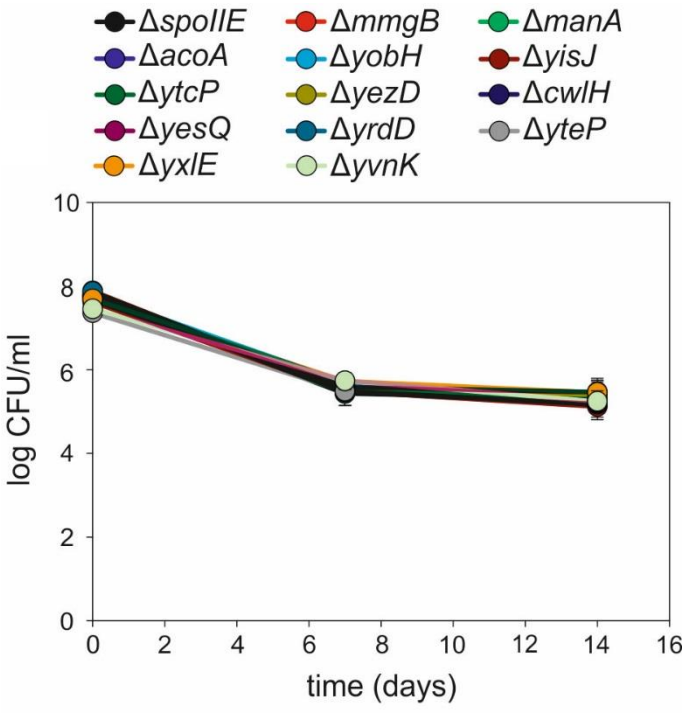

Figure 4.13 Survival of mutants identified by transcriptomics.

All cultures were set up in accordance with the starvation assay. Survival of mutants identified by microarray analysis. Graphs represent the average of 3 experiments with standard deviation.

Table 4.1 Up-regulated gene deletions tested for their effect on starvation survival

Gene	Function	Fold up-regulation (14 days/log)	Fold up-regulation (14 days/stat)
acoA	acetoin utilization	8.5	12.7
yteP	unknown	6.1	11.3
yesQ	uptake of rhamnose oligosaccharides	2.1	7.0
yrdD	unknown	3.1	5.1
mmgB	mother cell metabolism	32.5	4.4
yezD	unknown	3.4	4.1
yisJ	unknown	17.2	3.0
yobH	unknown	40.2	3.0
manA	mannose utilization	2.7	2.6
yxlE	unknown	2.6	2.2
ytcP	unknown	3.2	2.2
yvkN	unknown	6.1	2.1
cwlH	cell wall metabolism	3.1	2.6

4.2.7 *Competition*

During starvation experiments a GASP phenotype has been reported in *E. coli*, whereby mutations acquired during starvation allow the bacterial culture to outcompete a culture new to the stationary phase conditions when the populations are mixed (Finkel, 2006). This prompted the investigation as to whether under our conditions *B. subtilis* accumulated any beneficial mutations. Based on aberrant colony morphology types appearing from a 14 day starved culture, a strain was isolated and subjected to genome sequencing. This analysis revealed a single-nucleotide polymorphism resulting in a T1481C nucleotide exchange in the beta-subunit of the RNA polymerase. After discovering this mutation, the growth behaviour of the strain was assessed in SMM. Strain (Δ*spoIIE divIVA-gfp rpoB**), the *rpoB* mutant strain showed a slower growth rate compared to the parental Δ*spoIIE divIVA-gfp,* or BSB1 wild type strain (Figure 4.14A). It is worth mentioning that reduced growth rates have been observed for *rpoB* point mutations before (Maughan et al., 2004).

Next, the ability of the Δ*spoIIE divIVA-gfp rpoB** (*rpoB**) strain to survive under starvation conditions was assessed, however, no detectable difference in starvation survival was observed between *rpoB** and the parental Δ*spoIIE divIVA-gfp* strain (Figure 4.14B). Observing no benefit of the point mutation under normal conditions, the ability of the *rpoB** strain to compete against a strain containing only a *spoIIE* deletion under starvation conditions was analysed. For this aim, both strains were cultivated as defined by the starvation assay, followed by mixing the cultures after 4 days of starvation. The fraction of the mixed population representing each strain was determined by two methods: (a) antibiotic resistance on NA plates, and (b) GFP fluorescence. The *spoIIE* deletion of both strains was linked to erythromycin resistance, however, the *divIVA-gfp* of the *rpoB** strain, was linked to chloramphenicol resistance, a property which allowed the determination of CFU for both individual strain on NA plates supplemented with chloramphenicol. In addition, the DivIVA-GFP marker encoded within the *rpoB** strain provided the means to distinguish between the mixed strains using fluorescent microscopy combined with cell counting.

The CFU-analysis carried out immediately after mixing revealed that approximately 59 % of the population was Δ*spoIIE* while 41 % was Δ*spoIIE divIVA-gfp rpoB** (Figure 4.14C). The development of a fractional CFU representing each

subpopulation was monitored for 10 days of incubation. However, after only 3 days of incubation, the population was over 98 % in favour of Δ*spoIIE divIVA-gfp rpoB**. On day 7 the entire population was Δ*spoIIE divIVA-gfp rpoB** (Figure 4.14C). This was also the case for day 10, Δ*spoIIE* cells were no longer detectable (Figure 4.14C). The GFP results show a very similar trend. Although, while the antibiotic competition results revealed 100 % of the population to be Δ*spoIIE divIVA-gfp rpoB**, the GFP results highlighted a small portion of detectable Δ*spoIIE* cells, approximately 0.5 % of the population (Figure 4.14D). As a result it appears the *rpoB* mutation does provide a benefit during competition.

Due to the presence of the chloramphenicol resistance marker in the Δ*spoIIE divIVA-gfp rpoB** the observed outcompeting might not be due to the *rpoB* point mutation, but emerge as a side-effect of the chloramphenicol resistance marker, or as a potential competitive advantage of DivIVA-GFP. However, in the absence of chloramphenicol, chloramphenicol acetyltransferase should have no impact on survival. To test this possibility, the competition experiment was repeated for a combination of a single deletion Δ*spoIIE* strain, and Δ*spoIIE divIVA-gfp* strain (Figure 4.14E and F). Again, the cultures were set up in accordance with the starvation assay and, on day 4 of starvation, the cultures were mixed. The population fractions were determined both by CFU using selective plates, and by GFP fluorescence. Upon mixing, the cultures were roughly 50:50 with slightly more Δ*spoIIE.* After 3 days of incubation, there was a shift towards 60:40 in favour of Δ*spoIIE*. In the long term, however, Δ*spoIIE divIVA-gfp* did appear to be able to outcompete Δ*spoIIE* and reached approximately 90 % of the population after 14 days (Figure 4.14E). In comparison the results obtained from the GFP-based fluorescence counting revealed only a small difference in the population after 14 days. In this case, a gradual increase in the Δ*spoIIE divIVA-gfp* subpopulation reaching just over 60 % of the population on day 14 was observed (Figure 4.14F). The reason for this discrepancy is not clear. Nevertheless, it seems that these data suggest that, while *rpoB** does exhibit reduced growth rates in the presence of ample supply of nutrients, there is no benefit for starvation survival. However, the mutation does provide a competitive advantage upon starvation-triggered slow growth.

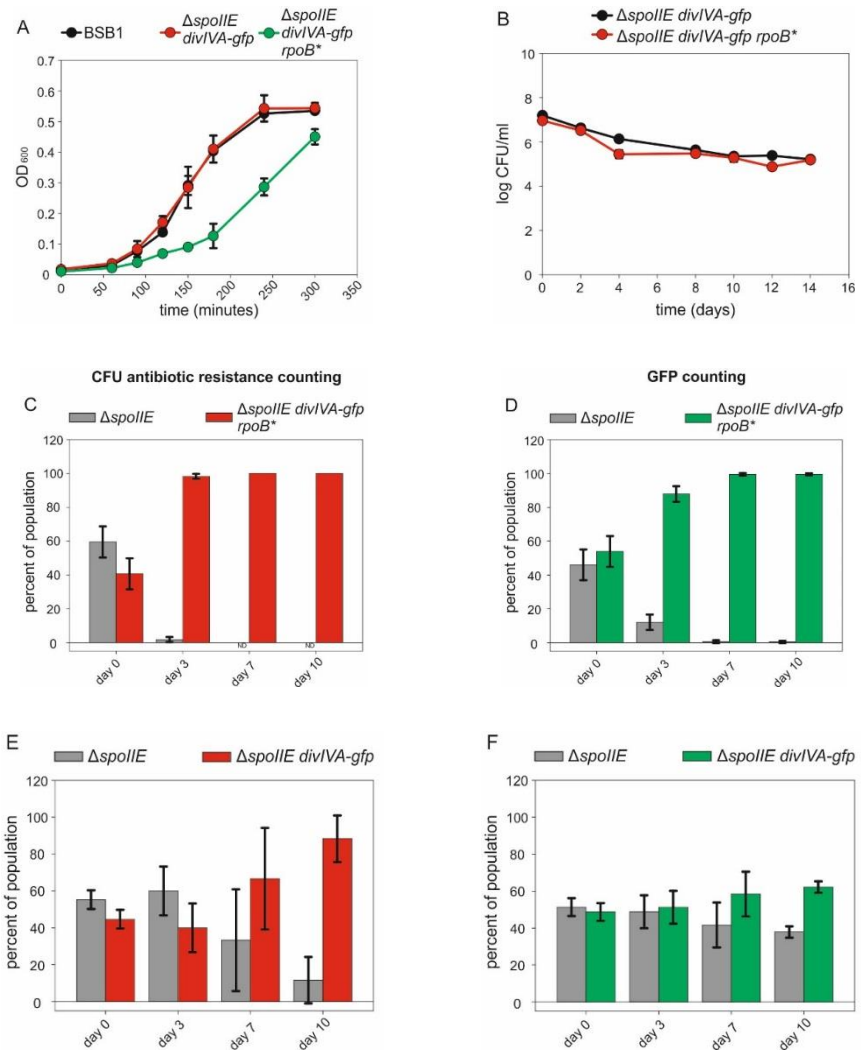

Figure 4.14 Analysis of *rpoB* point mutation.

A) Strains BSB1, Δ*spoIIE divIVA-gfp* and Δ*spoIIE divIVA-gfp rpoB** were cultured in SMM, cells were diluted in fresh SMM and the OD was measured for 5 hours. The graph represents the average and standard deviation of 2 independent experiments. B) Cultures Δ*spoIIE divIVA-gfp* and Δ*spoIIE divIVA-gfp rpoB** were set up in accordance with the starvation assay. The graph represents the average and standard deviation of CFU measured in 2 independent experiments. C/D) Δ*spoIIE* and Δ*spoIIE divIVA-gfp rpoB** cultures were set up according to the starvation assay, on day 4 of starvation the cultures were mixed (day 0). E/F Δ*spoIIE* and Δ*spoIIE divIVA-gfp* cultures were set up according to the starvation assay, on day 4 of starvation the cultures were mixed (day 0). C/E) For fractional CFU determination based on antibiotic selection the colonies were picked and streaked on to appropriate antibiotic plates to determine the % of the population. ND, represents no detectable colonies. D/F) For GFP determination samples were taken periodically and cells were visualised by fluorescent microscopy. To avoid bias, phase contrast images were used to count approximately 100 cells after which the number of fluorescent cells was determined using the corresponding fluorescent images. The graphs represent the average and standard deviation of 3 independent experiments.

4.3 Discussion

4.3.1 *Starvation survival of B. subtilis without sporulation*

Previous work has demonstrated that a subpopulation of non-sporulating *B. subtilis* cells can survive while the majority of the population either undergoes lysis or sporulates (Veening et al., 2008b). It has remained unclear, however, how long these cells can survive, and which mechanism they utilise in order to survive. Long term incubation of wild type (sporulation capable) *B. subtilis* strains showed that a small portion of the population were still vegetative cells, even after 100 days the population was never 100 % spores, revealing a remarkable capacity of these cells to survive for extended periods of time. This was further highlighted by the long term survival of *B. subtilis* cells incapable of sporulating (Δ*spoIIE*). The drawback of the spore is the response time to nutrient sensing, the germination of a spore requires approximately 60 minutes (Pandey et al., 2013), as a consequence vegetative cells still present in the starved culture are more readily able to utilise nutrients, should they arise. Since the wild type cells are still able to produce an endospore over time, it is likely that these cells use released nutrients to start the sporulation pathway. This also explains why there was a decrease in CFU with a strain unable to germinate after producing spores at the end of the two weeks starvation period. The rapid decline in CFU during the first few days of incubation and then plateauing of the CFU has been observed in other starvation studies involving different non-sporulating bacteria, including *S. aureus*, *E. coli* and *F. columnare* (Arias et al., 2012; Finkel, 2006; Watson et al., 1998).

The restoration of membrane potential observed during starvation suggests these cells are not in a dormant state and are still metabolically active. In further support of this, these cells are able to induce gene expression and protein synthesis. Previous studies have shown that starved cultures of *S. aureus* and *E. coli* are not in a dormant state but are active (Finkel, 2006; Finkel and Kolter, 1999; Watson et al., 1998). In addition, the *B. subtilis* cells examined here also undergo slow growth, indicated by a detectable increase in cell length upon inhibition of cell division. The measured increase was between 0.6 and 1 µm over a period of 48 hours. This suggests that the cells are growing with a remarkably slow rate for *B. subtilis* and exhibit an estimated doubling time of 2 – 4 days (assuming an average cell length of 1.5 µm, figure 4.5) instead of the normal 20 – 40 minutes doubling time achieved in standard laboratory conditions. Although, a doubling time of days has not been

document in *B. subtilis* before, such times have been observed for other bacteria (Hoehler and Jorgensen, 2013), *Treponema pallidum* has a doubling time of over 30 hours (Lafond and Lukehart, 2006).

Regulation of cell division has been shown to be tightly connected to nutrient availability (Wang and Levin, 2009). For a bacterial cell to divide into two identical daughter cells, it must replicate the entirety of its genome in an energy-intensive process. Under optimal growth conditions with abundant nutrients, *B. subtilis* utilises multifork replication characterised by initiation of multiple parallel rounds of chromosome replication (Yoshikawa et al., 1964). This type of chromosome replication allows the cell cycle to be completed in a time window which is less than the time required to fully replicate the entire chromosome. However, during low nutrient conditions the initiation of genome replication is inhibited by preventing DnaA from initiating new rounds of DNA replication (Wang and Levin, 2009). As a consequence, upon the very slow growth observed with long term starvation survival the cells are likely to initiate DNA replication only once per division cycle, which was observed for the marker frequency analysis.

Over the first 2 days of incubation, the average cell length decreased by approximately 1 µm, giving raise to cells with an increasingly coccoid morphology. Morphological changes are a common phenomenon observed as a response to starvation conditions. Especially a cell shrinking phenomenon which has been observed in several starvation studies (Lange and Hengge-Aronis, 1991; van Overbeek et al., 1995; Watson et al., 1998; Yao et al., 2012). The reduction in cell size results in an increase of the surface area-to-volume ratio, which has been postulated to aid nutrient uptake (Gottschal, 1992). In addition to this, a smaller cell would have a lower maintenance requirement, therefore requiring fewer nutrients in order to survive (Chien et al., 2012). Both of these would be beneficial traits to a bacterium during times of nutrient limitation, and are likely to contribute to the surprising ability of *B. subtilis* to survive upon long term nutrient exhaustion.

4.3.2 *Gone but not forgotten: using dead to survive*

A common trend observed across various experiments was a clear change over the first few days of starvation. These include a decrease in CFU, an increase in metabolic activity of the remaining cells, reduction of the average cell length, and re-initiation of slow cell growth. The decrease in CFU during the first week of incubation

suggests that the cells are either unable to form colonies on NA and exhibit a so-called VBNC state (Oliver, 2005), or have lysed or are dead but intact.

Although no experiments were performed to directly address if lysis was occurring, clear debris indicating cell lysis was microscopically visible. Furthermore, experiments carried out after 4 days of incubation required the medium to be concentrated at least 1000 fold via centrifugation in order to obtain a cell density allowing microscopic analysis. For these reasons, the lysis of the majority of the cell population within the first 4 days is assumed, and would suggest that the cells are not in the VBNC state (Ramamurthy et al., 2014). In good agreement, an increase in free protein concentration was observed between days 0 and 4. Removal of the nutrients released by the lysed cells resulted in a further decrease in cell viability. In contrast when 3 week starved *B. suis* cultures were washed and resuspended into fresh salt solution, no decrease was observed in the CFU (Al Dahouk et al., 2013). Taken together this would indicate that the metabolic activity and slow growth of the remaining *B. subtilis* cell population is indeed supported by nutrients released by lysis.

The importance of the proteins released from lysed cells is further highlighted by the observed decrease in survival ability of a strain deficient in 8 extracellular proteases normally produced by *B. subtilis*. Intriguingly, none of the tested 8 proteases had an important contribution themselves, suggesting that during long term starvation the generic ability to degrade extracellular proteins rather than a specific activity is essential to support growth.

B. subtilis has a known differentiation phenotype during which it kills its brethren in order to use the nutrients released from lysed cells, a so-called cannibalism phenotype (Gonzalez-Pastor, 2011). The operons associated with this phenotype, *skf* and *sdp,* are under the control of Spo0A~P. At low levels of Spo0A~P, this pathway is induced and has the ability to delay sporulation through the release of nutrients (Gonzalez-Pastor et al., 2003). However, the deletion of *spo0A* had no effect on survival and classical cannibalism-phenotype is therefore unlikely to trigger the observed cell lysis. The lysis of cells witnessed at the onset of starvation might occur from the inability of cells to repair sustained damage. However, it is also possible that a concept known as PCD is occurring (Bayles, 2014). PCD is a well-known property of eukaryotic systems and also exists in certain bacteria (Bayles, 2014). One PCD mechanism linked to sporulation is already known for *B. subtilis*

(Lewis, 2000). When the spore has matured within the mother cell, the mother cell must be sacrificed in order for the spore to be released. This is achieved by the hydrolysis of peptidoglycan by autolytic enzymes LytC and CwlC (Allocati et al., 2015). Therefore, it is not unthinkable to believe another PCD system could exist within *B. subtilis*.

4.3.3 *The benefits of starvation*

In the natural environment it is likely for bacteria to be exposed to nutrient limitation on a frequent basis. After all, their very own growth can lead to the exhaustion of nutrients (Bernhardt et al., 2003). It is common for a survival strategy to provide resistance to more than one type of stress (Setlow, 2006). Persisters, for instance, are dormant cell types that can survive nutrient poor conditions and their dormancy also allows them to tolerate normally lethal antibiotic concentrations (Wood et al., 2013), a benefit during “antibiotic warfare” between different bacterial and fungal species (Hibbing et al., 2010). Antibiotic tolerance is not automatically triggered by nutrient starvation and cases are known in which bacteria have remained sensitive to antibiotics during starved conditions, most likely as a result of an incomplete dormancy (Watson et al., 1998). In the case of non-sporulating *B. subtilis* cells, the very slow growth mode adopted upon nutrient starvation resulted in an intermediary tolerance towards ampicillin. The cells starved for 7 – 14 days were still affected by ampicillin, but less so than fast growing cells. This suggests that, as a population, the long-term starved cells are able to better tolerate cell wall targeting antibiotics. This is most likely due to their slow growth which implies low cell wall turnover, rendering the cell wall lytic action of ampicillin less effective.

The ability of the starved and slowly growing cells to tolerate high concentrations of oxidative damage triggering compound paraquat is remarkable. Oxidative stress caused by paraquat is due to the development of superoxide through redox reactions (Carr et al., 1986). Intriguingly, upon starvation (day 0) the cells exhibit an increased sensitivity towards paraquat whereas in later stages of starvation (day 7 and 14) a full tolerance is witnessed. A likely reason for this difference lies in the energy state of the cells, the better energised cells adapted to the slow growth being more capable of active repair of oxidative damage. This hypothesis is supported by the reduced GFP fluorescence detected on day 0 for the IPTG inducible GFP strain. This implies cells would be unable to readily produce new proteins in response to sensing superoxide or hydrogen peroxide generated from

paraquat (Mostertz et al., 2004). However, the slowly growing starved cells exhibit tolerance beyond that found in optimally energised logarithmically growing cells. A plausible explanation for this apparent discrepancy could lie in the above discussed long generation time accompanied with the low rate of replication initiation. The increased time window between the individual cell divisions would provide the cell more time to repair deleterious damage inflicted upon DNA, a benefit which could potentially explain the observed phenomenon.

The slow growth state observed could also provide an advantage over sporulating cells. The sporulation process requires time and nutrients, approximately 7 hours (Piggot and Hilbert, 2004). Additionally, the germination of the spore also requires at least 1 hour (Pandey et al., 2013), but is only stimulated in the presence of abundant nutrients, implying a spore could remain dormant for years if this threshold is not achieved. However, the nutrients present could be enough for the slow growth to occur. In the time it takes for a spore to germinate, upon nutrient induction, the available nutrients could have been consumed by either the slow growers or other present bacteria. Therefore, as a survival mechanism sporulation is beneficial due to its robustness, however, in order to compete for newly available nutrients the spore could have a disadvantage due to the time required for the spore to germinate.

It has been observed for nutrient-starved *E. coli* and *Salmonella* populations that mutations occur which allow them to outcompete cultures that are new to the starvation conditions (Finkel, 2006). This has been referred to as the GASP phenotype (Bacun-Druzina et al., 2007). After 14 days a single point mutation in RNA polymerase beta subunit (*rpoB**), resulting in an amino acid exchange (L494S), was identified. This mutation seems to allow the strain to outcompete the parental strain under conditions examined here. Mutations in *rpoB* have been previously shown to have an effect on doubling time resulting in slower growth (Maughan et al., 2004). Indeed, slow growth was observed for the strain carrying *rpoB**. In addition to slow growth, *rpoB* mutations have been known to cause resistance to rifampin, and cause the overproduction of bacilysin (Tojo et al., 2015). Therefore, it is possible that the *rpoB** strain is able to produce an antibiotic that the parental strain has no resistance to or overproduce an antibiotic resulting in a high enough concentration to kill the parental strain.

A discrepancy was observed between the two forms of analysis, antibiotic and GFP, for the *divIVA-gfp* competition assay. Determining the percent of the populations by antibiotic resistance indicated 90 % of the population was Δ*spoIIE divIVA-gfp* while GFP analysis revealed only approximately 60 % of the population to be Δ*spoIIE divIVA-gfp.* At this stage the reason for this discrepancy remains unclear. It is worth noting though, that the direct observation and cell counting based on GFP-fluorescence represents a more direct approach which is less likely to introduce experimental bias.

4.4 Future Work

The experiments presented here have shown the capacity of non-sporulating *B. subtilis* cells to survive adverse nutrient conditions for an extended period of time (over 100 days). A clear regulatory mechanism was not identified for the slow growth phenotype observed during these experiments. It is plausible that there is no specific differentiation pathway as observed with the other phenotypic differentiations of *B. subtilis*. However, there is potentially an as yet undiscovered differentiation pathway as *B. subtilis* is known to utilise a bet-hedging strategy in order to promote survival.

An interesting finding was the discovery that under these conditions the cells were undergoing slow growth. It would be interesting to know if DNA replication and cell division occur in the same manner as exponentially growing cells or are these processes also slowed down. Fluorescent time-lapse microscopy of the cell division machinery for these slow growing cells could provide insight into how these mechanisms occur.

The identification of a point mutation in *rpoB* which appears to provide the strain with an ability to outcompete the parental strain under nutrient deprived conditions, the mechanism by which this is achieved was not discovered. It is possible this mutation allows for the production of an antibiotic which the parental strain cannot produce. A simple halo test could give an indication to an antibiotic being produced.

Chapter 5. How dissipating membrane potential leads to cell death

5.1 Introduction

The emergence of bacterial antibiotic resistance has become a major healthcare problem in recent years (Shah, 2013). As a consequence, the World Health Organisation (WHO) has declared steps to reduce the spread and development of antibiotic resistance as a high global priority. Due to the emergence of superbugs such as MRSA (Dantes et al., 2013; Kock et al., 2010; Turnidge et al., 2009), we are already faced with the dire need for new antibiotics and treatments. Great efforts are currently directed towards discovery and development of new antibiotics. After an initial discovery, it can take a decade before the drug can be used to treat infections. In contrast, bacterial antibiotic resistance can emerge within a few years, a development which was clearly seen in the case of a completely synthetic new antibiotic (linezolid) (Gu et al., 2013).

In addition to resistance development, the presence of bacterial phenotypes that are able to tolerate otherwise lethal concentrations of antibiotics also pose a risk to effective antibiotic treatment. For instance persister cells, a dormant phenotype, well characterised in *E. coli* although also present in other bacteria, and have been attributed to the reoccurrence of infections (Dawson et al., 2011). Due to their dormant state they are able to survive antibiotic treatment as the majority of antibiotics target actively growing cells (Wood et al., 2013). Therefore, in addition to finding antibiotics that are effective against “superbugs” we are also in need of drugs that can be used to combat these dormant phenotypes.

A more thorough understanding of the mechanism of action of an antibiotic is needed in order to pre-emptively predict and counteract resistance development. The major cellular targets of the mostly used antibiotics are well known. These include the cell wall synthesis, protein synthesis, and DNA replication machineries (Figure 1.5). However, secondary cellular consequences of these compounds might be unclear although they can contribute to the potency (Kohanski et al., 2010). For over a decade there has been speculation that antibiotics not only unfold their antibacterial potential by inhibiting their specific cellular target, but also trigger oxidative stress, a process contributing to cellular death (Albesa et al., 2004). The generality of this mechanism is very controversial (Dwyer et al., 2014; Feld et al., 2012; Kang et al.,

2012; Liu and Imlay, 2013), but remains a viable hypothesis for certain classes of antibiotics.

The membrane potential is essential for bacterial life as it allows the bacteria to perform essential cellular functions such as ATP synthesis. The majority of cellular ATP is synthetized by F_1F_o ATP synthase, a membrane embedded protein complex which converts ADP to ATP using energy stored in the transmembrane PMF (Dimroth et al., 2000). The PMF is composed of a transmembrane proton gradient (ΔpH) and a transmembrane electric potential (ΔΨ). The proton gradient is established by the ETC through the transport of protons across the cytoplasmic membrane (Sazanov, 2015). In addition to establishing a chemical gradient of protons (ΔpH), the movement of protons across the membrane also induces an electric potential (ΔΨ) (Mitchell, 1961; Saraste, 1999). The resulting potential difference across the membrane is positive at the exterior of the cell, and can reach values above 100 mV (Hosoi et al., 1980; Te Winkel et al., 2016; Zaritsky et al., 1981). In addition to ATP synthesis, these separate factors are used to energise essential cellular processes such as ion and nutrient transport, and protein secretion. Cellular organisation has also been shown to depend on the membrane potential (Strahl and Hamoen, 2010). Due to the central and essential role the membrane potential, it is an enticing antimicrobial target (Hurdle et al., 2011). Numerous antimicrobial compounds target the membrane and result in dissipation of membrane potential. The mechanism through which this is achieved range from ion carrier activity to the formation of discrete pores or interference with the permeability barrier function of the membrane by other means. A major drawback of these compounds is their poor bacterial selectivity resulting in toxicity towards eukaryotic cells, thus they are not used in treatment (Gottlieb et al., 2003). However, this is not fundamentally always the case as demonstrated by the clinical use of membrane targeting, and membrane potential dissipating antibiotics daptomycin and polymyxin B (Humphries et al., 2013; Zavascki et al., 2007).

Aims of this chapter

The experiments presented in this chapter are aimed at investigating the effects of membrane dissipating compounds on dormant (stationary phase) *B. subtilis* cells. These cells are used as a model system for persister cells.

5.2 Results

5.2.1 *Killing triggered by dissipation of the membrane potential*

As a model for persister cells, stationary phase *B. subtilis* cells, which could not undergo sporulation (Δ*spoIIE*), were used to investigate the killing mechanism involved upon membrane potential dissipation. Persister cells are a dormant phenotype that can tolerate otherwise lethal concentrations of antibiotics (Dawson et al., 2011). A common trait between stationary phase *B. subtilis* cells and persister cells is their tolerance of antibiotics including the bactericidal antibiotic ampicillin. Two compounds were used in order to dissipate membrane potential, CCCP and valinomycin. Due to the specific mechanism of action of valinomycin, a carrier for K^+ ions across the membrane, the specific and full dissipation of the membrane potential relies on the presence of equimolar concentrations of K^+ in the cytoplasm and in the medium (Bhattacharyya et al., 1971; Te Winkel et al., 2016). In addition, both CCCP and valinomycin are pH dependent in their action. For these reasons, a tryptone and yeast extract based medium buffered with HEPES to pH 7.5, and containing a high concentration of KCl (300 mM) was used throughout this chapter (Te Winkel et al., 2016). The resultant medium is referred to as valinomycin medium.

The bactericidal activity of both CCCP and valinomycin was assessed on stationary phase *B. subtilis* cells. Overnight cultures of sporulation deficient *B. subtilis* cells (Δ*spoIIE*) were used to prevent the formation of endospores during the 10 hour incubation. Cultures were treated with 100 µM of CCCP or 100 µM valinomycin, and the changes in cell viability were monitored over a 10 hour time window with CFU measurements. Over the first 4 hours of incubation there was only a minor reduction of viability and a 50 % reduction of viability was only observed after approximately 6 hours (Figure 5.1A). The reduction of viability increased to over 90 % after 8 hours of incubation, and after 10 hours the viable population had reduced by approximately 90 – 99 % and 99.9 % for CCCP and valinomycin, respectively. The reduction in CFU was not reflected in the OD of the cultures (Figure 5.1A and B).

As previously stated, the membrane potential is important for the generation of ATP. Therefore, the levels of ATP were measured during the 10 hour treatment period. For untreated cells, a gradual reduction of ATP after 2 hours of incubation was observed. However, ATP levels from cultures treated with CCCP and valinomycin decreased at a clearly faster rate (Figure 5.1C). After 2 hours the ATP

level was less than 10 nM/OD_{600}. There was a further gradual decrease for the remainder of incubation. The low levels of ATP during treatment indicate membrane potential was dissipated for the duration of the 10 hour incubation.

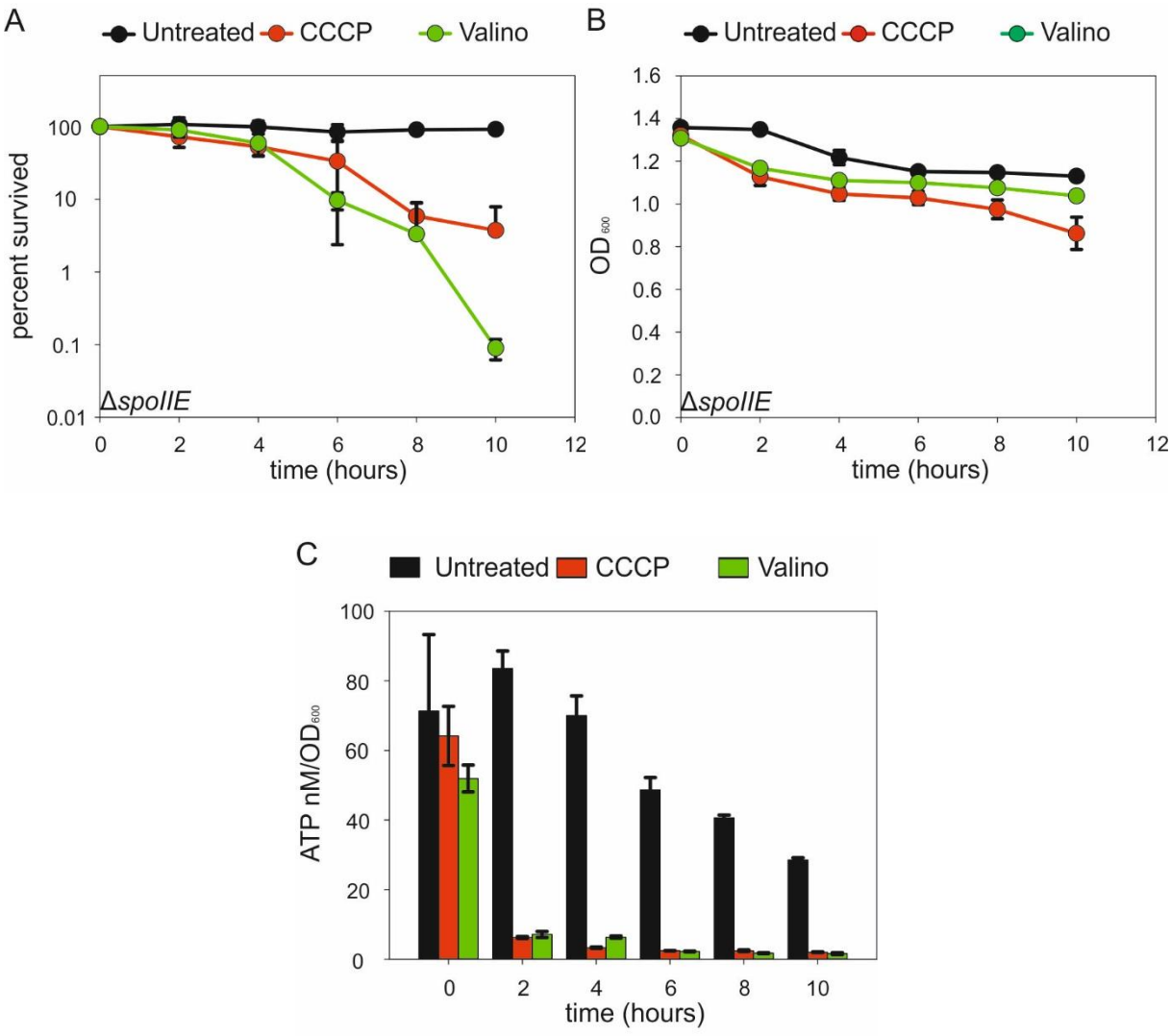

Figure 5.1 Effect of CCCP and valinomycin on stationary phase *B. subtilis* cells. Sporulation deficient *B. subtilis* cells (Δ*spoIIE*) were cultured in valinomycin media at 37 ºC overnight. Cultures were then incubated for 10 hours either with or without 100 µM CCCP or 100 µM valinomycin (valino). A) The percent survival of the population was determined every 2 hours through plating serial dilutions on NA. B) The OD_{600} over 10 hours was measured every 2 hours using a plate reader. Graphs represent the average and standard deviation of 3 independent experiments. C) Intracellular levels of ATP. Samples from 10 hour experiments were taken every 2 hours and flash frozen in order to determine ATP levels.

5.2.2 *Autolysins*

The difference observed between CFU and OD indicated that the CCCP and valinomycin-triggered killing is unlikely to be solely caused by cell lysis. However, autolysis caused by cell wall lytic enzymes has been reported to occur upon de-energisation of *B. subtilis* cells (Jolliffe et al., 1981). *B. subtilis* has several autolysins, many of which are encoded on prophage-regions of the chromosome (Smith et al., 2000). The major vegetative autolysins are LytC, LytD, LytE and CwlO (Blackman et al., 1998; Hashimoto et al., 2012; Smith et al., 2000). Autolysins are involved in a tightly regulated hydrolysis of peptidoglycan, an activity which is essential for both cell elongation and division (Typas et al., 2012). It has been observed that antimicrobial compounds induce autolysis (Falk et al., 2010; Lacriola et al., 2013). The decrease in percent survival observed could thus be a result of membrane potential dissipation-triggered autolysis. To test this possibility, the survival of a multiple autolysin mutant was assessed (Δ*lytCDEF*). This strain has been shown earlier to exhibit a reduced extend of autolysis (Danevcic et al., 2016; Lacriola et al., 2013). Deletion of the main autolysin genes had no impact on survival in the presence of either CCCP or valinomycin (Figure 5.2A and B). Thus, under these experimental conditions cell lysis is not the cause for the bactericidal activity of CCCP and valinomycin.

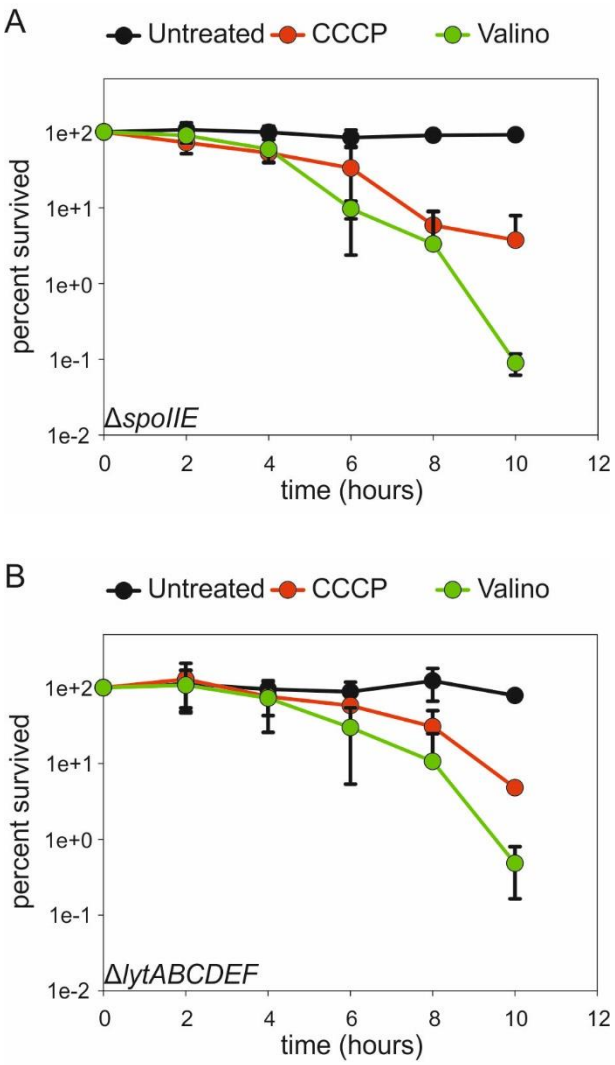

Figure 5.2 The effect of autolysin deletions.

Stationary phase *B. subtilis* cells were incubated for 10 hours either with or without 100 µM CCCP or 100 µM valinomycin (valino). Periodic sampling was performed in order to determine the CFU through serial dilution and plating on NA. The plates were incubated overnight at 37 ºC where colonies were counted the following morning. A) Δ*spoIIE* and B) Δ*lytABCDEF*. Graphs represent the average and standard deviation of 2 independent experiments.

5.2.3 *Investigating cell death by oxidative stress*

5.2.3.1 *SOS response*

Since the reduction in percent survival was not caused by cell lysis, another factor must be contributing to the cell death. The CFUs during experimentation were determined by plating on NA. Upon colony counting a difference in colony size was observed for cultures treated with either CCCP or valinomycin while untreated cultures generated uniform colonies. The different colony sizes suggested that the cells could be accumulating damage and, as a result, require a varying degree of time to restore robust growth in order to form a colony. Possibly, a loss of membrane potential could result, somehow, in extensive DNA damage (SOS response). One of the key proteins in the SOS response is RecA. Interestingly, a *recA* null mutant was highly sensitive for CCCP or valinomycin (Figure 5.3A and B). This supports the notion that DNA damage is incurred through dissipating the membrane potential.

5.2.3.2 *Production of ROS*

A natural cause of DNA damage is an increased level of ROS. However, an increase in ROS would be counter intuitive since the production of ROS is associated with an active respiratory transport chain. For mitochondria it has been shown that dissipation of the membrane potential by CCCP actually reduces ROS production (Miwa and Brand, 2003). Therefore, it is difficult to envision how membrane depolarisation would create ROS in bacterial cells. In studies where ROS production by classic antibiotics has been examined, ROS scavengers have been utilised to promote cell survival (Kohanski et al., 2007). Therefore, the experiments were repeated in the presence of different ROS scavengers, tiron (10 mM) and thiourea (150 mM) (Novogrodsky et al., 1982; Taiwo, 2008). Interestingly, the addition of 10 mM tiron, a superoxide scavenger, completely restored the viability for both valinomycin and CCCP treated cells, suggesting that ROS is formed (Figure 5.4A and B). Surprisingly, the addition of 150 mM thiourea, a hydroxyl radical scavenger, had the complete opposite effect and resulted in a complete loss of detectable colonies within 8 hours (Figure 5.4C). Tiron primarily neutralizes superoxide radicals (Taiwo, 2008), suggesting the superoxide anion contributes to cell death after dissipation of membrane potential. To confirm this, the intracellular levels of superoxide were measured in cells using a superoxide sensitive fluorescent probe (Oxyburst Green). Oxyburst Green was added and cells were observed by

fluorescence light microscopy at 15 minutes and 135 minutes after addition of either CCCP or valinomycin. When using the wild type (Δ*spoIIE*) there was no fluorescence detected even for the positive control (Figure 5.5A), therefore, a *sodA* deletion background was used in combination with Δ*spoIIE*. SOD enzymes are involved in the removal of superoxide, SodA is the major cytoplasmic SOD in *B. subtilis* (Inaoka et al., 1999). When using a Δ*sodA* background strain, detectable levels of fluorescence were observed for the positive control paraquat (Figure 5.5B). Importantly, the presence of either CCCP or valinomycin also increased the fluorescence, confirming that dissipation of the membrane potential generates superoxide. This was further underlined by the reduced fluorescence in the presence of tiron (Figure 5.5C).

The results with the Δ*sodA* background suggested that this background should be highly sensitive for CCCP and valinomycin. This was indeed the case as shown in figure 5.6A and B. *B. subtilis* contains two additional SOD enzymes, SodF and SodC. Neither of these appeared to be required for survival (Figure 5.6A, C and D), which is not surprising as SodA is the major superoxide dismutase within the cell (Inaoka et al., 1999). The removal of superoxide is achieved by converting it into hydrogen peroxide another potentially damaging ROS, therefore, the absence of the peroxide-sensing regulator PerR, which regulates *katA* expression and other genes of the Per regulon, was examined (Herbig and Helmann, 2001). An increase in sensitivity was observed for the Δ*perR* strain (Figure 5.6E), for that reason a strain lacking the main vegetative catalase, KatA, was assessed, however, no increased sensitivity was observed (Figure 5.6F). These findings are in line with the ROS scavenger data, which revealed that only the superoxide scavenger tiron was effective and not the hydroxyl radical scavenger thiourea (Figure 5.4B and C).

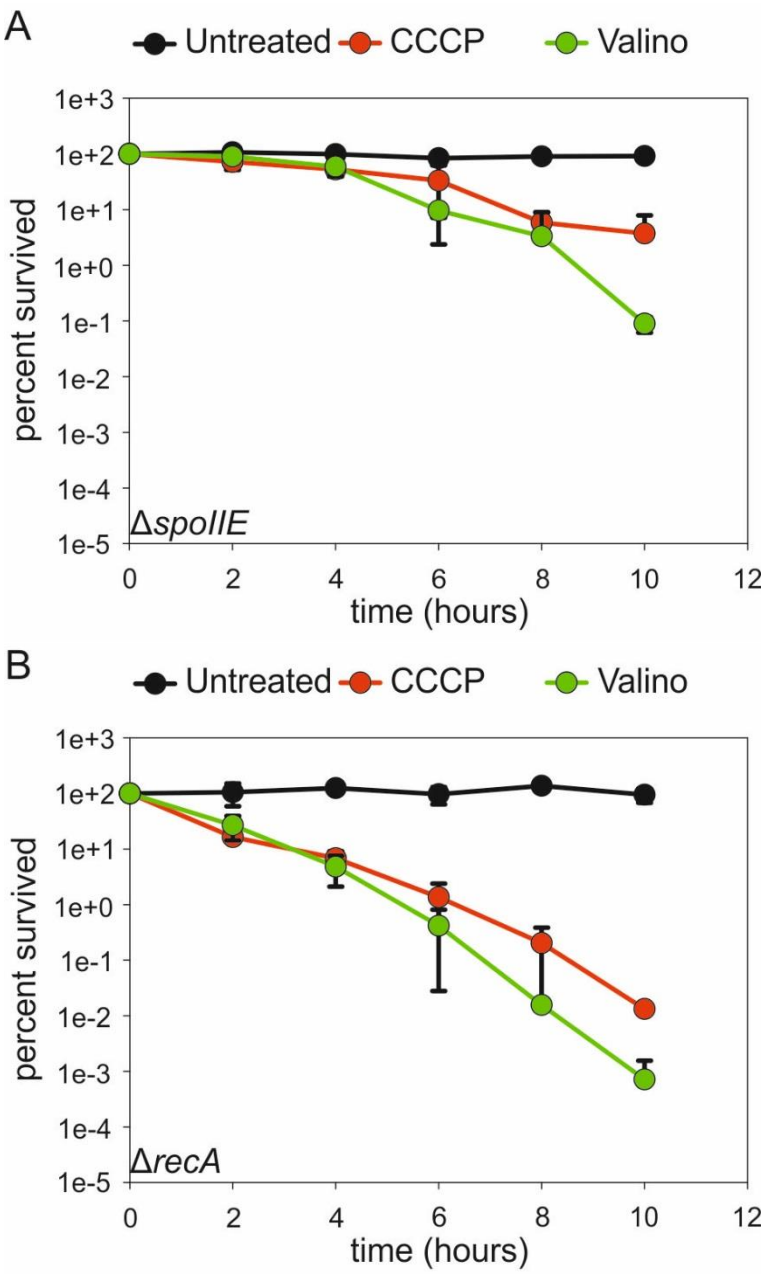

Figure 5.3 The effect of SOS response impairment.

Cultures were grown overnight at 37 ºC in valinomycin media. Cultures were then incubated for 10 hours either with or without 100 µM CCCP or 100 µM valinomycin (valino). Periodic sampling was performed in order to determine the CFU through serial dilution and plating on NA. A) Δ*spoIIE* and B) Δ*recA*. Graphs represent the average and standard deviation of 2 independent experiments.

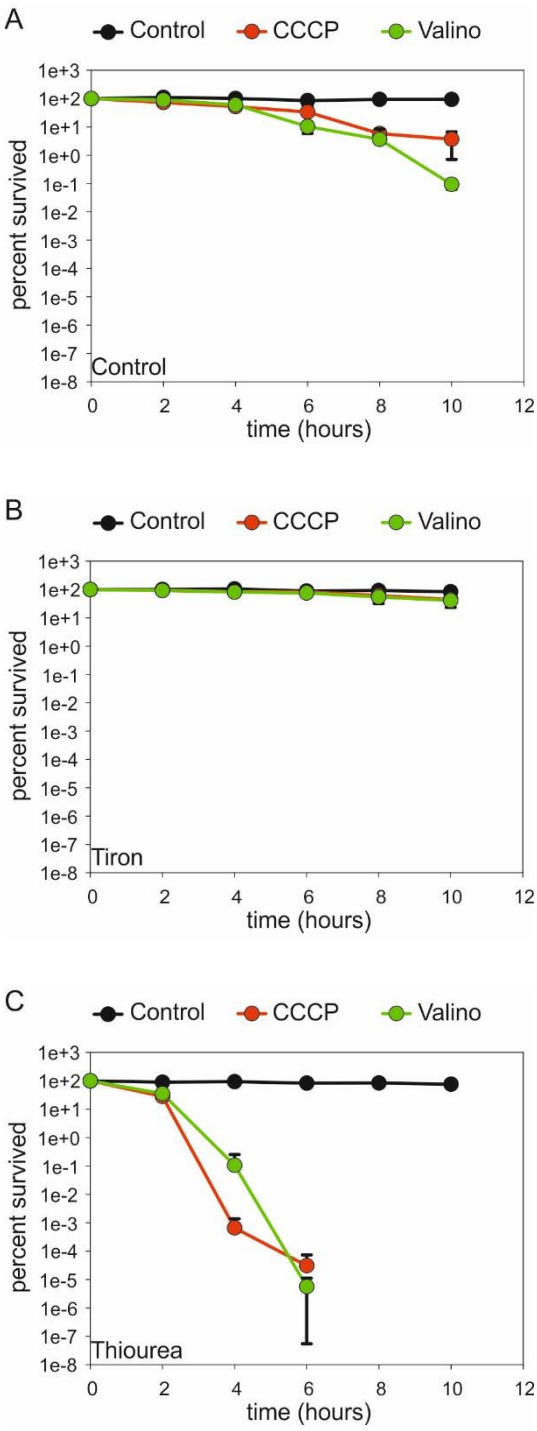

Figure 5.4 Survival in the presence of ROS scavengers.

Cultures of Δ*spoIIE* were grown overnight at 37 ºC in valinomycin media. Cultures were then incubated for 10 hours either with or without 100 µM CCCP or 100 µM valinomycin (valino). A) No additional compound was added, B) 10 mM tiron was added to all cultures, and C) 150 mM thiourea was added to all cultures. Periodic sampling was performed in order to determine the CFU through serial dilution and plating on NA. Graphs represent the average and standard deviation of 2 independent experiments.

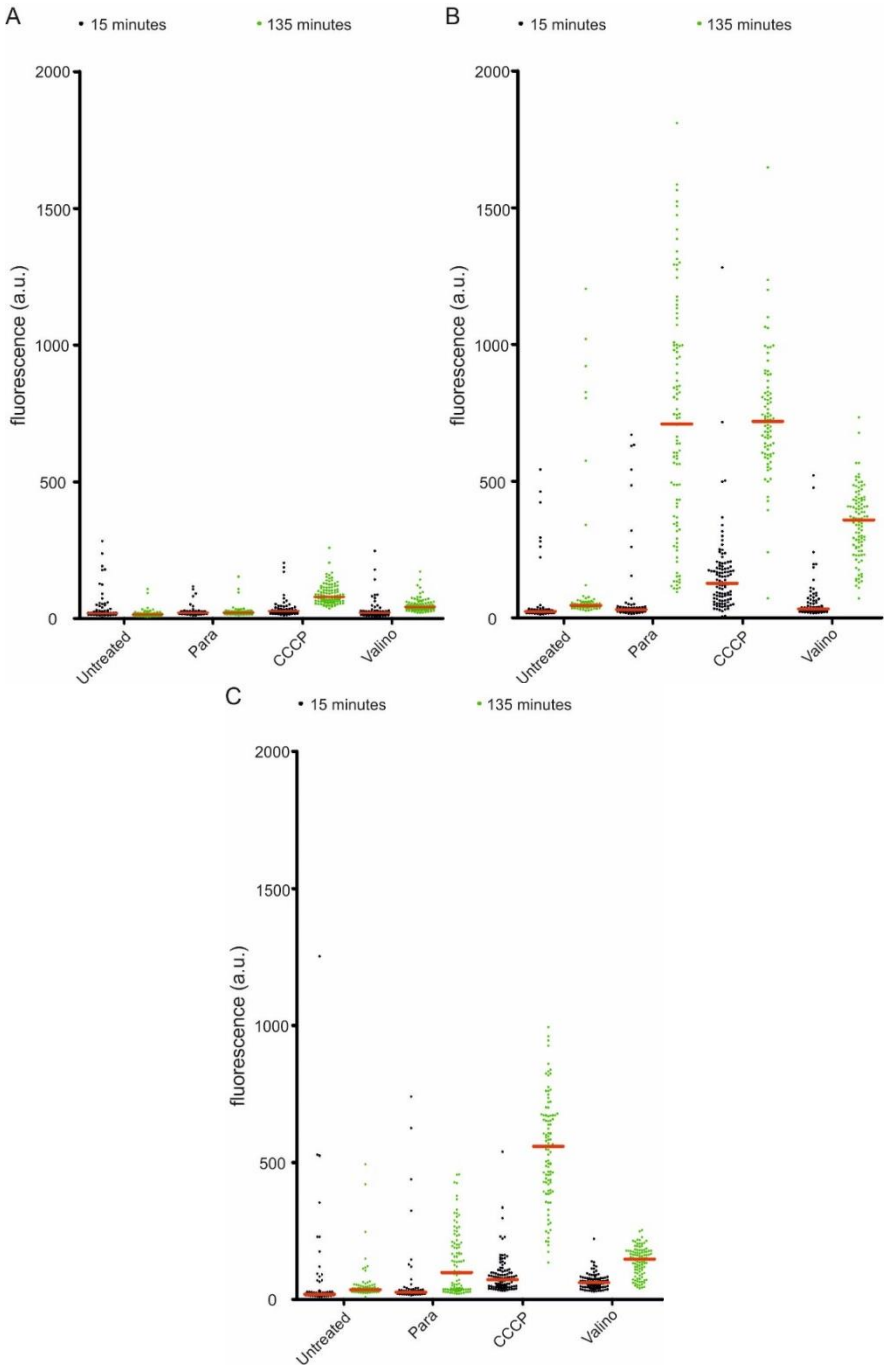

Figure 5.5 Intracellular superoxide levels.

Cultures were grown overnight at 37 ºC in valinomycin media. Cultures were then incubated for 2 hours either with or without 100 µM paraquat (para) or CCCP or valinomycin (valino). A) Δ*spoIIE,* B) Δ*sodA*, C) Δ*sodA* cultures with 10 mM tiron. At 0 and 2 hours, samples were incubated with 50 µM oxyburst green for 15 minutes at 37 ºC with aeration. The excess probe was removed by centrifugation and resuspension, and the fluorescence detected using a Nikon Eclipse Ti fluorescent microscope. Fluorescence intensities for approximately 100 cells were determined using ImageJ. Depicted is a column scatter plot with the calculated media value.

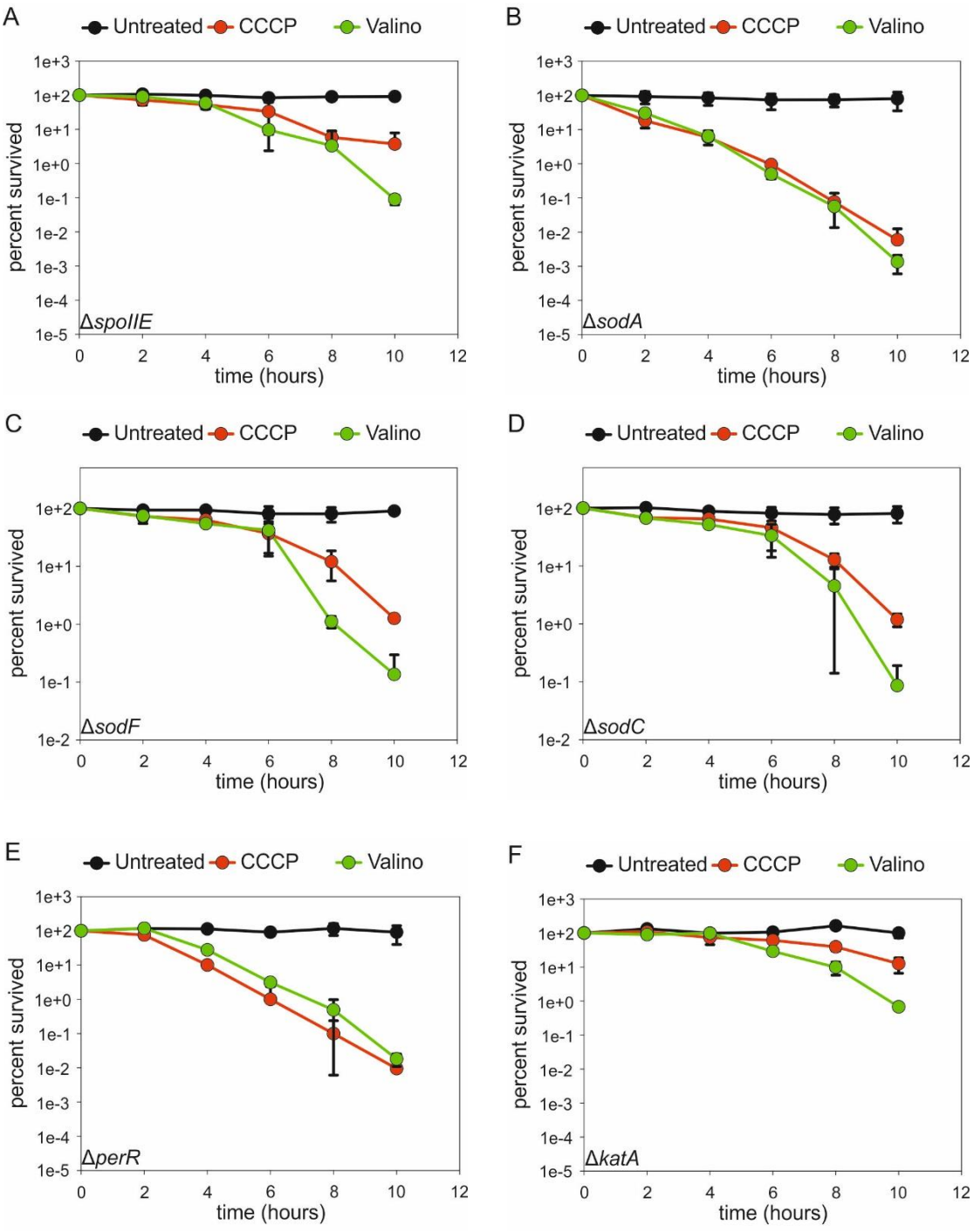

Figure 5.6 The effect of ROS repair and removal mutants on survival.

Cultures were grown overnight at 37 ºC in valinomycin media. Cultures were then incubated for 10 hours either with or without 100 µM CCCP or 100 µM valinomycin (valino). Periodic sampling was performed in order to determine the CFU through serial dilution and plating on NA. A) Δ*spoIIE,* B) Δ*sodA*. C) Δ*sodF*. D) Δ*sodC*. E) Δ*perR* and F) Δ*katA*. Graphs represent the average and standard deviation of 2 independent experiments.

5.2.3.3 *Anaerobic conditions*

The formation of ROS is something all aerobic organisms must cope with as a by-product of oxidative phosphorylation. As a consequence, mechanisms have evolved to cope with a low level production of ROS from the respiratory chain. In the absence of oxygen, no ROS can be formed. For this reason, the classical experiment to test if ROS is responsible for the antibiotic-triggered killing is to repeat the experiments under anoxic conditions. In the case of depolarisation-triggered killing, this experiment is complicated because *B. subtilis* is an “almost” obligate aerobe, and can only grow anaerobically after a prolonged adaptation time either by anaerobic respiration, or by fermentation (Cruz Ramos et al., 2000; Nakano and Zuber, 1998). Nevertheless, to test if membrane depolarisation results in loss of viability under anoxic conditions, the cells initially were cultured under the same conditions as all previous experiments. However, upon addition of CCCP or valinomycin, the cells were transferred to an anaerobic chamber, followed by an incubation of 10 hours. Unfortunately, the chamber could not be opened without the introduction of oxygen. For this reason, the viability was only determined for two time points, 0 and 10 hours. Incubation of cells under anaerobic conditions, by itself, resulted in an approximately 99.9 % reduction in culturability of the untreated culture. An additional approximately 1000 fold reduction was observed for cultures treated with either CCCP or valinomycin (Figure 5.7A).

Since killing was observed under anaerobic conditions, selected deletions and scavengers were examined under anaerobic conditions, in order to verify if the observed supressing or sensitising effects were still at place. For this aim, the survival of a Δ*sodA* strain, and the effect caused by the addition of the superoxide scavenger tiron, were examined under the anoxic conditions. The *sodA* deletion strain showed similar results compared to the Δ*spoIIE* strain with approximately 99.9 % of the untreated population dying as a consequence of the transition to anoxic conditions. CCCP treated cells showed the same 1000 fold decrease in viability as observed under anoxic conditions. However, only a 100 fold additional reduction was observed for valinomycin treated cells (Figure 5.7B). When 10 mM tiron was added to the cultures under anaerobic conditions, an increase in survival was again observed. With no valinomycin or CCCP present only 90 % of the cultures died instead of the 99.9 % observed for all other strains and conditions examined under anaerobic conditions. CCCP treated cells showed a similar trend to the untreated sample, only

a further small reduction was observed (Figure 5.7C). Valinomycin showed a 100 fold decrease in survival compared to the untreated sample. With the addition of 150 mM thiourea under anaerobic conditions, the cells without CCCP or valinomycin showed the same decrease in survival as the Δ*spoIIE* and Δ*sodA* strains; 99.9 % of the population died. For CCCP treated cells, only a 100 fold increase in killing was observed (Figure 5.7D) which is 10 fold lower than without thiourea. Valinomycin showed the same 1000 decrease in survival with or without the presence of thiourea (Figure 5.7A and D).

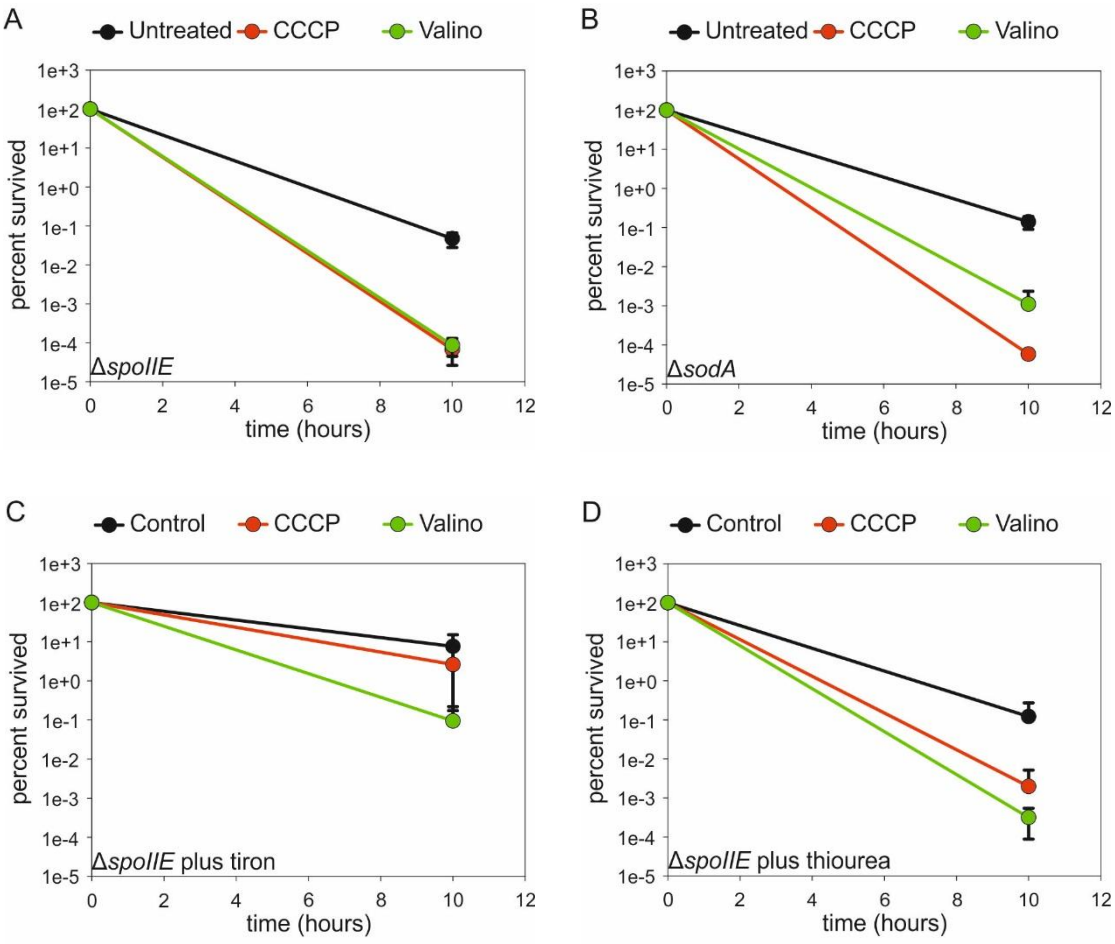

Figure 5.7 Incubation under anaerobic conditions.

Cultures were grown overnight at 37 ºC in valinomycin media. Cultures were then incubated for 10 hours either with or without 100 µM CCCP or 100 µM valinomycin (valino). A) Δ*spoIIE*, B) Δ*sodA*, C) Δ*qcrA*, D) Δ*spoIIE* cultures with 10 mM tiron, and E) Δ*spoIIE* cultures with 150 mM thiourea. Sampling was performed at hour 0 and hour 10, in order to determine the CFU through serial dilution and plating on NA. The plates were incubated overnight at 37 ºC where colonies were counted the following morning. Graphs represent the average and standard deviation of 2 independent experiments.

5.2.3.4 *Inhibition of the electron transport chain*

The results from the anaerobic experiment were indecisive whether aerobic respiration is involved in superoxide production upon dissipation of the membrane potential. As an alternative way to assess this, the addition of the cytochrome *bc1* (complex III) inhibitor antimycin A was assessed (Slater, 1973). The addition of 200 µM of antimycin A was, by itself, enough to reduce cell survival, and approximately 90 % of the population had lost viability at hour 6. After this, no further loss in viability was observed (Figure 5.8A and B). The mixture of CCCP and antimycin A resulted in an increase in cell death by approximately 10,000 fold. In contrast, there was no cumulative effect when antimycin A and valinomycin were jointly present (Figure 5.8A and B).

To further analyse the interplay between valinomycin and antimycin-triggered killing, a concentration series of antimycin A was analysed in the presence and absence of valinomycin. Four concentrations of antimycin A were examined, 50 µM, 100 µM, 200 µM and 400 µM, with 0 µM as a control. Antimycin A only caused a reduction in CFU at 100 µM and above. With valinomycin and antimycin A both present, the same overall reduction in survival was observed regardless of the antimycin A concentration (Figure 5.9A, B, C, D and E). Due to the lack of a cumulative killing affect in the presence of valinomycin and antimycin A, this data suggests valinomycin could be having an effect on complex III, the target of antimycin A.

In order to determine if the of accumulative killing is specific for antimycin A, another ETC inhibitor, potassium cyanide (KCN), was tested in combination with valinomycin. The target of KCN is the cytochrome *c* oxidase (complex IV) (Way, 1984), which is located further downstream in the ETC. In the presence of KCN, a similar reduction in survival of approximately 99.9 % was observed (Figure 5.9F). However, with both KCN and valinomycin present, a clear additive effect was observed (Figure 5.9F). As a consequence the use of ETC inhibitors gave a hint that complex III might be a source for ROS upon membrane potential dissipation.

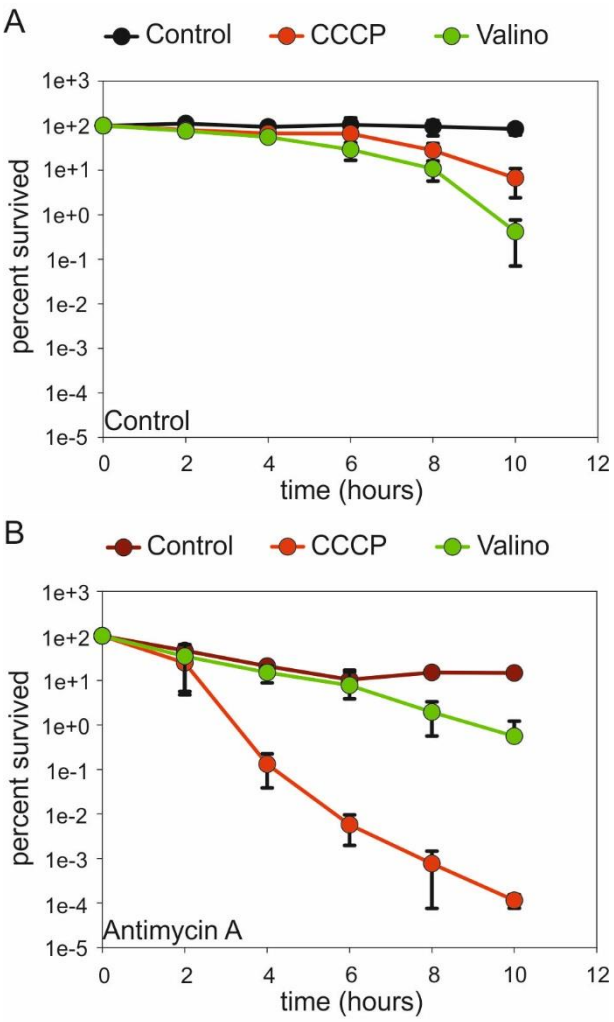

Figure 5.8 Electron transport chain inhibitor.

Cultures of Δ*spoIIE* were grown overnight at 37 ºC in valinomycin media. Cultures were then incubated for 10 hours either with or without 100 µM CCCP, 100 µM valinomycin (valino), and/or 200 µM antimycin A. A) Cultures were untreated with antimycin A, and B) 200 µM antimycin A was added to all cultures. Periodic sampling was performed in order to determine the CFU through serial dilution and plating on NA. Graphs represent the average and standard deviation of 2 independent experiments.

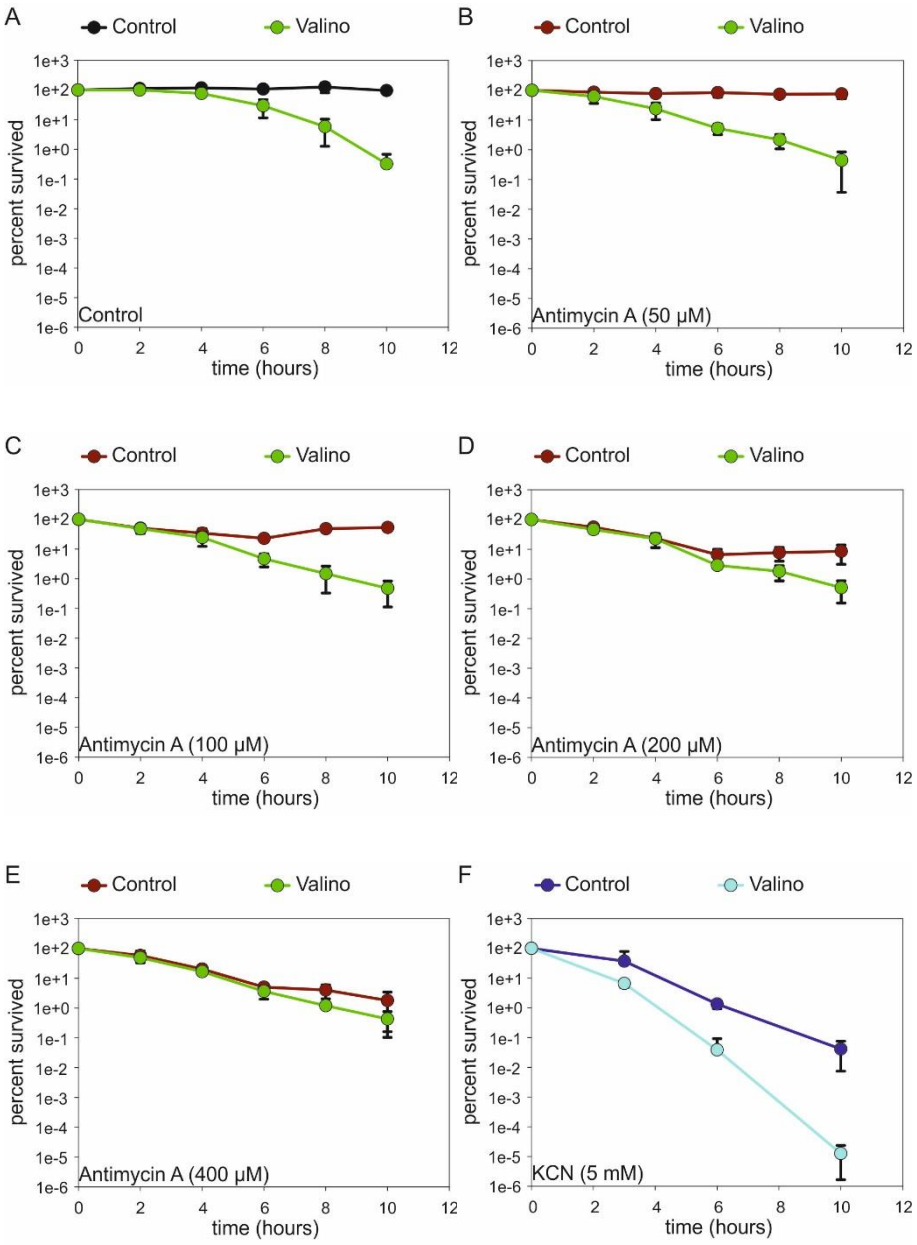

Figure 5.9 The effect of different concentrations of antimycin A in the presence of valinomycin.

Cultures of Δ*spoIIE* were grown overnight at 37 ºC in valinomycin media. Cultures were then incubated for 10 hours either with or without 100 µM valinomycin (valino), where appropriate concentrations of antimycin A or potassium cyanide were added. A) Control, only valinomycin added. B) 50 µM of antimycin A was added to both cultures. C) 100 µM of antimycin A was added to both cultures. D) 200 µM of antimycin A was added to both cultures. E) 400 µM of antimycin A was added to both cultures. F) 5 mM of KCN was added to both cultures. Periodic sampling was performed in order to determine the CFU through serial dilution and plating on NA. Graphs represent the average and standard deviation of 2 independent experiments.

5.2.3.5 *Respiration mutants*

Due to results suggesting that superoxide play a role in cell death upon dissipation of membrane potential, additional sources of ROS were investigated within the respiratory pathway. Known points of superoxide production are complex I and complex III of the ETC, and glycerol-3-phosphate dehydrogenase, and in the Kreb cycle 2-oxoglutaratedehydrogenase (Odh) (Drose and Brandt, 2008, 2012; Mailloux et al., 2014; Orr et al., 2012) as schematically shown in figure 1.7.

Genetic deletions of these potential ROS sources were tested including, *ndh* (NADH dehydrogenase) (Gyan et al., 2006), *glpD* (glycerol-3-phosphate dehydrogenase) (Holmberg and Rutberg, 1992), *qcrA* (menaquinol:cytochrome *c* oxidoreductase) (Yu et al., 1995b) and *pdhB* (pyruvate dehydrogenase beta subunit) (Blencke et al., 2003) mutants. Additional mutants examined were, *sdhC* (succinate dehydrogenase) (Hagerhall et al., 1992) and *qoxB* (cytochrome *aa3* quinol oxidase subunit I) (Santana et al., 1992). These mutants provided coverage over all most all the ETC components.

Deleting *glpD*, glycerol-3-phosphate dehydrogenase involved in glycerol utilisation, had no effect on survival in the presence of either compound (Figure 5.10A and B). The absence of pyruvate dehydrogenase beta subunit (*pdhB*) which links glycolysis and the TCA cycle, did not improve survival either. In fact, for CCCP the sensitivity was increased and the CFU was no longer detectable after 10 hours (Figure 5.10C). Curiously, there was only a marginal extra decrease in survival for valinomycin.

The deletion of genes involved in the ETC, specifically complex I (*ndh* - NADH dehydrogenase), complex II (*sdhC* - succinate dehydrogenase) and cytochrome *aa3* (*qoxB* - quinol oxidase subunit I), did not mitigate the effect of either compound. While these deletions had little to no effect on survival in the presence of valinomycin, there was a considerably higher sensitivity with CCCP (Figure 5.10D, E and F). The final deletion mutant tested with regard to respiration and the ETC was *qcrA* (menaquinol:cytochrome *c* oxidoreductase, iron-sulphur subunit). This strain showed an intriguingly opposite effect. When tested with valinomycin, the Δ*qcrA* cultures showed a clear increase in survival by approximately 100 fold (Figure 5.10G). There was no detectable effect on survival in the presence of CCCP. The majority of gene deletions involved in respiration resulted in an increased sensitivity

to CCCP while having no effect on valinomycin treatment (Figure 5.10H). One deletion was shown to improve survival in the presence of valinomycin (Δ*qcrA)*, possibly indicating a source of the ROS previously observed. Intriguingly, this was complex III, the same location highlighted by the antimycin A experiments.

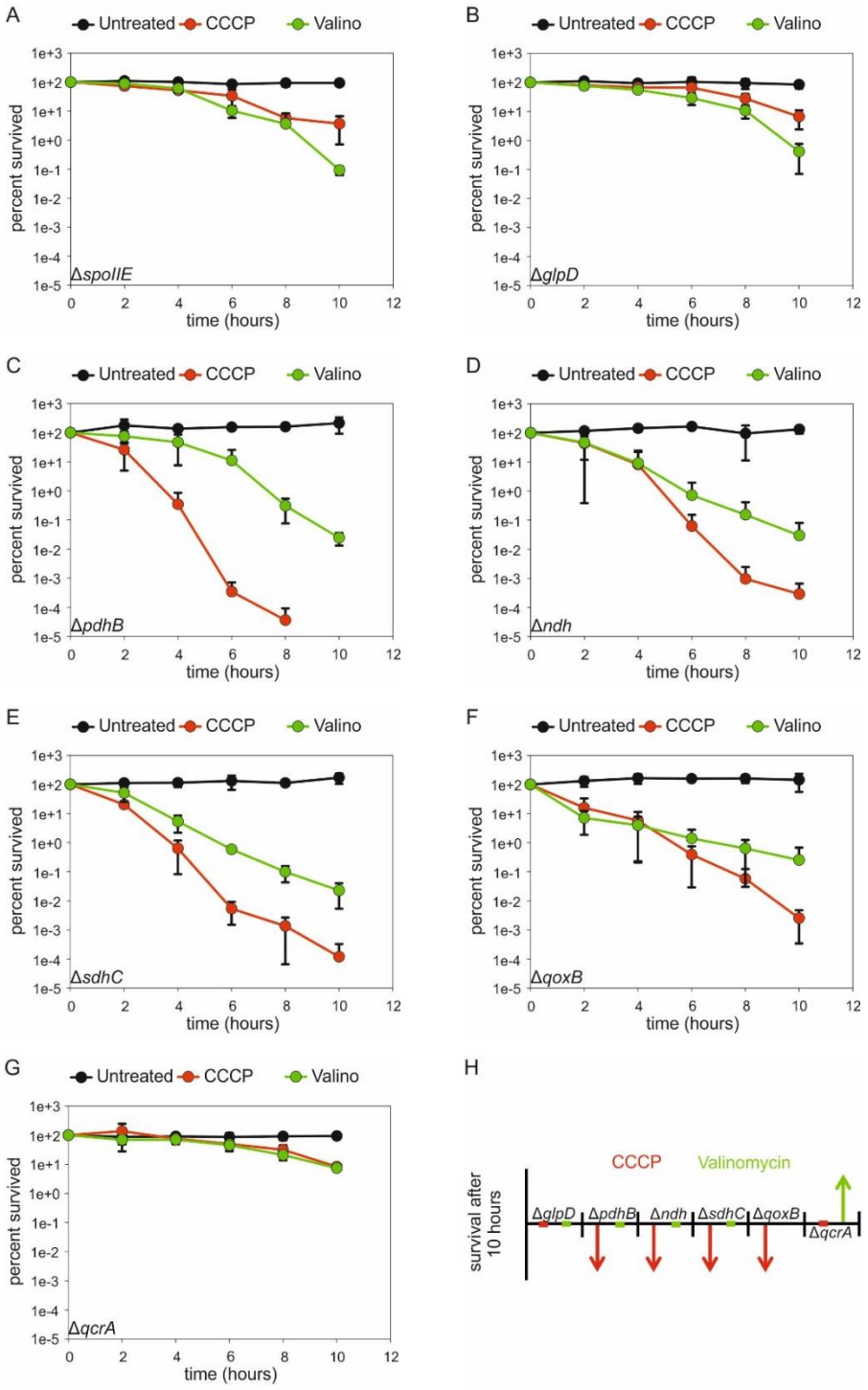

Figure 5.10 Respiration deletion mutants.

Cultures were grown overnight at 37 ºC in valinomycin media. Cultures were then incubated for 10 hours either with or without 100 µM CCCP or 100 µM valinomycin (valino). Periodic sampling was performed in order to determine the CFU through serial dilution and plating on NA. A) Δ*spoIIE,* B) Δ*glpD,* C) Δ*pdhB*, D) Δ*ndh*, E) Δ*sdhC*, F) Δ*qoxB* and G) Δ*qcrA*. Graphs represent the average and standard deviation of at least 2 independent experiments. H) Graphical summary of the positive (up-arrow), negative (down-arrow) or no effect (box) that gene deletions have on survival for CCCP (red) and valinomycin (green) after 10 hours.

5.2.3.6 *Iron chelators*

Menaquinol:cytochrome *c* oxidoreductase contains an iron-sulphur subunit. Release of Fe^{2+} can create ROS by means of Fenton reactions, this takes place when H_2O_2 and Fe^{2+} react to form Fe^{3+}, HO• and hydroxide (OH^-). Additionally, superoxide can contribute to the level of Fe^{2+} present by reacting with Fe^{3+} to generate Fe^{2+} (Kehrer, 2000). Two iron chelators were examined for their effect on survival FerroZine and 2,2-bipyridyl (Baichoo et al., 2002; Fish, 1988). FerroZine had no influence on survival in the presence of CCCP or valinomycin, and the killing curves were comparable to those in the absence of FerroZine (Figure 5.11A and B). In contrast, 2,2-bipyridyl sensitised the cells for CCCP and valinomycin and a further decrease in survival by approximately 100 fold was observed (Figure 5.11C). The decrease in CFU was apparent after 4 hours with a reduction of approximately 90 % of the population. These data suggest that ROS is not generated through Fenton chemistry and in fact removal of Fe^{2+} can prove to be detrimental to survival upon membrane potential dissipation.

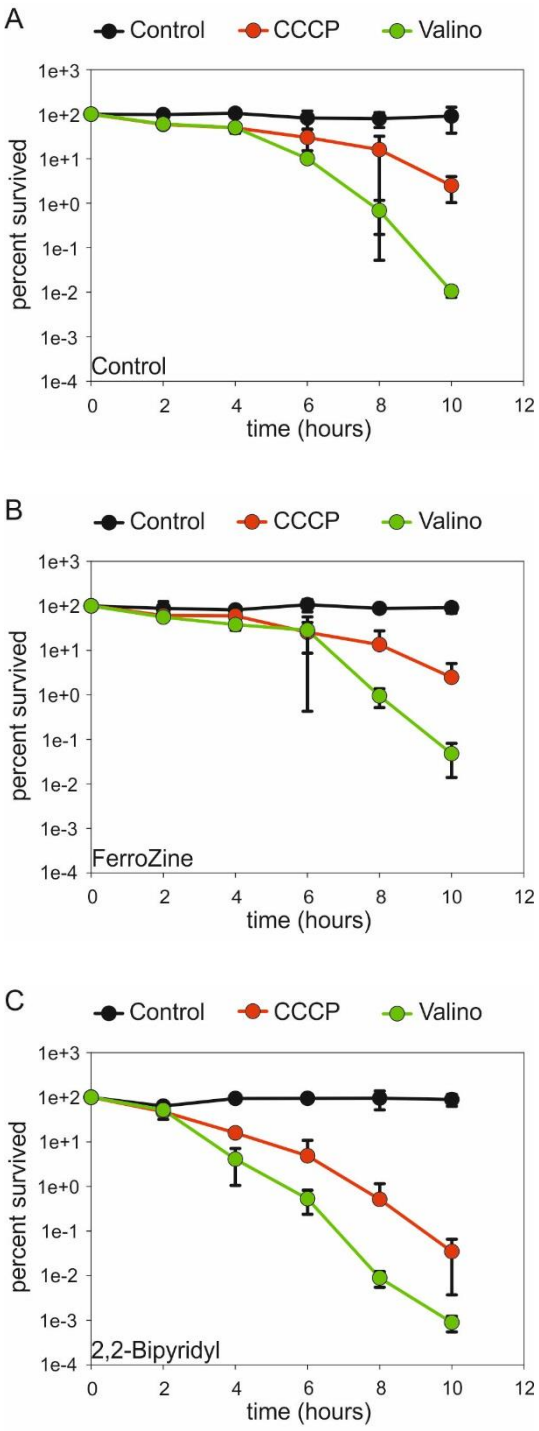

Figure 5.11 Effect of iron chelators.

Cultures of Δ*spoIIE* were grown overnight at 37 ºC in valinomycin media Cultures were then incubated for 10 hours either with or without 100 µM CCCP or 100 µM valinomycin (valino). Where appropriate, 500 µM of FerroZine or 2,2-bipyridyl was also added. A) Untreated, B) 500 µM FerroZine added to all cultures, C) 500 µM 2,2-bipyridyl added to all cultures. Periodic sampling was performed in order to determine the CFU through serial dilution and plating on NA. Graphs represent the average and standard deviation of 2 independent experiments.

5.2.3.7 *The regulator Spx*

So far, evidence has been provided to show that dissipation of the membrane potential results in the formation of superoxide leading to cellular damage. Oxidative stress is incurred when a cell can no longer cope with the level of ROS being generated. If dissipation of the membrane potential is causing oxidative stress deletion of the oxidative stress regulator, Spx, would resulted in an increased sensitivity. Indeed, as shown in figure 5.12 a Δ*spx* strain was more sensitive to both CCCP and valinomycin.

The sensitivity of the *spx* deletion strain prompted further investigation. Spx is a negative and positive regulator of many genes involved in the oxidative stress response (Zuber, 2009). These include *bshA*, *B1*, *B2* and *C* which are involved in the biosynthesis of bacillithiol (Gaballa et al., 2013). Bacillithiol aids to maintain cellular redox balance and is potentially the substitute for glutathione, a common thiol in eukaryotes and other bacteria (Masip et al., 2006). Additionally, *msrAB*-operon is up-regulated by Spx (You et al., 2008). MsrA and MsrB are methionine sulfoxide reductases involved in regeneration of methionine, and restoration of protein function after oxidative damage. Due to their role in oxidative stress, these gene deletions were investigated further. However, none of the individual deletions had an effect on survival after 10 hours (Figure 5.13A, B, C, D, E, F and G).

Spx itself is subject to post-translational regulation via proteolytic regulation by ClpX and ClpP (Nakano et al., 2002). For this reason, single deletions of both *clpP* and *clpX* were investigated. Deletions of either *clpP* or *clpX* both resulted in an overall increase in survival for valinomycin treated cultures (Figure 5.14A, B and C). Approximately a 100 fold increase in percent survival after the 10 hour treatment period was observed for the *clpP* deletion (Figure 5.14A and B). Deletion of *clpX* had, with an observed 10-100 fold increase, a slightly lower effect (Figure 5.14A and C). Surprising these deletions did not appear to improve survival for CCCP treatment (Figure 5.14 A, B and C).

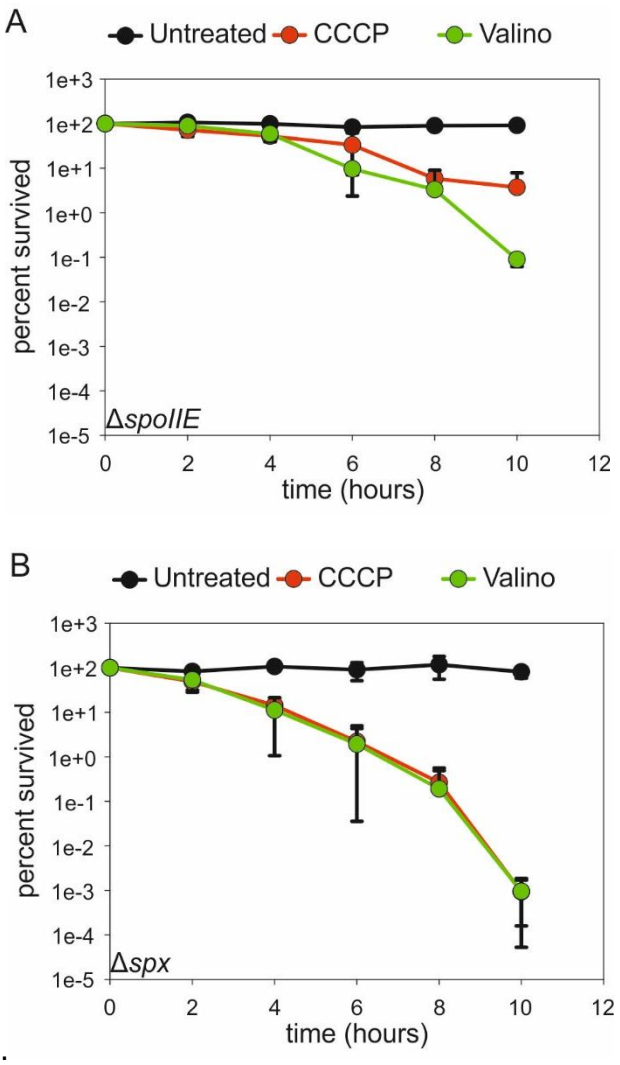

Figure 5.12 Deletion of the oxidative stress master regulator.

Cultures were grown overnight at 37 ºC in valinomycin media. Cultures were then incubated for 10 hours either with or without 100 µM CCCP or 100 µM valinomycin (valino). Periodic sampling was performed in order to determine the CFU through serial dilution and plating on NA. A) Δ*spoIIE* and B) Δ*spx*. Graphs represent the average and standard deviation of 2 independent experiments.

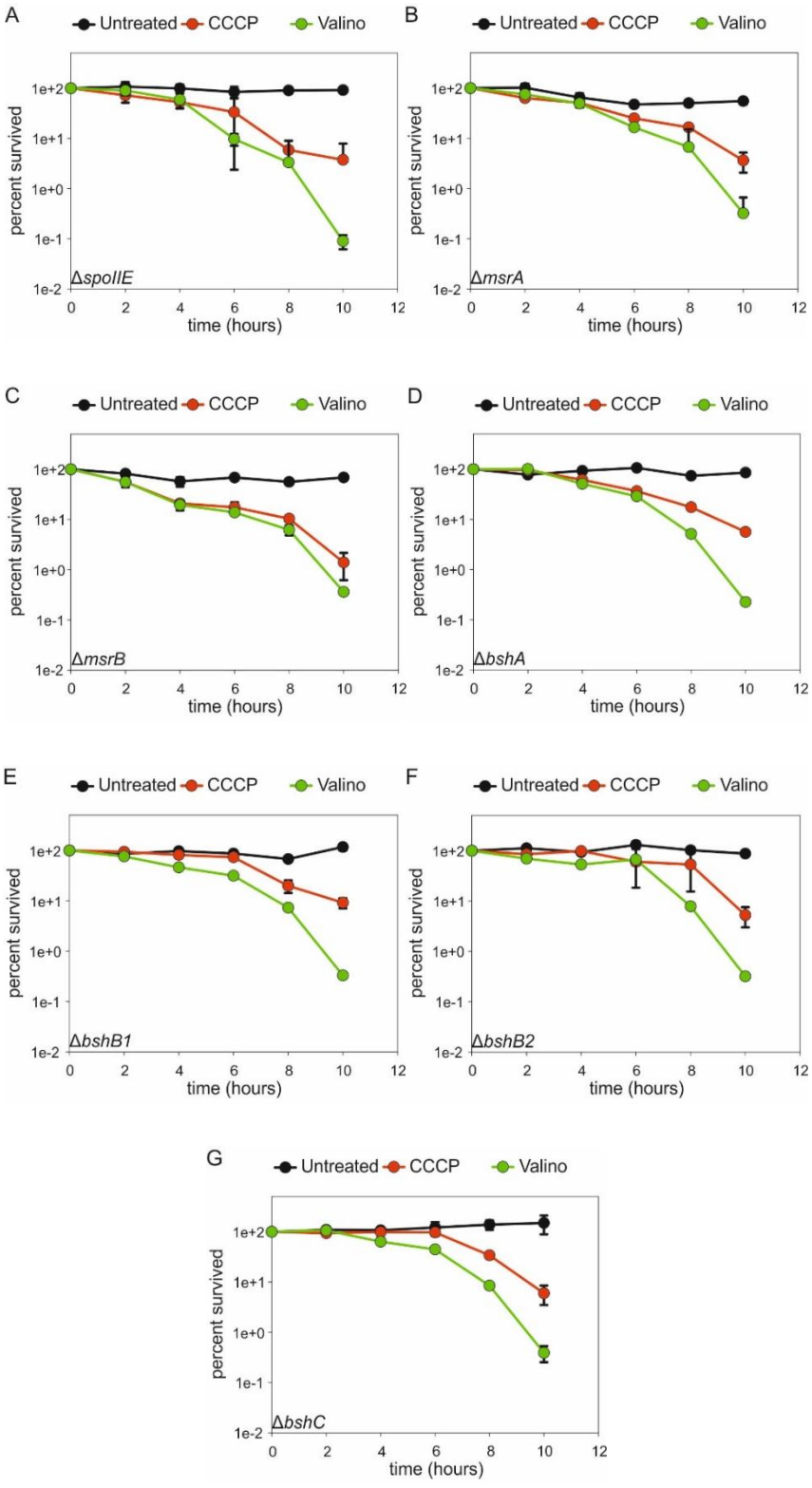

Figure 5.13 Spx up-regulated genes.

Cultures were grown overnight at 37 ºC in valinomycin media. Cultures were then incubated for 10 hours either with or without 100 µM CCCP or 100 µM valinomycin (valino). Periodic sampling was performed in order to determine the CFU through serial dilution and plating on NA. A) Δ*spoIIE*, B) Δ*msrA*, C) Δ*msrB*, D) Δ*bshA*, E) Δ*bshB1*, F) Δ*bshB2* and G) Δ*bshC*. Graphs represent the average and standard deviation of at least 2 independent experiments.

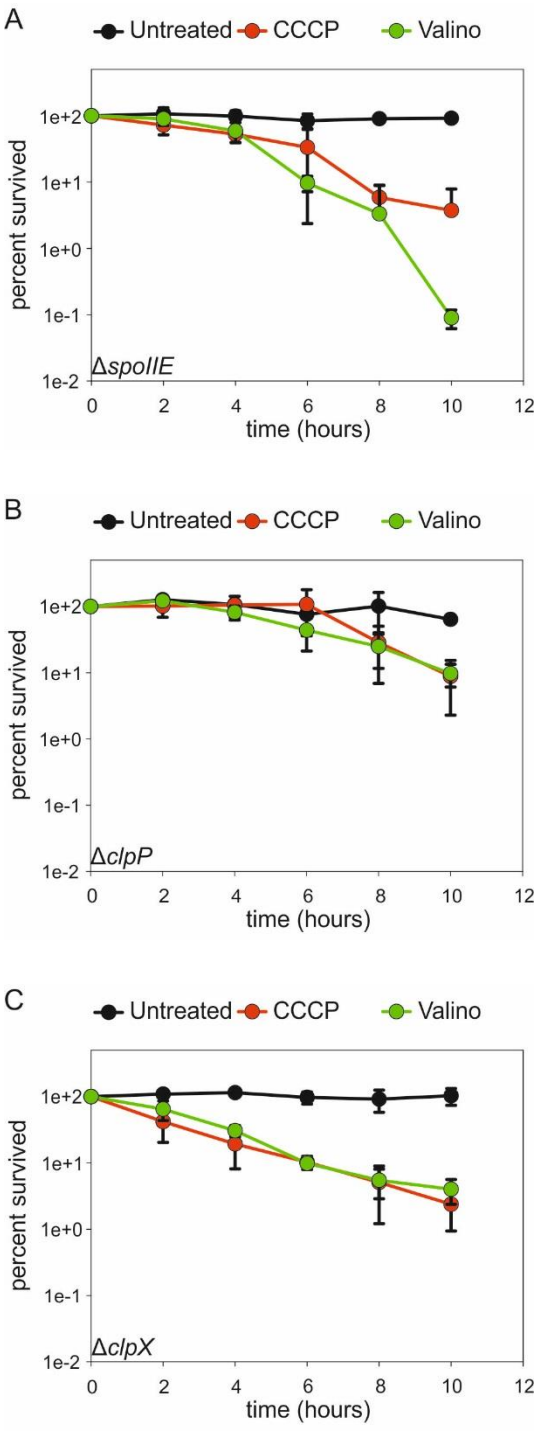

Figure 5.14 Degradation of Spx.

Cultures were grown overnight at 37 ºC in valinomycin media. Cultures were then incubated for 10 hours either with or without 100 µM CCCP or 100 µM valinomycin (valino). Periodic sampling was performed in order to determine the CFU through serial dilution and plating on NA. A) Δ*spoIIE*, B) Δ*clpP*, C) Δ*clpX*. Graphs represent the average and standard deviation of at least 2 independent experiments.

5.3 Discussion

5.3.1 *Cell death not due to lysis*

Previous studies have shown the effectiveness of compounds that dissipate the membrane potential in killing bacteria (Cui et al., 2012; de Carvalho et al., 2011). Understanding the general mechanism through which the bactericidal activity is established is important because these compounds could be effective even against the dormant cell types.

Under the conditions analysed here, using stationary phase *B. subtilis* cells as a model for non-growing bacteria, a reduction in culturability was observed over the 10 hour treatment period. For the proton ionophore CCCP approximately 90 – 99 % of the population died. The potassium carrier valinomycin resulted in even more effective killing with a 99.9 % reduction in population viability. The main reduction in CFU occurred between 4 to 6 hours of treatment indicating that cell death does not occur immediately following the membrane depolarisation which takes place practically immediately (Te Winkel et al., 2016). A possible reason for this could be that cells have a small store of energy they can utilise in order to remain energised even in the absence of membrane potential. However, this is unlikely since the ATP levels reduced to very low levels already after 2 hours. Another possibility is that dissipation of membrane potential is not lethal directly due to the energy starvation but rather results in some sort of slowly accumulating cellular damage.

When OD was measured for the population, only a small decrease for valinomycin treated cultures was observed, a reduction which does not match the 99.9 % cell death observed in the CFUs. In addition, deletion of autolysins which are responsible for de-energisation triggered lysis in *B. subtilis* had no effect on survival, further supporting the notion that cell death is not due to lysis. Since the cells remain intact, but are unable to form colonies on rich media, the possibility that the cells are in a VBNC state (Oliver, 2005) cannot be formally ruled out.

5.3.2 *Is ROS causing cell death*

A common source of damage within a bacterial cell is caused by ROS, and there is currently a debate as to whether ROS production is induced by antibiotic treatment. Evidence has been provided in support and opposition of this phenomenon (Dwyer et al., 2014; Feld et al., 2012; Kohanski et al., 2007; Liu and

Imlay, 2013; Paulander et al., 2014). Studies conducted in *E. coli* have shown that DNA repair mutants (Δ*recA*) and deletions of genes involved in oxidative stress are more sensitive to antibiotic treatment (Feld et al., 2012; Kohanski et al., 2007). In our experiments, a decrease in cell survival was observed for *B. subtilis* cells containing similar deletions upon membrane depolarisation. The decrease in survival observed for the Δ*recA* strain indicated cells were sustaining DNA damage after membrane potential dissipation, supporting ROS damage. Further to this, deletion of genes involved it ROS protection resulted in a decrease in survival, most notably Δ*sodA* and Δ*spx*. This supports the conclusion that dissipation of the membrane potential results in the formation of ROS resulting in cell death.

In previous work it was shown that the presence of iron chelators or hydroxyl radical scavengers including thiourea reduced killing by antibiotics like ampicillin, kanamycin and norfloxacin (Kohanski et al., 2007; Wang et al., 2010). Surprisingly, however, under the experimental conditions examined here, thiourea resulted in a dramatic decrease in CFU. Hydroxyl radicals are reported to be the most reactive form of ROS. Therefore, it is unclear why thiourea would cause an increase in sensitivity to membrane potential dissipation. ROS species such as H_2O_2 have been associated with cell to cell signalling, however, no such function has been reported for hydroxyl radicals (Marinho et al., 2014). Nevertheless, the possibility exist that hydroxyl radicals are involved in a so far unknown signalling process which is essential for cell survival, although this seems unlikely due to their very high reactivity. It appears more likely that thiourea is not only scavenging hydroxyl radicals but also has an additional unknown role within the *B. subtilis* cell. The sensitising effect of thiourea appears to be specific for aerobic conditions as the increased killing is not observed under anaerobic conditions. Due to the oxygen-dependency of the hypersensitivity observed with thiourea, the formal possibility that scavenging hydroxyl radicals is the cause for the decreased viability cannot be ruled out either.

Another difference with previous studies of ROS generation by antibiotics was the decrease in survival observed in the presence of the iron chelator 2,2-bipyridyl. Iron is an essential nutrient and is a cofactor for several proteins (Helmann, 2014). The removal of iron could thus inhibit the function of proteins which require iron as a cofactor, and which are involved in survival upon membrane potential depolarisation. The difference observed between 2,2-bipyridyl and Ferrozine could be due to several factors, including iron chelation efficiency and accumulation within cells.

Also in support of the ROS hypothesis was the reduction in survival observed for the superoxide dismutase-deficient Δ*sodA* strain. In contrast, no difference in survival was seen for the catalase-deficient Δ*katA* strain. This indicates that cell death was directly or indirectly triggered by accumulation of superoxide rather than hydrogen peroxide. This was further supported by the observed mitigation of cell death by the superoxide scavenger tiron and the increased fluorescence of the superoxide probe Oxyburst Green. Such probes have frequently been used as evidence to support ROS generation triggered by antibiotics (Dwyer et al., 2014). However, the specificity of the fluorescent redox probes has been questioned (Liu and Imlay, 2013). In the case of *B. subtilis*, no fluorescence was detected in the presence of SodA. In contrast, clearly detectable levels were measured with the specific positive control paraquat in the absence of SodA, thus providing strong experimental support for the superoxide-specificity of the assay.

The generation of ROS is due to aerobic respiration which cannot take place under anaerobic conditions. As a consequence, no ROS should be generated under anoxic conditions. However, studies have shown that antibiotics are still able to kill under anaerobic conditions (Liu and Imlay, 2013). Indeed, dissipation of membrane potential under anaerobic conditions still resulted in a reduction in the CFU, indicating that ROS is not the only reason for cell death. Anaerobic respiration uses nitrite as an electron acceptor instead of oxygen and generation of reactive nitrogen species (RNS) is possible (Hartig and Jahn, 2012). Therefore, under anaerobic conditions the cell death could be triggered by accumulation of damage caused by RNS instead of ROS. A limiting factor for the anaerobic experiments performed in this thesis was the removal of oxygen from the anaerobic chamber which was accomplished using AnaeroGen 2.5L (Miller et al., 1995). The used catalyst is able to deplete the oxygen concentration to below 0.1 % within 2.5 hours. For this reason, the residual generation of ROS cannot be fully ruled out. Moreover, *B. subtilis* is capable of anaerobic respiration and fermentation, provided enough time to adapt (Nakano and Zuber, 1998). Therefore, due to the lack of adaptation time to anaerobic conditions it is impossible to say if under the conditions tested here *B. subtilis* was undergoing anaerobic respiration, additionally the lack of a nitrate source in the medium would also suggest that *B. subtilis* was unable to achieve anaerobic growth. In order to counteract these experimental problems, *B. subtilis* could be grown under steady anaerobic conditions providing full adaptation to nitrite as a terminal electron

acceptor, followed by treatment with CCCP or valinomycin. This however, requires specific equipment currently not available at the CBCB.

Spx regulates over 100 genes, and is a global regulator of oxidative stress response (Rochat et al., 2012). Therefore, the decrease in survival witnessed for this deletion lends more credence to the ROS hypothesis. However, we were unable to identify a specific gene under Spx control that would be responsible for the observed reduced survival. The prime candidates tested, BshA, BshB1, BshB2, BshC, MsrA and MsrB, had no effect on survival. Other candidates potentially involved in this process are *trxA* and *trxB*, which encode thioredoxin and thioredoxin reductase, respectively (Nakano et al., 2003). Both of these genes are essential and therefore cannot be deleted. However, it would be possible to construct *trxA* and *trxB* strains under the control of an inducible promoter to test their contribution. Of course, it is conceivable that the reduction in culturability observed for Δ*spx* could be by any one of the other >100 genes of the Spx-regulon. In agreement with the specific role of the Spx-regulon, the deletion of *clpP* or *clpX*, which are involved in proteolytic degradation of Spx (Nakano et al., 2002), resulted in an overall increase in survival for the 10 hour treatment period. It has been shown that deletion of either of these genes results in the accumulation of Spx (Nakano et al., 2002), thus resulting in an increased expression of genes under the control of Spx.

5.3.3 *ROS generation location*

In the ETC electrons are transported from complex I and II to complex III through reduction and oxidation of menaquinone (Borisov and Verkhovsky, 2015). Complex III (cytochrome *bc1* complex) transfers the electrons to cytochrome *c* by a process known as the Q-cycle. Reduced menaquinone is oxidised at the Q_P site (Rieske protein – Fe-S protein) releasing 2 electrons. One of these electrons is directed to cytochrome *c* while the second one partially reduces menaquinone bound at the Q_n site. This forms the first half of the Q-cycle (Cramer et al., 2011). The second half is the same reaction with a freshly reduced menaquinone releasing 2 electrons, one for cytochrome *c* and the second one to fully reduce the menaquinone bound at the Q_n site thereby triggering its release. For *B. subtilis* it has been postulated that menaquinone-dependent electron transfer within the ETC is somehow dependent on the PMF (Azarkina and Konstantinov, 2002).

The Fe-S cluster Rieske protein above is encoded by *qcrA,* and forms complex III of the ETC in combination with QcrB (cytochrome *b*) and QcrC (cytochrome *c*) (Yu et al., 1995a). Deletion of *qcrA* resulted in an increase in survival for valinomycin treated cultures, suggesting that the cytochrome oxidase pathway (menaquinone → cytochrome *bc1* complex → cytochrome *c* → cytochrome *c* oxidase) (Borisov and Verkhovsky, 2015) is possibly the main source of superoxide. Dissipation of membrane potential in mitochondria causes the release of cytochrome *c* from the membrane (Gottlieb et al., 2003), if the effect is the same in *B. subtilis* when treated with valinomycin this would interrupt the electron flow and stall further oxidation of menaquinone at the Q_P site. Delocalisation of cytochrome *c* has been observed in *B. subtilis* in the presence of membrane dissipating compounds gramicidin S or MP196 (Wenzel et al., 2014), providing credence to this hypothesis. Therefore, the dissipation of membrane potential appears to effect electron transfer through the cytochrome oxidase pathway, specifically between cytochrome *bc1* and cytochrome *c*, and not at the level of menaquinone oxidation reduction as previously described (Azarkina and Konstantinov, 2002). A possible reason for this difference is the growth state of the cells. It has been suggested that the cytochrome oxidase pathway is not expressed until stationary phase (Winstedt and von Wachenfeldt, 2000). Our study used cultures well into stationary phase, whereas Azarkina and Konstantinov used exponential phase cultures.

The proposed mechanism above could also explain the why there was no cumulative killing in the presence of antimycin A and valinomycin. The hypersensitivity of cells to antimycin A and CCCP is not completely unexpected because this combination has been shown to increase ROS production in mitochondria (Carriere et al., 2004). It should be highlighted though, that the observed contribution of complex III of the ETC in valinomycin-triggered killing, which is likely caused by formation of superoxide, is surely not the sole cause for the observed viability. Due to the multitude of processes affected by the loss of membrane potential, it is not unreasonable to assume that cell death is achieved by numerous contributing factors.

Valinomycin specifically dissipates membrane potential by acting as a carrier for K^+ ions while CCCP does so by transporting H^+ ions. As a consequence, CCCP dissipates ΔpH as well as membrane potential (Bhattach.P et al., 1971; Kasianowicz et al., 1984). The most likely reason for the difference observed between the two

compounds lies in this difference. The increased sensitivity of Δ*ndh* (NADH dehydrogenase), Δ*pdhB* (pyruvate dehydrogenase beta subunit), Δ*sdhC* (succinate dehydrogenase), Δ*qoxB* (cytochrome *aa3* quinol oxidase subunit I) strains suggests that cell death is not due to superoxide formation from a single source. Although the dissipation of membrane potential impairs the generation of ATP by ATP synthase (Dimroth et al., 2000), low amounts of ATP can still be produce upstream in the respiration pathway (Santana et al., 1994). However, as a result of the deletions examined here the redox balance could be affected decreasing the yield of ATP resulting in the decrease in survival observed for Δ*ndh*, Δ*pdhB*, Δ*sdhC*, Δ*qoxB* strains in the presence of CCCP. Since no mutant was found to improve survival in the presence of CCCP, it is difficult to hypothesise where the potential source of ROS might lie. Since CCCP also dissipates ΔpH it would be worth investigating the specific effects of ΔpH dissipation on the analysed respiration mutants. This could be achieved by using nigericin which, at low concentrations, only dissipates ΔpH without affecting membrane potential (Farha et al., 2013). However, the data presented here suggests dissipating membrane potential could be and effective treatment against persisters.

5.4 Future work

The membrane potential is important for cellular function, ATP synthesis, protein localisation and ion uptake, to name a few. Therefore the dissipation of membrane potential affects various cellular processes, some of which may have a role in causing cell death. Evidence has been provided that ROS contributes to cell death, however, it is not the only factor. The exact source of the superoxide radicals was not determined, however, there is evidence suggesting complex III of the ETC as the source. Additional mutants of complex III should be assessed for their impact on survival. Additionally, the point of electron inhibition should be assessed, previous work indicates the inhibition of electron flow to occur at the level of menaquinone reduction and oxidation, however, data present here indicates complex III as the point of inhibition, possibly due to the delocalisation of cytochrome *c*. Western blotting could be performed to assess cytochrome *c* delocalisation.

The analysis of deletion mutants revealed the importance of the oxidative stress response protein Spx in survival upon membrane depolarisation. However, the specific genes under the regulation of Spx that caused the increased sensitivity were not identified. It is possible that a combination of several genes that are up-regulated, are responsible for the observed sensitivity. A DNA microarray analysis of cultures after 30 minutes of treatment could highlight important genes for surviving membrane potential dissipation. In addition, this analysis could highlight additional processes of a cell that are dependent or controlled by membrane potential, for instance proteins within the ETC, thereby revealing potential new antibiotic targets. A challenge in these experiments would be to identify suitable controls because the cellular consequences of membrane potential dissipation are so pleiotropic. One potential control would be specific inhibition of the F_1F_o ATP synthase complex with N,N′-Dicyclohexylcarbodiimide (DCCD). The comparison between valinomycin and DCCD would provide the means to distinguish between ATP-specific effects from those caused by depolarisation.

The sensitivity to both CCCP and valinomycin was not removed under anaerobic conditions indicating that ROS is not the only factor contributing to the cell death. However, it is possible that under these conditions the death is caused by RNS. This possibility should be investigated through utilising RNS scavengers and/or fluorescent probes sensitive to RNS. Additionally genes deletions of genes involved

in RNS removal and repair could be assessed for their impact survival under anaerobic conditions.

Chapter 6. General discussion and summary

6.1 Starvation assay

A starvation assay was developed for *B. subtilis* with similar features to already published assays which have tested the starvation survival of different bacterial species (Arias et al., 2012; Britos et al., 2011; Byrd et al., 1991; Watson et al., 1998). The assay provided a robust experimental framework for a controllable way of testing starvation survival in a well-reproducible manner. The development of the assay highlighted the high capacity of *B. subtilis* to survive in different starvation conditions. This is perhaps unsurprising due to the various habitats from which *B. subtilis* has been successfully isolated.

The major limitation of this assay and for the majority of starvation assays utilised in these types of studies is that they are closed and stable systems. In the natural environment, the environmental conditions are in constant flux, and the level and type of stresses occurring at any particular moment varies. Although the purpose of these assays is to evaluate the starvation survival for a given bacteria, even the stress from starvation can be more severe in a natural habitat due to the combination of multiple simultaneous stresses affecting the bacterium. In the framework of this PhD-project, methods were tested in order to overcome the closed system limitation by using dialysis. This turned out to be a challenging method with reproduction-difficulties caused by a high likelihood of contamination from other ubiquitous bacteria. Nevertheless, the developed assay allowed testing for the purpose for which it was designed, but it should be remembered that additional factors such as the presence of multiple species of bacteria, nutrient influxes from different sources, and the presence of over flow metabolites would also affect the starvation survival in the natural environment.

6.2 Starvation survival of *B. subtilis*

The starvation survival of bacterial species which are incapable of sporulation has been well documented (Arias et al., 2012; Britos et al., 2011; Byrd et al., 1991; Finkel, 2006; Watson et al., 1998). Additionally, it is well known that the endospore, generated by such genera as *Bacillus* and *Clostridium*, can survive for many years under unfavourable conditions (Gerding et al., 2008; Thomas, 2012). During a time-lapse microscopy experiment with *B. subtilis,* it was observed that a small population

of cells did not undergo lysis or sporulation (Veening et al., 2008b). However, their viability capacity was unknown.

In this dissertation, non-sporulating cultures of *B. subtilis* were demonstrated to survive prolonged incubation under nutrient deprivation conditions for periods of over 100 days. Not only were non-sporulating cultures able to survive, but cultures with the ability to sporulate were shown to contain a fraction of vegetative cells. This indicates that even when presented with continuous starvation, sporulation is not adopted by 100 % of the population. A bet-hedging strategy employed by *B. subtilis* gives the population a greater chance of survival by having multiple phenotypes present at any given time. This allows certain phenotypes to take advantage of fluctuating conditions (Lopez et al., 2009). Within the natural environment of *B. subtilis*, periods of limited nutrient availability are likely to occur for extended periods of time. The survival of these slow growing cells could be advantageous to the population as a whole as they have the potential to utilise transient small amounts of nutrients which spores would be incapable of due to their long germination time. Although the long term survival of *B. subtilis* is attributed to endospore formation, it is now apparent that long term survival during nutrient exhaustion is a more complex process.

The observed survival appears to be dependent upon the nutrients released from lysed cells. These nutrients allow the bacteria to grow at a much slower rate with division times of days as opposed to 20 – 40 minutes of exponentially cells normally growing in the laboratory. This lysis has been observed for the non-sporulating *S. aureus* (Watson et al., 1998), however, it is still unclear if the lysis is a type of active adaptation mechanism linked to PCD (Allocati et al., 2015). The slow growth of the starved cells appears to give the bacteria an increased tolerance to additional stresses such as antibiotic treatment and oxidative stress. This would be beneficial to cells in the natural environment, as in soil, given the fierce competition for nutrients taking place between bacteria and fungi, a battle to a large extend conducted using antimicrobial and antifungal compounds.

In the framework of these studies, a point mutation in *rpoB* was isolated with a selective advantage. Competition assays suggested that this *rpoB** mutation allowed the encoding cells to outcompete a *B. subtilis* strain lacking this mutation under nutrient deprived conditions. A similar phenotype known as growth advantage in stationary-phase (GASP), by which old cultures are able to outcompete younger

cultures, exists in several species (Bacun-Druzina et al., 2007; Finkel, 2006; Martinez-Garcia et al., 2003). Whether *rpoB** represent a *bone fide* GASP-phenotype was not directly tested, however, it is likely that the GASP phenomenon also occurs in *B. subtilis*.

The survival mechanisms of *B. subtilis* have been studied in great detail, and various differentiation pathways and resulting phenotypes have been identified (Lopez et al., 2009). To date, all these phenotypes have been observed upon logarithmic growth phase, or during a relatively early (in the framework of this work) stationary phase. Evidence presented here suggests the survival mechanisms of this species could be even more complex and possibly contain more survival mechanisms operating in parallel to sporulation. Therefore, the survival beyond the "death phase" should be examined in greater detail.

6.3 Membrane potential dissipation

The development of antibiotic resistance to many of the commercially used antibiotics, and the development of "super bugs" such as MRSA has become an large burden to healthcare systems (Moellering, 2012; Ventola, 2015a, b). In addition, dormant phenotypes such as persisters which are able to tolerate otherwise lethal concentrations of antibiotic treatment are likely to cause re-occurring infection (Wood et al., 2013). Thus, the development of new antibiotics, especially ones able to eradicate non-growing bacteria is of great importance.

Over the last decade, research has investigated the possibility that antibiotics cause an increase in ROS production, a secondary mechanism which contributes to cell death (Kohanski et al., 2007). There is evidence both for and against this phenomenon and it remains a hotly debated topic (Dridi et al., 2015; Dwyer et al., 2014; Keren et al., 2013; Kohanski et al., 2010; Kohanski et al., 2007; Liu and Imlay, 2013). Dissipation of membrane potential by antimicrobial agents has proven to be an effective means of killing bacteria. Although dissipation of membrane potential results in cell death, the exact mechanism by which this occurs is largely unknown. Using stationary phase *B. subtilis* as a model for the persister phenotype enabled the analysis of cell death triggered by membrane potential dissipation. Previous studies in *B. subtilis* have indicated membrane potential is required to facilitate electron movement within the ETC, however, the exact step at which inhibition occurs has

remained unclear, although it was postulated to be at the level of menaquinone reduction (Azarkina and Konstantinov, 2002; Schirawski and Unden, 1998).

Two compounds, CCCP and valinomycin, resulted in a reduction in culturability through a process which did not involve cell lysis. The killing effect of these compounds was increased for deletions involved in oxidative stress such as *sodA*, *perR*, *recA* and *spx*. In addition, preventing the degradation of Spx by removing either ClpX or ClpP increased the overall survival of the population. This suggests that genes induced by Spx are important for survival upon membrane depolarisation. The high sensitivity of Δ*sodA* indicates that superoxide contributes to cell death, a conclusion further supported by the ability of a superoxide scavenger to increase survival rates. Although evidence supported the involvement of ROS in cell death, the lethality of these compounds was not negated by the absence of oxygen (anaerobic conditions). The reason for this could be that, under these conditions, reactive nitrogen species are formed instead, no sufficiently anaerobic conditions could be achieved, or, simply, that anaerobically incubated *B. subtilis* is very sensitive towards one of the other pleiotropic consequence of membrane depolarisation.

Analysis of genes involved in respiration pathways revealed a surprising difference in the cell death caused by CCCP and valinomycin. This might be explained by the different mechanism of action between the two compounds (ionophore for H^+ and K^+, respectively) (Bhattach.P et al., 1971; Kasianowicz et al., 1984).The cytochrome *c* oxidase pathway turned out to be especially important for the lethality triggered by valinomycin. The accumulative killing observed for valinomycin in the presence of cyanide but not antimycin A indicated that the cytochrome *bc1* complex is likely involved in cell death triggered by membrane potential dissipation with valinomycin. Therefore, if ROS indeed is a significant contributor to cell death, as the data presented in this thesis suggests, the cytochrome *bc1* complex is the most likely source. However, this still requires further investigation. Lastly, the *qcrA* deletion did not, by any means, restore cell viability to the level observed in untreated cells. This suggests that there are multiple contributors to cell death, which is unsurprising due to the many cellular processes which rely upon membrane potential.

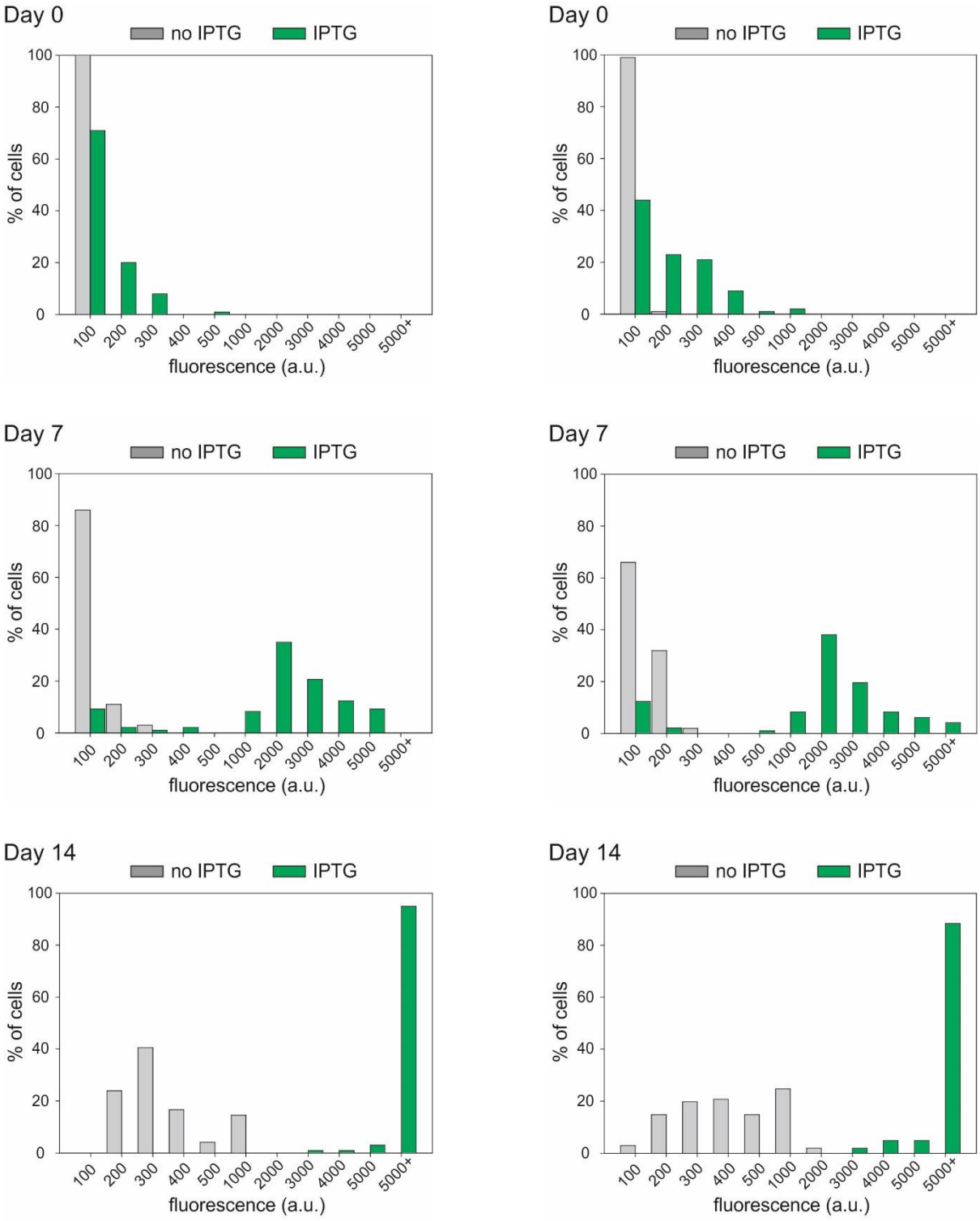

Figure 7.1 Inducing *gfp* during starvation incubation.

On days 0, 7 and 14 samples were taken and incubated with and without IPTG for 4 hours, after which fluorescent images were acquired using a Ti Nikon microscope, and the GFP fluorescence levels measured using ImageJ. Approximately 100 cells were counted to determine the average GFP levels within a population. Graphs represent two repeat experiments.

Table 7.1 Up-regulated genes of 14 day of cultures compared to exponential and stationary phase cultures

Gene	Fold up-regulation (14 days/log)	Fold up-regulation (14 days/stat)
abfA	1.9	1.8
abn2	1.7	1.8
abnA	3.1	2.6
acoA	**8.5**	**12.7**
acoB	**4.8**	3.4
acoC	2.0	2.3
acoL	1.8	1.7
acoR	1.8	1.5
amiE	1.8	1.6
araA	1.9	2.0
araB	2.3	2.2
araD	2.6	2.0
araE	2.1	2.0
araL	2.4	2.2
araM	2.8	1.9
araN	2.1	2.3
araP	3.6	2.1
araQ	3.6	2.5
bglH	2.0	1.8
bglP	1.6	1.7
bhlA	1.6	**5.1**
conB	1.9	2.5
conG	1.7	3.3
cotM	1.7	1.8
cotT	1.6	3.7
cotU	2.8	1.6
cwlH	3.1	2.6
cwlT	2.5	2.2

cycB	1.7	1.7
czcD	2.6	2.7
dtpT	1.7	1.8
exuT	2.1	3.1
ganA	1.7	1.5
ganB	2.9	3.4
ganP	1.9	1.7
ganQ	2.0	1.9
gerAB	2.8	1.9
gerPA	1.5	2.0
glnM	**5.9**	1.7
glpF	1.8	1.9
glpT	1.6	1.7
glvR	1.6	1.5
hutG	1.8	1.7
hutH	2.4	2.2
hutI	1.7	1.8
hutM	1.9	1.7
hutU	1.8	1.8
iolC	1.5	1.8
iolE	1.7	2.0
iolF	1.9	2.0
iolG	1.9	2.0
iolH	1.8	2.5
levD	1.7	3.6
licA	2.3	1.7
ligB	2.6	1.7
lplA	1.9	2.6
lplB	1.8	2.0
manA	2.7	2.6
manP	1.8	1.8
mdxF	1.7	1.5
mhqN	2.7	1.7
mmgB	**32.5**	**4.4**

mtlF	1.9	1.6
murP	1.7	1.6
murQ	1.5	1.9
murR	1.6	1.8
nicK	4.7	**3.9**
pdaB	**7.6**	1.6
rbsA	2.5	2.0
rbsB	2.5	1.9
rbsC	3.5	2.1
rbsD	3.1	2.3
rbsK	1.7	1.7
rbsR	1.5	1.8
rhiF	2.5	**4.3**
rhiG	2.1	**7.0**
rhiL	1.7	**4.0**
rhiN	1.8	1.6
rsgI	1.6	1.5
rsiV	1.7	3.0
sat	1.9	1.9
sda	1.6	2.1
sigI	2.0	2.3
sigV	2.8	3.6
sigY	2.4	1.8
sigZ	1.7	1.6
slrA	1.6	1.5
sodF	3.0	1.8
spoVD	1.6	1.5
sqhC	1.9	1.5
sspH	2.5	1.9
sspJ	3.4	1.7
sunT	1.5	2.5
trkA	1.6	1.7
uxaC	2.5	3.0
uxuA	2.0	2.1

xhlB	1.7	3.0
xkdB	1.8	3.5
ybcF	2.5	1.5
ybcH	3.1	2.3
ybgB	1.7	1.8
ycbF	1.9	1.5
ydcO	1.9	2.2
ydfK	1.5	2.4
ydfQ	2.2	3.5
ydfR	**19.7**	2.7
ydzN	1.5	2.5
ydzR	4.3	3.6
ydzT/1	2.2	1.6
yesL	1.7	2.4
yesM	1.5	2.4
yesW	2.1	1.6
yesX	1.9	1.5
yesY	2.0	1.5
yezD	3.4	**4.1**
yfhS	1.8	**3.9**
ygaB	1.8	1.7
yhaJ	2.1	1.6
yhaR	1.7	1.8
yhcU	1.6	2.0
yhfM	1.8	1.6
yhzF	2.9	2.2
yisJ	**17.2**	3.0
yitE	**15.6**	2.2
yjaZ	2.3	2.1
yjdB	2.3	2.0
yjmB	4.6	**4.4**
yjmC	**4.8**	2.3
yjmD	3.3	2.2
yjmF	2.1	1.6

yjzK	1.8	**3.9**
ykjA	3.4	2.0
ykoP	3.8	2.1
ykzB	2.3	1.8
ykzD	2.6	2.3
ykzH	1.9	1.7
ykzP	1.8	**3.9**
ykzV	2.5	1.6
ynaE	1.5	1.7
yngG	2.5	1.8
yngH	2.6	1.6
yngHB	**28.0**	2.1
yngI	2.4	2.1
ynzJ	1.8	2.2
yoaQ	3.1	1.9
yoaS	2.0	1.8
yobD	1.5	3.2
yobH	**40.2**	3.0
yobN	3.4	1.9
yodQ	3.1	1.8
yojB	1.8	1.6
yomG	1.6	2.5
yomO	2.1	2.0
yonG	1.6	2.1
yopE	2.2	1.9
yorQ	2.4	**10.1**
yosR	2.9	2.9
yosU	2.1	1.9
yoyB	2.0	1.6
yoyI	2.1	1.5
yozU	**5.8**	1.9
yozY	1.7	1.7
yppG	1.7	1.7
ypzI	3.0	1.6

yqaB	2.0	1.6
yqaD	2.9	2.2
yqaG	**8.0**	1.6
yqaI	1.8	**3.9**
yqaJ	2.7	3.3
yqaL	**12.0**	2.3
yqaN	3.3	2.0
yqaQ	2.2	3.3
yqcI	3.0	1.7
yqdA	1.8	3.9
yqfQ	**7.7**	1.6
yqfZ	2.1	1.8
yqhR	1.8	2.0
yqhV	1.6	**5.1**
yqzO	1.8	3.9
yraF	4.6	2.5
yraG	**35.4**	1.5
yraL	1.6	2.1
yrbG	1.6	2.0
yrdB	2.3	2.5
yrdD/2	3.1	**5.1**
yrdD/3	2.3	**5.1**
yrdF	2.7	2.2
yrdR	**15.6**	1.6
yrhP	1.9	1.7
yrkD	1.6	1.7
yrkE	2.4	2.0
yrkJ	1.7	1.8
yrzE	2.1	3.1
yrzM	1.9	2.7
yrzP	2.6	1.6
ytcP	3.2	2.2
ytcQ	2.1	2.0
yteP	**6.1**	**11.3**

yteR	3.8	**4.6**
yteS	**7.3**	**5.2**
yteT	2.3	2.0
yteU	2.2	1.8
yteV	3.0	2.1
ytlA	2.4	1.6
ytvI	1.8	2.7
yubC	4.7	3.7
yutC	1.8	1.6
yvfR	1.8	2.4
yvfS	3.5	1.6
yvkN	**6.1**	2.1
yvnA	1.9	1.9
yvzI	1.9	3.5
ywcB	2.0	1.6
ywcI	3.0	1.5
ywzD	1.6	2.9
yxlC	2.6	2.1
yxlD	2.6	2.2
yxlE	2.6	2.2
yxlF	1.8	1.7
yxlG	1.9	1.8
yxzL	2.7	3.3
yyaC	2.0	3.0
yycO	2.4	1.5
yycP	2.1	1.7
yycQ	1.9	1.5
yyzG	1.8	1.8

Chapter 8. References

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