Co-ordination of DNA replication initiation and chromosome segregation during sporulation

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Abstract

The essence of viability and fitness of an organism is dictated by proper transmission of the genetic material from the parent to the progeny. Therefore in all organisms, processes of DNA replication and chromosome segregation are highly coordinated to match growth and cell division. Spore development in the model organism *Bacillus subtilis* provides a tractable system for studying a number of cellular processes including DNA replication and chromosome segregation.

In the early stages of sporulation, DNA replication initiation is tightly regulated so that a single cell replicates only once and the diploidy is maintained. This is achieved through regulation of the interaction between the initiator protein DnaA and specific DNA elements arrayed at the origin of replication. DnaA assembles at the origin and promotes DNA unwinding and the assembly of a replication initiation complex. SirA is a DnaA-interacting protein that interacts with the N-terminal domain of DnaA and inhibits initiation of DNA replication in diploid *Bacillus subtilis* cells committed to sporulation. However the underlying mechanism by which SirA regulates the chromosome copy number in sporulating cells remains elusive. In the study presented here, I report that the *in vivo* examination of GFP-SirA localisation indicates that the protein accumulates at the replisome in sporulating cells. Also taking advantage of the newly released SirA crystal structure in complex with the N-terminal domain of DnaA (domain I), I show that the SirA localisation is dependent on the integrity of the interface identified in the co-crystal structure. This suggests that SirA accumulation at the replisome is likely a through a direct interaction with DnaA.

In order to test the significance of inhibition of reinitiation events during spore development, I examined the chromosomal origin segregation in sporulating cells defective in regulating DnaA. I found that cells that lack regulators of DnaA; SirA, Soj or YabA exhibit

reduced fidelity in chromosomal origin trapping in the forespore compartment. I also provide evidence that the origin trapping defect observed in the cells lacking DnaA-regulators; SirA and YabA is a direct result of loss of regulation of DnaA. Finally, I show that Soj plays a DnaA-independent role in chromosomal origin trapping, for which its ability to localise at the septum seems to be required.

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Chapter 1-Introduction

1.0 Coordination of cellular processes are vital for growth and viability

The bacterial cell cycle involves overlap of critical cell cycle processes (Haeusser and Levin, 2008, Nordstrom et al., 1991). For such a system to generate viable and fit progeny a high level of organisation and coordination is vital. The focus of the work presented here is the coordination of initiation of DNA replication and chromosome segregation during sporulation of *B. subtilis.* Hence, Chapter 1 is dedicated to giving an overview of the key players implicated in initiation of DNA replication and the regulatory systems overseeing replication initiation. In addition it will cover the chromosome organisation and the known components of the chromosome segregation machinery during both the vegetative growth and sporulation of *B. subtilis*. Where appropriate a brief overview of *E. coli* counterparts is presented.

In order to paint a clear picture of orderly and highly organised processes involved in assuring stable and accurate maintenance of the genetic information throughout generations in bacteria, first, a brief and general overview of *B. subtilis* life cycle during vegetative growth and spore development is given.

1.1 Bacterial cell cycle

Prior to cell division, a bacterial cell must complete a number of processes in order to produce viable and fit progeny (Figure 1.1.1). New-born cells need to replicate and segregate their chromosomes while doubling their cellular content before precisely positioning the cell division machinery. Coordination and timely onset of these processes is critical for proper growth and viability (Haeusser and Levin, 2008).

In eukaryotic cells, cell cycle progression is tightly regulated by checkpoints that only allow entry into a new phase upon successful completion of the previous one (Elledge, 1996). In contrast, the bacterial cell cycle includes an overlapping set of loosely linked parallel steps (Nordstrom et al., 1991).

Our foremost understanding of the bacterial cell cycle is obtained from studies focused on two of the best characterised model organisms: *Escherichia coli* and *Bacillus subtilis*. Here the key events will be briefly discussed with the emphasis on *B. subtilis* during vegetative growth and spore development.

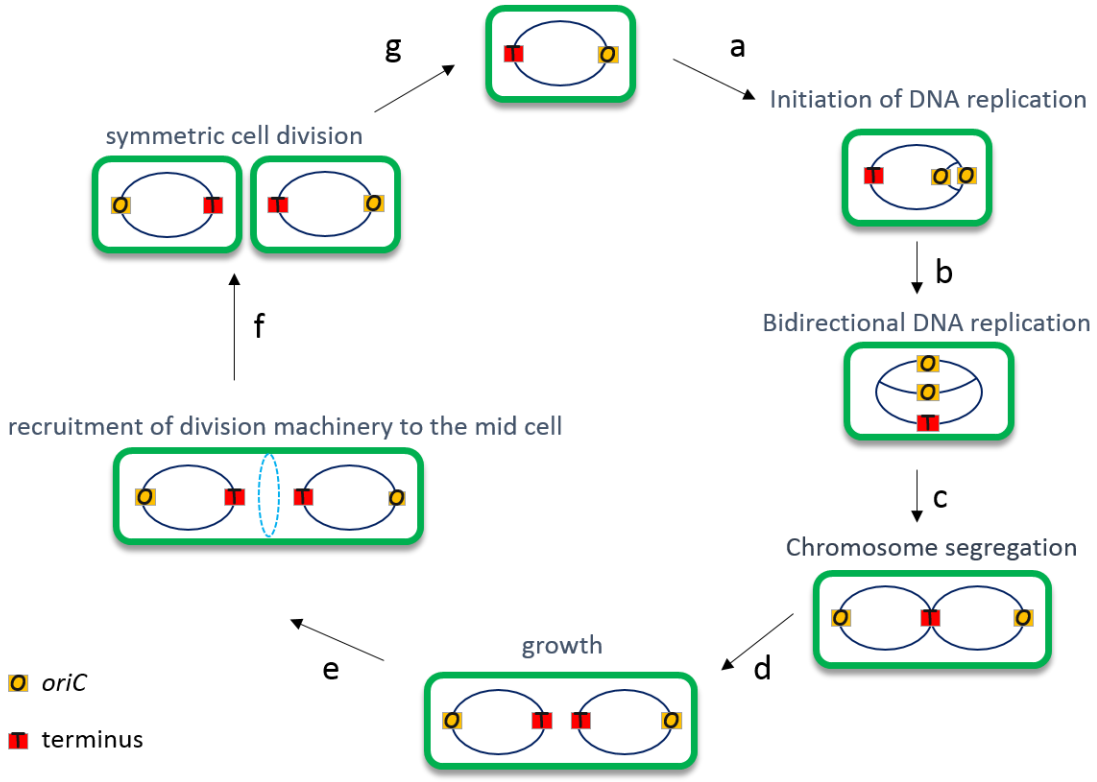


Figure 1.1.1. Simplified schematic diagram of *B. subtilis* vegetative growth cycle. It is worth noting that in *B. subtilis* cells are born with two intact copies of the chromosome however in this figure for simplisity cells are shown with only one copy of the chromosome **a)** A nascent cell initiates DNA replication from the *oriC* **b)** From the *oriC* the bidirectional DNA replication leads to duplication of both chromosomal arms (left and right arms). **c) T**he DNA replication occurs simultaneously with segregation of the newly synthesised chromosomal origins towards the cell quarter positions. **d)** Segregation of the remainder of the chromosomes follows as cells grow in size and mass. **e)** In cells that have completed chromosome segregation and doubled in size and mass, the cell division machinery is targeted to the midcell. **f)** Symmetric cell division produces daughter cells of similar size.

1.1.1 DNA replication cycle

The majority of bacteria contain a single circular chromosome with a defined origin of replication (*oriC*). In bacteria duplication of the genetic material can be divided into three steps: initiation of DNA replication, elongation and termination (Sherratt, 2003). Initiation of DNA replication requires recruitment and assembly of a highly conserved DNA replication initiator protein, DnaA (DnaA and initiation of DNA replication will be discussed in detail below). DnaA facilitates unwinding of the DNA duplex at *oriC* and recruits components of the initiation complex including the replicative DNA helicase that would be loaded on to the single stranded DNA (ssDNA) through the action of helicase loader. This is followed by expansion of the ssDNA region by the replicative helicase and loading of the DNA primase and primer synthesis at the site of unwound DNA. DNA polymerase III is then recruited to the primed sites from where the bidirectional replication of the chromosome proceeds (Mott and Berger, 2007, Katayama et al., 2010). Upon completion of chromosome duplication the replication machinery dissociates from the chromosome after encountering specific termination proteins bound near the terminus region (Bussiere and Bastia, 1999).

1.1.2 Chromosome compaction and segregation

In bacteria, DNA replication is coupled with chromosome compaction, organisation and segregation. As the sister chromosomes are synthesized they are separated and the new origin regions are segregated towards the opposite cell poles, providing a path and a destination for the remainder of the chromosomes to follow. Negative supercoiling and nucleoid associated proteins play a key role in compaction and organisation of the

chromosomes which is critical for accurate chromosome segregation (Wang et al., 2013, Sherratt, 2003, Nielsen et al., 2006).

1.1.3 Cell growth and division

Prior to division cells double in size and mass. The starting point of cell division in most bacteria involves polymerisation and assembly of a tubulin-like GTPase FtsZ into a ring-like structure (called the Z-ring) precisely at the midcell. Z-ring formation leads to recruitment of the remaining components of the cell division machinery to facilitate constriction of the Z-ring and septum biosynthesis, thus giving rise to two daughter cells of equal size (Harry et al., 2006, Romberg and Levin, 2003, Ben-Yehuda and Losick, 2002b, Peters et al., 2007).

1.2 DnaA the master initiator of DNA replication

The viability and the fitness of all organisms across the kingdoms of life is tied to accurate transmission of genetic information. Failure to control initiation of DNA replication can lead to improper cell growth, impaired chromosome segregation, and is often associated with disease or death. Hence, initiation of DNA replication and chromosome copy number is tightly regulated to ensure inheritance of genetic material by progeny. Throughout the three kingdoms of life specific initiator proteins interact with DNA and facilitate initiation of DNA replication.

ORC in eukaryotes, Orc1/Cdc6 in archaea, and DnaA in bacteria serve as the DNA replication initiator proteins. Phylogenetic analysis of eukaryotic, archaeal and bacterial replication initiator proteins has revealed a common structural motif - a AAA+ nucleotide binding domain, suggesting a common evolutionary heritage amongst the replication initiator proteins (Mott and Berger, 2007, Wigley, 2009, Kawakami and Katayama, 2010).

1.2.1 DnaA structure and function

DnaA is a multidomain protein that orchestrates DNA replication initiation from the bacterial origin of replication. DnaA is a member of the AAA+ (ATPases associated with diverse cellular activities) protein superfamily and is made up of four distinct domains (Kaguni, 2006)(Figure 1.2.1). Domain I is known to have a number of interaction partners, including replication regulators and DNA helicase (Seitzz et al., 2000; Abe et al., 2007). Domain II is thought to be a flexible linker, that may allow for nuances in regulatory control (Molt et al., 2009). Domain III harbours the AAA+ motif that binds and hydrolyses ATP,

mediates DnaA oligomerisation (Erzberger et al., 2006) and binds single stranded DNA (thus aiding duplex unwinding) (Duderstadt et al., 2011). Domain IV contains a helix-turn-helix (HTH) motif that facilitates sequence-specific recognition of DnaA-boxes (5'-TTATCCACA-3') within replication origins (Fujikawa et al., 2003, Messer, 2002).

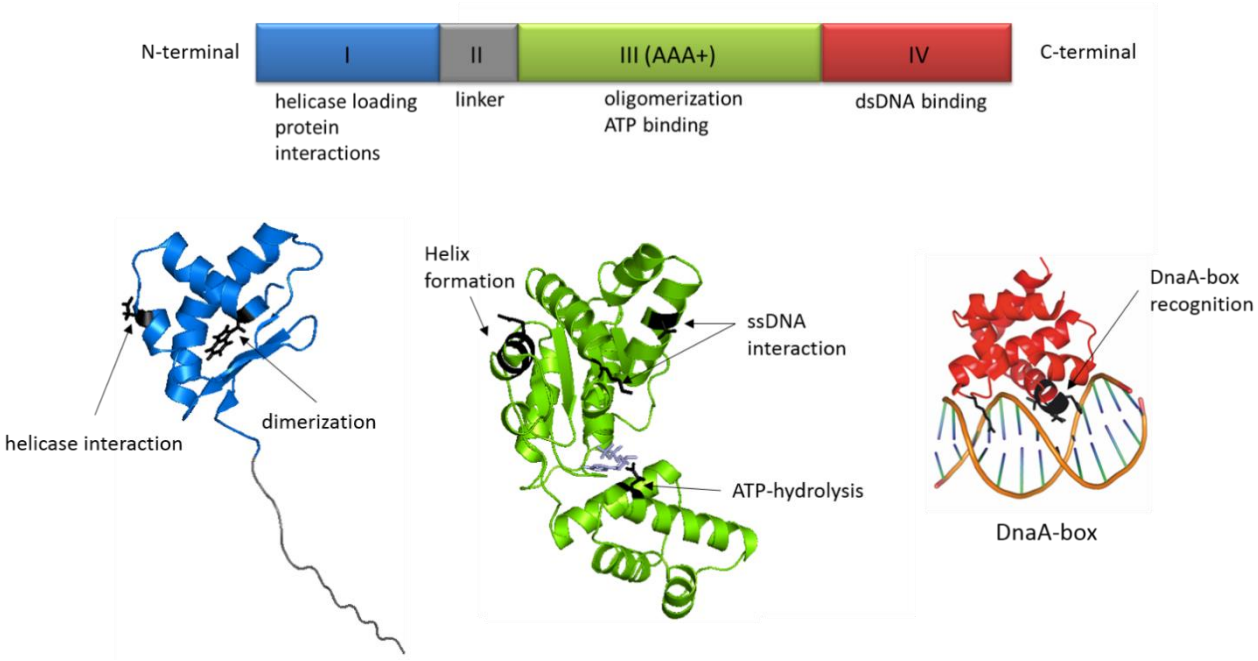


Figure 1.2.1. DnaA structural domains. A schematic representation of four structural domains of DnaA and the corresponding crystal structures are shown (PDB IDs: *E. coli* domain I – 2E0G (Abe et al., 2007), *A. aeolicus* domain III – 2HCB (Erzberger et al., 2006) and *E. coli* domain IV – 1J1V (Fujikawa et al., 2003)). Arrows highlight residues implicated in functions specifically served by each domain.

1.2.2 Initiation of DNA replication-duplex unwinding

Initiation of DNA replication in bacteria requires step-wise recruitment of DnaA molecules to the origin of replication (Figure 1.2.2B). This process ultimately leads to DnaA assembling into an active initiation complex that opens the DNA duplex to facilitate deposition of the replication machinery. DnaA and DnaA-box sequences are highly conserved throughout the bacterial kingdom. However, the *oriC* region is notably diverse amongst different species in terms of the overall number, arrangement, and orientation of DnaA-interaction motifs. Therefore, it has been proposed that cognate DnaA proteins are adjusted for optimal efficiency within different species (Messer, 2002).

Our current understanding of DnaA and its interaction with *oriC* is mainly originated from studies using the model organism *E. coli*. Here there are several sequence elements within the replication origin that are crucial for duplex unwinding by DnaA, amongst which are: DnaA-boxes, sequence motifs called 'I-sites' and the AT-rich DNA unwinding element (DUE) (Bramhill and Kornberg, 1988, Schaper and Messer, 1995, Grimwade et al., 2000, Ryan et al., 2002). DnaA exhibits the highest affinity for DnaA-boxes and it is thought to frequently occupy the DnaA-boxes that most closely resemble the consensus sequence throughout the cell cycle (Nievera et al., 2006). I-sites are sequence elements interspersed among the DnaA-boxes within the *oriC*. The nucleotide sequence of I-sites subtly deviate from DnaA-boxes and therefore DnaA exhibits a lower affinity towards them (Grimwade et al., 2000, Ryan et al., 2002). Adjacent to the array of DnaA-boxes and I-sites is the AT-rich DNA unwinding element (DUE). The DUE region consists of three 13-mer repeats called ATP-DnaA boxes. Unlike the high affinity DnaA-boxes that are bound by DnaA throughout the cell cycle, interaction of DnaA with I-sites and the ATP-DnaA boxes is highly dependent on

nucleotide binding state of DnaA (McGarry et al., 2004, Speck et al., 1999, Speck and Messer, 2001). In its ATP-bound state DnaA is cooperatively recruited onto the low affinity binding sequences to drive oligomerisation into a large nucleoprotein complex visible by electron microscopy (Funnell et al., 1987).

The initial DnaA oligomerisation at *oriC* requires domain IV interacting with double stranded DNA (dsDNA) (Figure 1.2.2B). However, the ATP-dependent oligomerisation of DnaA promotes a shift in DnaA/DNA interaction from domain IV binding double stranded DNA to domain III interacting with one strand of DNA at the DUE region (or in proximity of DUE) (Speck and Messer, 2001, Ozaki et al., 2008, Ozaki and Katayama, 2012). This is achieved by formation of a distinct ATP-dependent right-handed helical filament which promotes unwinding of the DNA duplex within the DUE region (Figure 1.2.2C). This reaction generates the single stranded substrate onto which the replicative helicases will be loaded, thereby promoting assembly of the replisomal components onto the open complex and setting DNA replication into motion (Erzberger et al., 2006, Duderstadt et al., 2011).

1.2.3 *B. subtilis oriC* structure

B. subtilis contains a bipartite origin of replication composed of a number of DnaA-boxes arranged into three clusters; *incA*, *incB* and *incC* (Moriya et al., 1988). *incAB* box clusters are located 1.4kb upstream from the *incC* region. The *dnaA* gene is positioned downstream of *incAB* (*incA* and *incB* flank the *dnaA* promoter) and upstream the *incC*. Adjacent to the *incC* region a 27mer AT-rich DUE region is found, near/at which the DNA unwinding by DnaA has been recorded (Figure 1.2.2A) (Moriya et al., 1994, Moriya et al., 1985, Krause et al., 1997). DnaA interacts with highest preference with *incB* region and to a

lower degree to *incA* (Krause et al., 1997). *In vitro* DnaA bound to the DnaA-boxes at *incA* and *incB* interact with *incC*-bound DnaA molecules leading to formation of a loop between the intervening regions (Moriya et al., 1999, Krause et al., 1997).

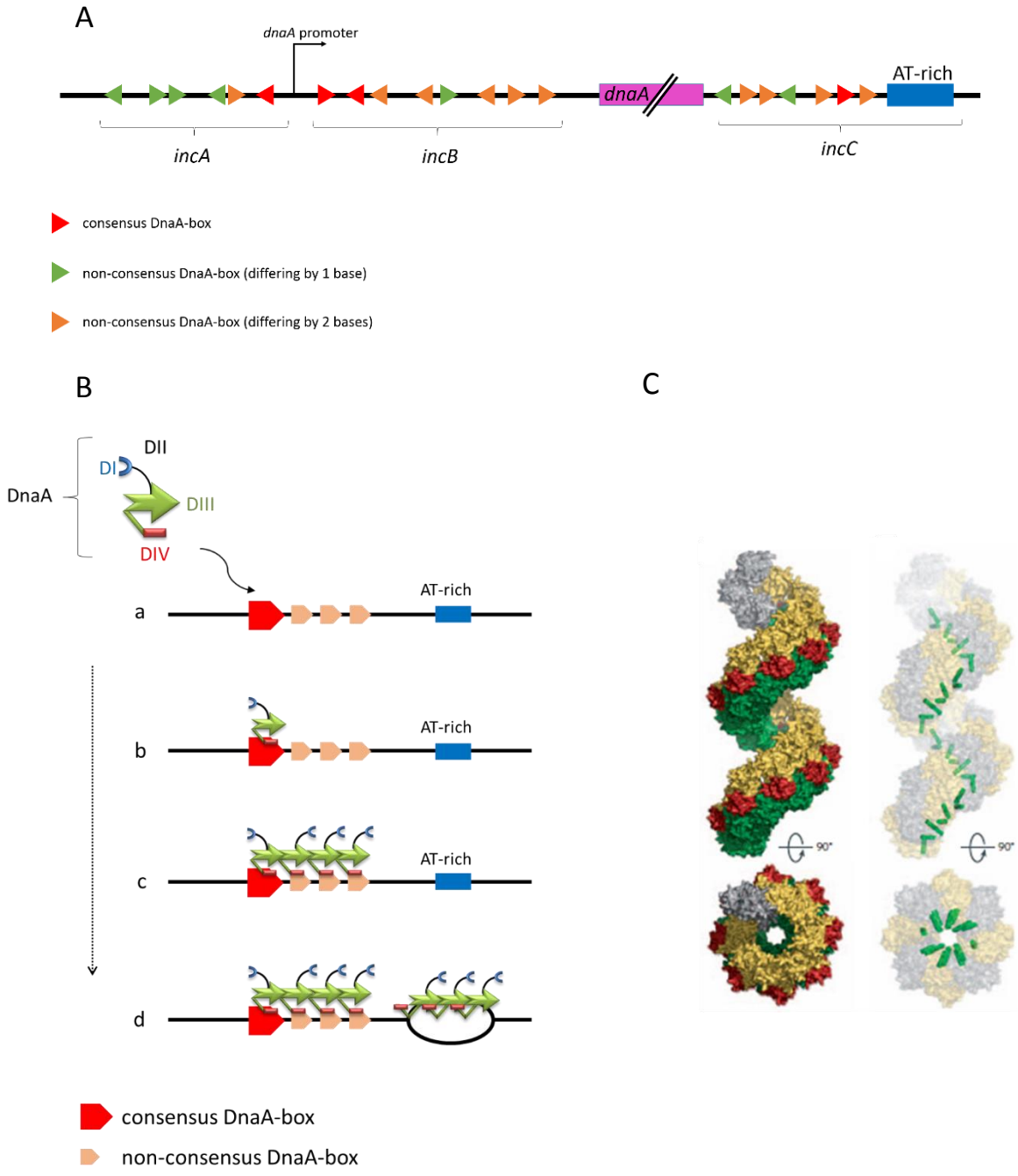

A
dnaA promoter
AT-rich
dnaA
incA
incB
incC
consensus DnaA-box
non-consensus DnaA-box (differing by 1 base)
non-consensus DnaA-box (differing by 2 bases)
B
DnaA
DI
DII
DIII
DIV
a
b
c
d
AT-rich
consensus DnaA-box
non-consensus DnaA-box
C
90°
90°

Figure 1.2.2. DnaA initiates DNA replication at *oriC*. A. Schematic diagram showing the *oriC* of *B. subtilis*. DnaA-box cluster regions of *incA*, *incB* and *incC* are highlighted. Consensus DnaA-boxes are shown as red triangles; non-consensus DnaA boxes deviating 1 base are shown as green triangles and non-consensus DnaA boxes deviating 2 bases are shown as orange triangles. Figure adapted from (Fukuoka et al., 1990).**B.** A simplified cartoon representation of DnaA oligomerisation at *oriC,* and DUE region unwinding is shown. For simplicity a consensus DnaA-box is shown in red and non-consensus DnaA-boxes are shown in orange (regardless of base deviation frequency). **a)** *oriC* contain several high affinity and low affinity DnaA-boxes and an AT-rich DUE region. **b)** DnaA binds to strong recognition sequences and acts as anchoring sites. **c)** Interaction of DnaA-ATP with the DnaA boxes leads to cooperative recruitment of additional DnaA-ATP proteins to the low affinity DnaA-boxes and oligomerisation of DnaA molecules. **d)** ATP-dependent ssDNA binding and DNA duplex unwinding. Figure adapted from Heath Murray (unpublished). **C.** DnaA-ATP right-handed helix composed of four symmetry-related DnaA tetramers is shown from side and axial views. Domain IV is shown in yellow, domain III in red and green, domain II in grey and domain I in blue (RCSB Protein Data Bank (PDP) ID 2HCB). Figure taken from(Mott and Berger, 2007).

1.2.4 DnaA directs assembly of initiation complex to the unwound DNA

Although DnaA is conserved in bacteria, many of the other initiation components are not. This is exemplified by differences in replication initiation observed between the model Gram-negative and Gram-positive organisms: *E. coli* and *B. subtilis*.

In *B. subtilis* DnaA assembly at the *oriC* is followed by sequential recruitment of two additional initiation proteins, DnaD and DnaB, which are required for deposition of the helicase loader:DNA helicase complex (DnaI:DnaC respectively) to the unwound DNA duplex. DnaD is recruited to the origin through an interaction with DnaA, moreover DnaD binding has been shown to be accompanied by pronounced bending of origin DNA. Unwinding of the duplex appears to be assisted by the formation of DnaD scaffolds, which may provide further anchorage points for DnaA (Zhang et al., 2008). DnaB is subsequently recruited, appearing to play a role in helicase loading along with the helicase loader DnaI (Velten *et al.*, 2003). DnaD and DnaB are not present in *E. coli* (Ishigo-oka *et al.*, 2001; Rokop *et al.*, 2004) and it should be noted that *B. subtilis* DnaB is unrelated to *E. coli* DnaB (helicase).

Loading of DNA helicase DnaC (DnaB in *E. coli*) to the DNA by a helicase loader DnaI (*E. coli* DnaC) leads to extension of the open complex to which the primase, DnaG is recruited (Rowen and Kornberg, 1978). This is followed by recruitment and assembly of the replisome on the unwound DNA and commencement of DNA replication (Katayama et al., 2010).

1.3 Regulation of DnaA

As stated before, failure to maintain and control chromosome copy number is frequently associated with growth defect or cell death. Regulation of DNA replication is mainly exerted at the initiation step when DnaA binds to the origin of replication.

In eukaryotes, multiple regulatory systems and checkpoints ensure that DNA replication is initiated once and only once per cell cycle (Elledge, 1996). By contrast in bacteria during rapid growth DNA replication can be reinitiated before the previous cycle of replication is complete, giving rise to multiple replication forks along each chromosome arm. Daughter cells thus inherit chromosomes that are already undergoing replication. This emphasises the need for precise mechanisms to control the frequency of initiation of DNA replication so that it matches the growth rate and nutrient availability (Murray and Koh, 2014). Regulatory mechanisms take the form of proteins and *cis*-acting DNA elements which typically act on either DnaA or *oriC.* Protein regulators vary between *genera*. Notably, *B. subtilis* and *E. coli* employ a range of replication regulators that lack known homologs in the other species (*E. coli* SeqA, Hda, *datA*; *B. subtilis* YabA, Soj, SirA, Spo0A)(Katayama et al., 2010; Briggs *et al.*, 2012), reflecting differences in their mechanisms of regulatory control.

1.3.1 DnaA regulation in *E. coli*

1.3.1.1 Negative regulation of DnaA activity by RIDA system

Regulation of DnaA in *E. coli* is mainly exerted by regulatory inactivation of DnaA (RIDA) system. RIDA system employs Hda (Katayama et al., 1998, Kato and Katayama, 2001), a protein that forms a tripartite complex with the β clamp (DNA polymerase sliding clamp processivity factor) and DnaA to stimulate ATP-hydrolysis by DnaA (therefore

generating initiation-inactive DnaA-ADP)(Nishida et al., 2002). The necessity of the DNA-loaded β clamp for activity of RIDA links the negative regulation of the DnaA function with active replication, a negative feedback system that is widely utilised by different bacterial species for inhibition replication initiation at improper time (*B. subtilis* YabA)(Kato and Katayama, 2001, Camara et al., 2003, Noirot-Gros et al., 2002).

1.3.1.2 Inhibition of DnaA interaction with the oriC *by SeqA*

In *E. coli* the origin of replication contains 11 repeats of the sequence GATC. The adenine residues of GATC sequences are methylated on both strands through the action of DNA adenine methylase (Dam)(Geier and Modrich, 1979, Campbell and Kleckner, 1990). However, after a new round of DNA replication is initiated the newly synthesized DNA strand remains transiently unmethylated, thereby generating hemi-methylated GATC sequence repeats to which SeqA preferentially binds. GATC sequences overlap with low-affinity DnaA-binding sites and when bound by SeqA prevent recruitment of DnaA-ATP, thereby inhibiting replication reinitiation in cells that have recently duplicated their origin of replication (Lu et al., 1994, Slater et al., 1995, Kang et al., 1999, Boye et al., 1996).

SeqA interaction with its binding sites at the *dnaA* gene is also thought to impose transcriptional regulation on DnaA expression. Transcription of *dnaA* is also subjected to autoregulation; DnaA interaction with DnaA-boxes that overlap the *dnaA* promoter inhibit further expression of the DnaA molecules. The importance of transcriptional regulation of *dnaA* is due to abundance of ATP in cells compared to ADP (cells contain about 10-times more ATP than ADP (Bochner and Ames, 1982)), which makes it more likely that newly synthesised DnaA molecules will bind ATP to produce initiation-competent DnaA (reviewed by Katayama 2010).

1.3.1.3 Titration of DnaA molecules away from the oriC *by* datA *locus*

DnaA molecules are sequestered away from the *oriC* through interaction with a specific chromosomal locus called *datA*. The *datA* locus is about 1 kb in size and includes five DnaA-boxes and an IHF (Integration host factor) binding site. IHF is a protein that enhances DnaA-binding to DNA by introducing bends in the DNA (IHF-binding sites are found in *E. coli oriC* and modulating interaction of DnaA molecules with the low-affinity sites). Several hundred DnaA molecules are estimated to accumulate at the *datA* locus, and deletion of *datA* leads to a modest increase in the frequency of DNA replication initiation (Kitagawa et al., 1996, Kitagawa et al., 1998, Messer and Weigel, 1997, Mott and Berger, 2007).

1.3.2 Regulation of DNA replication initiation in *B. subtilis*

1.3.2.1 DnaA also functions as a transcriptional regulator

Besides serving as the initiator protein, DnaA also serves as a transcriptional regulator for a number of genes including its own. In both *E.coli* and *B. subtilis* DnaA transcription is subjected to autoregulation in order to control availability of DnaA molecules within the cell. Genes under regulation of DnaA are involved in a variety of cellular processes (such as DNA replication, cell division, checkpoint proteins, DNA replication arrest response, DNA damage response etc.)(Messer and Weigel, 1997, Goranov et al., 2005).

The majority of genes under regulation of DnaA are involved in DNA replication arrest response. In fact, the transcriptional regulatory action of DnaA accounts for a major portion of *recA*-independent DNA replication stress response (Gon et al., 2006, Goranov et al., 2005). Like replication initiation, transcriptional regulatory function of DnaA is also

facilitated by the ATP-bound from of DnaA (Kaguni, 2006, Kurokawa et al., 2009). *B. subtilis* DnaA is thought interact with about 20 operons and directly regulate expression of more than 50 genes in response to perturbations in DNA replication. (Goranov et al., 2005). Chip-chip and Chip-PCR analysis of DnaA interaction with chromosomal regions during exponential growth of *B. subtilis* revealed 17 chromosomal loci (15 of which included genes involved in DNA replication stress response) at which DnaA interaction was enriched upon disturbation of DNA replication initiation and/or elongation. 6 of these chromosomal regions, each containing at least 9 potential DnaA interaction sites showed a more profound enrichment of DnaA molecules upon encounter of pertubutations in replication initiation or elongation (Breier and Grossman, 2009).

1.3.2.2 Regulation of DnaA by YabA

YabA was first identified in a genome-wide yeast two hybrid screen searching for factors that directly interacted with the known components of the DNA replication machinery. YabA was observed to interact with both DnaA and the DNA polymerase sliding clamp, DnaN (Noirot-Gros et al., 2002). Δ*yabA* deletion leads to increased number of chromosomes per cell while YabA overexpression leads to inhibition of DNA replication initiation, implying that YabA is a negative regulator of DNA replication initiation (Noirot-Gros et al., 2002, Noirot-Gros et al., 2006, Hayashi et al., 2005, Cho et al., 2008, Goranov et al., 2009). Localisation studies using fluorescently labelled YabA and DnaX (τ subunit of DNA polymerase) showed that YabA is bound to the replisome during DNA replication, most likely through interaction with DnaN. Moreover, it was shown that YabA mediates tethering of DnaA to the replication fork though association with DnaN (Soufo *et a l.*, 2008). These

findings collectively suggested that YabA acts, at least in part, by sequestering DnaA at the replication fork and inhibiting its interaction with the *oriC.*

Biochemical analysis of YabA using purified proteins revealed that YabA prevents ATP-dependent cooperative binding of DnaA to the *oriC* while in absence of YabA, DnaA is enriched at the *oriC* (Merrikh and Grossman, 2011). YabA interacts with domain III of DnaA but unlike Hda it does not promote ATP-hydrolysis by DnaA (Cho et al., 2008, Scholefield and Murray, 2013). In a recent study performed by Scholefield et al. using a specific assay detecting ATP-dependent DnaA oligomerisation it was reported that YabA interaction with domain III prevents oligomerisation and hence helix formation of DnaA at the *oriC* (Scholefield and Murray, 2013).

1.3.2.3 DnaD also serves as an anti-cooperativity factor for DnaA

As stated earlier (in section 1.2.4), DnaD is required for loading of replicative helicase to promote DNA replication initiation in *B. subtilis.* Intriguingly, it has been reported that DnaD also acts as a negative regulator of cooperative recruitment of DnaA to the *oriC*. Bonilla and Grossman showed that DnaD reduces the dissociation constant (K_d) of DnaA for DNA without perturbing the nucleotide-binding or hydrolysis of DnaA (Bonilla and Grossman, 2012). Recently DnaD was shown to interact with domain III of DnaA (overlapping with YabA binding determinants of DnaA) and to prevent DnaA helix assembly at the *oriC* (Scholefield and Murray, 2013).

1.3.2.4 Soj (ParA) inhibits DnaA oligomerisation at oriC

Soj is dynamic protein and a component of *B. subtilis* partitioning (Par) system (Soj and the Par system will be discussed in detail below, see section 1.4.2.2 for details). Soj can bind and hydrolyse ATP, with ATP-binding and hydrolysis triggering conformational changes in Soj (Leonard et al., 2005). ATP-bound Soj forms a dimer that stimulates DnaA-dependent initiation of DNA replication. ATP-hydrolysis promotes Soj dimer dissociation; monomeric Soj was shown to interact with domain III of DnaA and prevent DnaA helix formation at the *oriC* (Murray and Errington, 2008, Scholefield et al., 2012, Leonard et al., 2005). As well as regulating the initiation of DNA replication, the Par system also features a central role in chromosome segregation (Wang et al., 2013).

1.4 Chromosome organisation and segregation

1.4.1 Cytological organisation of bacterial chromosomes

Most bacteria contain a chromosome of 2-8 Mb in size, therefore in order for the chromosome to fit inside a bacterial cell it has to be compacted more than 1000-fold. Early studies of bacterial chromosome morphology, using electron microscopy analysis of gently lysed *E. coli* cells revealed that the bacterial chromosome is organised into a structure with interwound loops (plectonemic loops) projecting from a dense core. These studies proposed the rosette model for bacterial chromosome organisation that suggests the interwound loops of DNA are arranged into a bottlebrush-like structure by a nucleoid scaffold (Pettijohn and Hecht, 1974, Delius and Worcel, 1974, Worcel and Burgi, 1972, Kavenoff and Ryder, 1976, Kavenoff and Bowen, 1976).

Recent technical advances in the field, especially in live-cell imaging that allow tracking of multiple chromosomal loci during the bacterial cell cycle, have revealed that bacterial chromosomes are spatially organised in respect to the cells that they reside in. Cytological analysis of bacterial chromosomes have revealed two distinct organisation patterns often associated with bacterial chromosomes, "*ori-ter*" organisation and "left-*ori*-right" organisation (Figure1.4.1) (Wang et al., 2013). In the *ori-ter* organisation the origin is located towards the cell poles with the two replication arms (left arm and the right arm) lying behind it; this organisation pattern is observed in *Caulobacter crescentus* and *Vibrio cholera* chromosome I (Viollier et al., 2004, Fogel and Waldor, 2005, Fogel and Waldor, 2006). In left-*ori*-right pattern, the origin is located at midcell and the two replication arms lie in the opposite cell halves. The left-*ori*-right pattern is observed in slow growing *E.coli* (Nielsen et al., 2006, Wang et al., 2006).

Initial chromosome organisation studies conducted in *B. subtilis* reported that replication is initiated at/near midcell and that the nascent sister origins are rapidly segregated towards the nucleoid periphery, ultimately adapting a layout in which the origin regions were positioned towards the opposite cell poles and the termini localised at the midcell (*ori-ter ter-ori*) upon completion of DNA replication (Webb et al., 1997, Teleman et al., 1998). However, the timing and the mechanism of the origin movement from the poles to the midcell for initiation of DNA replication remained elusive.

It was found that the *B. subtilis* chromosome alternates between the *ori-ter* and left-*ori*-right organisation pattern depending on the stage of DNA replication and origin segregation (Figure 1.4.1) (Wang et al., 2014a). In this study, which also confirmed that *B. subtilis* maintains diploidy throughout its cell cycle, it was shown that during most of the vegetative growth cycle the chromosomes are maintained in *ori-ter* configuration with the origins positioned near the cell poles and termini at midcell leaving each left and the right replication arm pairs adjacent to each other. However, in contradiction to what was previously thought, the initiation of DNA replication occurs at this point when the origins are located near to the cell poles at the nucleoid periphery. This is followed by the movement of the newly duplicated sister origins (as a unit) to the middle of the nucleoid leading to a partial spatial resolution of the unreplicated right and the left arms on the opposite sides the each origins in left-*ori*-right pattern. The replication of the remainder of the chromosome is followed synchronously with the segregation of the origins away from each other and to the nucleoid periphery. Upon completion of the chromosome duplication the newly synthesised chromosome returns to the *ori-ter* configuration (Figure 1.4.1)(Wang et al., 2014a).

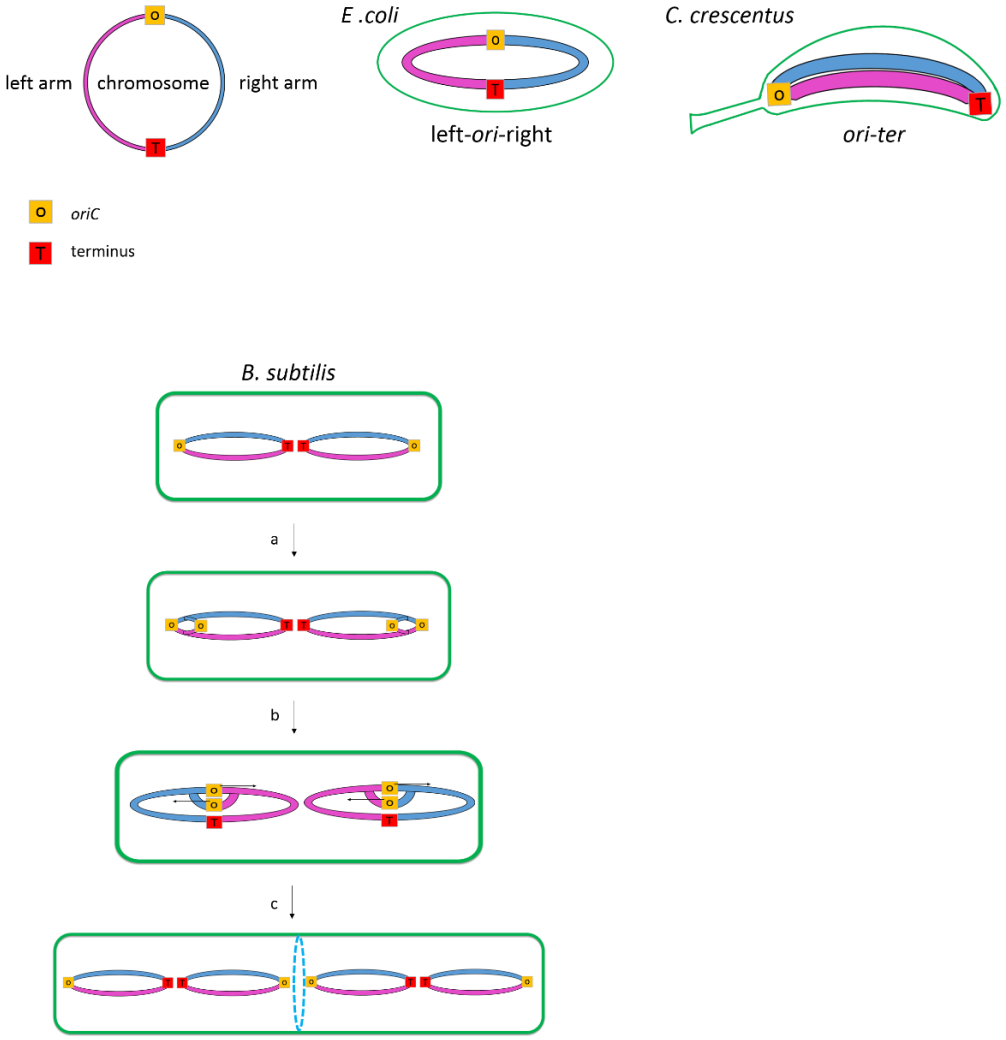


Figure 1.4.1. Schematic diagram showing left-*ori*-right and *ori-ter* chromosome organisation in *E.coli* and *C. crescentus* and oscillation between the two configurations during *B. subtilis* life cycle. a) In a new born *B. subtilis* cell the origin regions are positioned at the periphery of the nucleoid with termini at the midcell, at this point the left and the right arm of each chromosome are lined up adjacent to each other (*ori-ter ter-ori* configuration). The initiation of DNA replication takes place when the origin regions are at the nucleoid periphery. **b)** The duplicated sister origins are moved to the middle of each chromosome as a unit, each chromosome taking up the left-*ori*-right configuration, this is

followed with the elongation of the left and the right arms in synchrony with segregation of the origin regions towards the periphery of the each nucleoid. **c)** Upon completion of origin segregation the *ori-ter ter-ori* configuration is resumed. Figure adapted from Wang et al., 2014.

1.4.2 Chromosome compaction

1.4.2.1 Supercoiling and nucleoid scaffolding

The compaction of the bacterial chromosome is primarily achieved by negative supercoiling of the DNA duplex, which causes the drawing of the DNA on itself and formation of plectonemic loops by the DNA duplex. The homeostasis of DNA supercoiling is monitored and maintained by the antagonism of DNA gyrase and topoisomerases (Topo I); DNA gyrase introduces negative supercoils while Topo I relaxes them. DNA gyrase and another topoisomerase, Topo IV, are required for alleviation of positive supercoils introduced by DNA transactions that require DNA duplex unwinding such as DNA replication and transcription, and are enriched ahead of replication fork and transcription bubbles (Drlica, 1992, Luttinger, 1995, Nöllmann et al., 2007, Hsu et al., 2006, Tadesse and Graumann, 2006).

Although supercoiling is the principal mechanism of chromosome compaction it does not account for the level of chromosome condensation observed in bacterial cells. The interwound plectonemic loops of DNA are somewhat restrained by DNA-binding boundary elements (domainins) that stabilise the loops into topological domains providing a scaffold for the chromosome. Importantly, these chromosome scaffolds and domains are highly

dynamic to allow constrained mobility of chromosomal loci within the nucleoid, thus providing organisation and structure without imposing rigidity (Wang et al., 2013).

A number of elements have been identified to contribute to the chromosome compaction and organisation in bacteria, amongst which are the highly abundant small nucleoid-associated proteins (NAPs) and structural maintenance of chromosome (SMC) condensation complexes. The nucleoid-binding proteins interact specifically and non-specifically throughout the genome and facilitate DNA compaction and stabilisation of the plectonemic loops by bending the DNA and bridging chromosomal loci (Postow et al., 2004, Deng et al., 2005, Cui et al., 2008).

1.4.2.2 The structural maintenance of chromosomes condensation (SMC) complex

In all eukaryotes and most bacteria SMC complexes serve key functions in chromosome organisation and segregation. In eukaryotes SMC complexes, comprised of distinct pairs of SMC proteins, are involved in various processes of mitotic chromosome condensation, recombination, X chromosome dosage compensation, sister chromatid cohesion and DNA repair (Haering and Nasmyth, 2003, Nasmyth and Haering, 2005). Bacteria, however, only encode a single type of SMC protein that forms a homodimer in association with the kleisin family protein ScpA and a dimer of ScpB (Britton et al., 1998, Hirano and Hirano, 2004, Mascarenhas et al., 2002, Soppa et al., 2002). The bacterial SMC complex constrains and compacts the chromosome by bridging different sections of the DNA to stabilize the chromosome structure (Hirano, 2006).

In *B. subtilis* the SMC complex facilitates compaction of chromosomes and proper chromosome segregation. Deletion of genes encoding the components of the SMC complex leads to severe growth defects in rich media including temperature sensitivity, chromosome

disorganisation, and increased production of anucleated cells (Britton et al., 1998, Mascarenhas et al., 2002, Soppa et al., 2002, Jensen and Shapiro, 1999).

1.4.3 Chromosome segregation

As stated before (see section 1.1.2), in bacteria the generation of the sister chromosomes occurs concomitantly with chromosome compaction and segregation. As sister origins are replicated they are resolved and segregated towards opposite cell poles, leading the remainder of the chromosome to its destination. In *B. subtilis* the genome encoded Par proteins and the SMC complex cooperate to achieve proper chromosome organisation and chromosome segregation (Gruber and Errington, 2009, Sullivan et al., 2009, Wang et al., 2013, Wang et al., 2014a, Wang et al., 2014b).

1.4.3.1 The plasmid encoded and chromosomal Partitioning systems

The Par systems were first discovered as factors ensuring the stable inheritance of the low copy number plasmids in *E. coli* (Austin, 1983, Ogura and Hiraga, 1983, Gerdes et al., 1985). Later, orthologs of plasmid encoded *par* genes were identified as genome encoded elements in the majority of bacterial species from all branches of the phylogenetic tree (Gerdes et al., 2000, Livny et al., 2007, Austin, 1983). The genome encoded Par systems are thought to play a role in processes of nucleoid organisation, regulation of DNA replication, and chromosome segregation. The mode of action of plasmid encoded Par systems in ensuring the stable inheritance of low copy number plasmids has provided a model system to understand the role of chromosomal Par systems in chromosome organisation and segregation.

Par systems are composed of three essential elements; a partitioning sequence motif on DNA, a partitioning-site recognition/interaction protein and a nucleotide triphosphatase (NTPase). Par systems are divided into three classes based on the type of NTPase they possess; Walker type, actin-like or tubulin-like, each of which employ a distinct mechanism to segregate plasmids to the opposite cell poles. Par systems containing an actin-like ATPase or a tubulin-like GTPase utilize a filament formation mechanism by the cognitive NTPase to segregate DNA molecules (by either pushing plasmids through an extending filament formed by an actin-like ATPase or by "treadmilling-like" movement of plasmids through the tubulin-like GTPase filaments along the membrane and towards the cell poles)(Gerdes et al., 2010, Garner et al., 2007, Larsen et al., 2007).

The majority of plasmid encoded and chromosomal Par systems possess a Walker type ATPase generically termed ParA, a partitioning-site binding protein ParB and a partitioning-site DNA sequence motif *parS*. This class of Par systems seem to employ a distinct mechanism not reliant on filament formation to segregate DNA (Gerdes et al., 2010).

In vitro analysis of ParA proteins revealed that ParA forms an ATP-dependent dimer that interacts non-specifically with DNA. ParB interaction with ParA stimulates ATP-hydrolysis and dissociation from DNA. Localisation studies of ParA proteins have indicated ParB-dependent dynamic movement of ParA over the nucleoid. In a recent study investigating the mechanism of chromosome segregation by Sop system of F plasmid (composed of SopA (ParA), SopB (ParB) and *sopC* (*parS*)) using a cell free system it was shown that SopA facilitates plasmid segregation through a diffusion-ratchet mechanism (Vecchiarelli et al., 2013, Vecchiarelli et al., 2014). In this work a flow cell was coated with a

DNA-carpet to mimic the nucleoid and magnetic beads coated with *sopC*-coding DNA bound by SopB was used as a cargo plasmid. SopA (ParA) was added to the immobilised DNA and a magnetic field was applied to mimic the association of plasmid with the nucleoid. It was observed that the interaction of *sopC*- SopB coated beads with the DNA-associated SopA (resembling nucleoid associated ParA) mediated dissociation of SopA from the DNA-carpet to produce a depletion zone around the SopB-*spoC* bead that generated a concentration gradient of SopA molecules driving the movement of the bead (Vecchiarelli et al., 2013, Vecchiarelli et al., 2014).

1.4.2.2 The Partitioning system of B. subtilis

The Par system of *B. subtilis* consists of three elements: Soj (ParA), Spo0J (ParB) and *parS* (Figure 1.4.2.1A). Spo0J is a site-specific DNA-binding protein that contains a classical helix-turn-helix DNA-binding motif. Spo0J exhibits the highest affinity for *parS* sites, 16 base-pair palindromic DNA sequence elements that act as a nucleation site for Spo0J nucleoprotein complexes. The *B. subtilis* genome contains ten *parS* sites, 8 of which are clustered in the vicinity of the replication origin (Figure 1.4.2.1B). The *parS* sites most proximal to the *oriC* are most frequently bound by Spo0J (Breier and Grossman, 2007, Lin and Grossman, 1998, Murray et al., 2006). Upon interacting with a *parS* site, dimeric Spo0J spreads and bridges along the DNA into the neighbouring regions, creating a large nucleoprotein structure that extends several kilobasepairs surrounding a *parS* site (Murray et al., 2006). In a recent study (Graham et al., 2014) it was shown that a modest number of Spo0J molecules (~20 Spo0J dimers) interact with a single *parS* site and manage to form a nucleoprotein complex that spans several kilobases of DNA surrounding a *parS* site by

bridging a combination of long-range and short-range segments of DNA. This bridging activity of Spo0J could contribute to constraining of DNA loops and to defining the topological domains that organise the origin region (Graham et al., 2014).

In bacterial species clusters of *parS* sites are often found surrounding the origin of replication and are thought to provide a centromere-like function. Spo0J recruits the SMC complex to the *parS* sites at the origin of replication. Spreading of Spo0J to the non-specific flanking regions of the *parS* sites is required for enrichment of SMC complex at the *parS* sites, however, the mechanism of SMC recruitment by Spo0J is an outstanding question (Gruber and Errington, 2009, Sullivan et al., 2009).

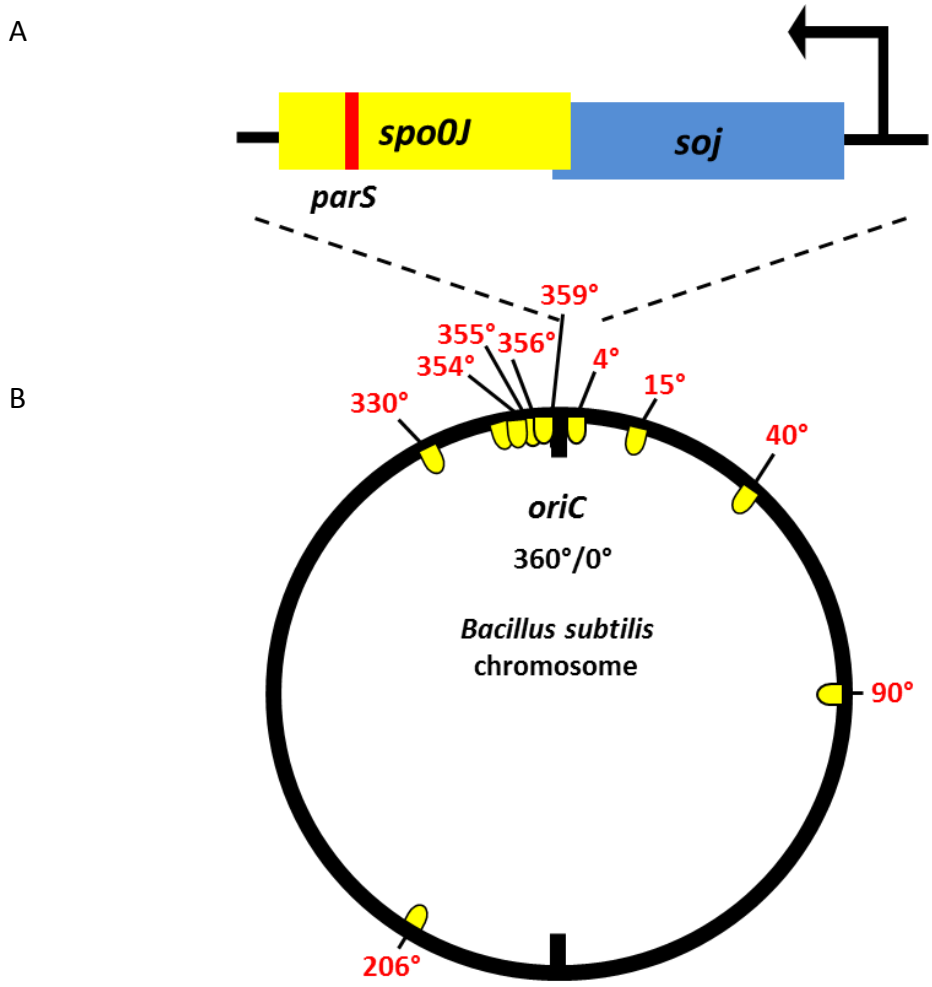


Figure 1.4.2.1. Par system of *B.subtilis.* A. *par* operon of *B. subtilis*. The *par* operon of *B. subtilis* encodes for Spo0J (ParB), Soj (ParA) and *parS*. **B.** 8 out of 10 *parS* site are clustered around the *oriC* on the *B. subtilis* chromosome. Spo0J interacts with all the *parS* sites with the closest *parS* sites to the *oriC* being most frequently bound by Spo0J. Adapted from Murray *et al.,*(2006)

Soj is a dynamic Walker-type ATPase that switches from a monomer to a dimer depending on its nucleotide bound state (Leonard et al., 2005). Monomeric Soj has been shown to act as a negative regulator of DNA replication initiation by inhibiting DnaA-helix formation. When bound to ATP, Soj forms a sandwich dimer that is capable of binding to DNA and stimulating DnaA-dependent replication initiation. Thus, the ATP-dependent conformational changes in Soj switches the activity of Soj from a negative regulator of DNA replication initiation to a positive regulator. Spo0J interacts with dimeric Soj and stimulates Soj ATP-hydrolysis activity, thereby promoting dimer dissociation and returning Soj to its monomeric conformation (Murray and Errington, 2008, Scholefield et al., 2011, Scholefield et al., 2012, Leonard et al., 2005).

Cytological studies of Soj using a GFP fusion (GFP-Soj expressed from its native locus) revealed that Soj localises at septa and as faint foci within cytoplasm (Figure 1.4.2.2). Spo0J is required for the GFP-Soj localisation, and in absence of Spo0J GFP-Soj colocalises with the nucleoid. The cytoplasmic GFP-Soj foci colocalise with the origin regions while the septal recruitment of Soj is the function of MinD protein, a component of the Min system of *B. subtilis*. In absence of MinD, GFP-Soj localisation appears as a defuse haze throughout the cell (Figure 1.4.2.2) (Murray and Errington, 2008, Autret and Errington, 2003).

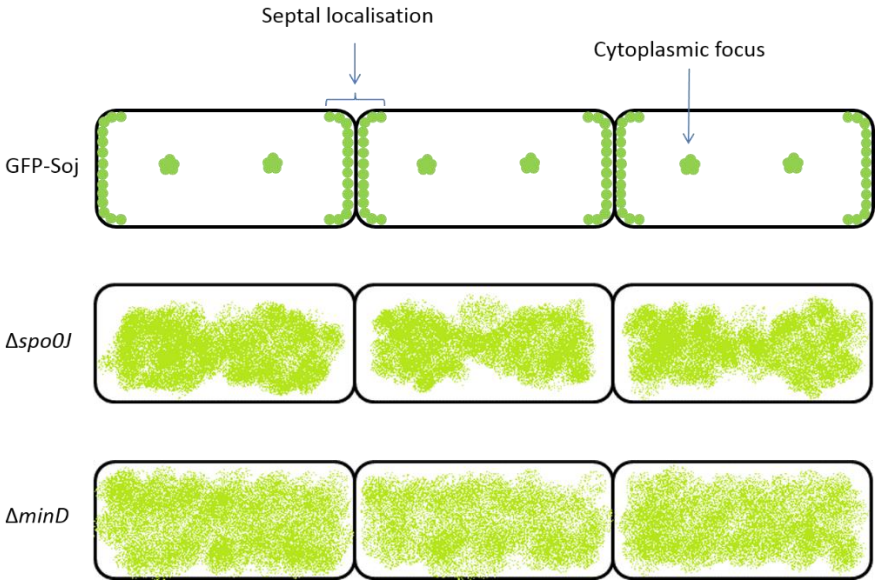


Figure 1.4.2.2. **GFP-Soj localisation in the wild-type, Δ*spo0J* and Δ*minD* mutants.** GFP-Soj localises at the septum and as punctate foci within the cytoplasm. GFP-Soj localisation is dependent on Spo0J, in the Δ*spo0J* mutant the GFP-Soj localises with the nucleoid. GFP-Soj localisation at the septum is dependent on MinD, in the Δ*minD* GFP-Soj appears defused throughout the cytoplasm. Figure adapted from Murray et al (2008).

1.4.2.3 Min system of B. subtilis

MinD is a component of the Min system, a highly conserved negative regulatory system that prevents anomalous cell division events occurring at the cell poles. In *B. subtilis* the Min system consists of MinD (a membrane-bound ATPase), MinC (an inhibitor of Z-ring assembly), DivIVA (the polar specificity factor, which differs between bacterial species), and MinJ (a bridging factor between DivIVA and MinD) (Bramkamp et al., 2008, Patrick and Kearns, 2008, Hu and Lutkenhaus, 1999, Edwards and Errington, 1997, Marston and Errington, 1999, Marston et al., 1998).

During vegetative growth, DivIVA and MinJ are targeted to the division sites and are retained at the cell poles after completion of cell division. DivIVA/MinJ sequesters MinCD complex to the cell poles, inhibiting polar cell division events that would otherwise lead to generation of small anucleated minicells (Cha and Stewart, 1997, Edwards and Errington, 1997, Marston et al., 1998). Interestingly, the Min system functions distinctively during sporulation, playing an important role in segregation of chromosomes (Thomaides et al., 2001).

1.4.2.4 Role of Soj and Spo0J in bulk chromosome segregation

Disruption of *spo0J* has a negative effect on chromosome organisation and segregation in rich media, most likely through loss of chromosome compaction in the absence of Spo0J and anomalous effects due to lack of SMC recruitment. The nucleoid positioning and the level of chromosome condensation is disrupted in about 20% of the population and there is also a 100-fold increase in the number of anucleated cells (Ireton et al., 1994, Sharpe and Errington, 1996, Autret et al., 2001).

A Δ*spo0J* mutant is also impaired in the initiation of sporulation, which can be restored by deletion of the *soj* gene. This was initially thought to be due to Soj functioning as an inhibitor for sporulation (Ireton et al., 1994). However, it was later shown by Murray et al that, in absence of Spo0J, accumulation of the Soj dimer leads to the activation of DnaA, which in turn stimulates expression of the checkpoint protein Sda (discussed in detail below see section 1.9.1). It is Sda that inhibits initiation of sporulation in cells with ongoing DNA replication (Burkholder et al., 2001, Murray and Errington, 2008, Veening et al., 2009).

Unlike most Par systems that require both ParA and ParB to stabilise the inheritance of the cognate genetic material (such as plasmid encoded Par systems and a number of chromosomal encoded Par systems including the Par systems of *C. crescentus* and *Pseudomonas aeruginosa*), no significant defects in bulk chromosome segregation are detected in a Δ*soj* mutant (Lin and Grossman, 1998, Yamaichi and Niki, 2000, Mohl and Gober, 1997, Toro et al., 2008, Vallet-Gely and Boccard, 2013). Based on these observations it was envisaged that Soj might only play a redundant role in chromosome organisation and segregation (Ireton et al., 1994, Wu and Errington, 2003). However, using more quantitative cytological analysis it was later shown that both Soj and Spo0J are required for the

separation and repositioning of newly replicated sister origins (Lee and Grossman, 2006, Wang et al., 2014a, Wang et al., 2014b). Furthermore, studies have revealed that Soj is specifically required for proper chromosomal origin segregation in sporulating cells (Sullivan et al., 2009, Wang et al., 2013, Wang et al., 2014a, Wang et al., 2014b).

1.4.2.5 *SMC complex and Par system facilitates efficient chromosome organisation and segregation*

As stated above (see section 1.4.1), *B. subtilis* chromosome organisation alternates between *ori*-*ter* and left-*ori*-right configurations depending upon the DNA replication state (Figure 1.4.1) (Wang et al., 2014a). The SMC complex and the Par system of *B. subtilis* cooperate in order to achieve the desired configuration at appropriate times during DNA replication/segregation cycle that yield optimal chromosome stability (Gruber and Errington, 2009, Sullivan et al., 2009, Wang et al., 2014a, Wang et al., 2014b).

The left-*ori*-right configuration is achieved through the action of SMC complex at the *oriC* (Wang et al., 2014a), which requires recruitment by Spo0J-*parS* nucleoprotein complexes (Gruber and Errington, 2009, Sullivan et al., 2009). Disruption of SMC complex (through inactivation SMC or ScpA or ScpB) or *spo0J* deletion renders cells unable to organise their chromosomes in the left-*ori*-right pattern (Wang et al., 2014a). SMC complex recruitment and function at *oriC* is particularly important for sister origin resolution and segregation during rapid cell growth. Furthermore, visualisation of chromosomal loci located on opposite arms of the chromosomes shows that inactivation of SMC complex leads to accumulation of chromosomes in left-*ori*-right configuration on top of each other. These findings suggest that DNA bridging and lengthwise compaction resulting from Spo0J-

dependent recruitment and enrichment of SMC complex at the origin of replication produces the driving force that moves the replicated origins away from each other.

Following SMC-dependent recruitment of duplicated origins to midcell, Soj is then required for efficient bidirectional segregation of the origins to the nucleoid periphery to re-establish the *ori-ter* configuration (Wang et al., 2014a). In the absence of Soj the migration of the origin regions towards the outer edges of the nucleoid is perturbed, with origin movement becoming slower and irregular. Even when origin segregation to the nucleoid periphery is achieved the origins were frequently found to return to the middle of the nucleoid in a Δ*soj* mutant (Wang et al., 2014a).

Chromosome segregation during the developmental pathway of spore development in *B. subtilis* has been intensively studied. Sporulation of *B. subtilis* provides a tractable system for chromosome segregation and has been long exploited for discovery and identification of bacterial chromosome segregation factors. In the work presented in this thesis, regulation of DNA replication initiation and chromosome segregation are studied during sporulation. The key players and the main events that are instrumental to spore development are described in the following section (for a detailed review of sporulation see review by Errington 2003).

1.5 Sporulation

A fascinating characteristic of the bacterial life cycle is its potential for adapting to environmental changes. Bacterial growth rate is highly dependent on nutritional availability and environmental conditions. Certain bacterial species have evolved alternative growth modes that result in highly specialised cell types capable of withstanding unfavourable conditions. Several bacterial species (such as *Bacillus subtilis*, *Clostridium botulinum, Myxococcus xanthus,* etc.) enter a sporulation pathway in response to signals such as nutrient limitation and population density. The resulting metabolically dormant and highly resistant spores can withstand harsh environments and survive for hundreds of years, resuming active metabolism upon encountering growth promoting stimuli.

The spore forming soil bacterium *Bacillus subtilis* provides a powerful model for studying cellular development and differentiation. In sporulating cells the distinct event of asymmetric cell division leading to cell type specific compartmentalisation, intercellular communication and compartment specific gene expression exemplify, in a simpler form, a number of fundamental developmental aspects of complex organisms.

Under growth promoting conditions *B. subtilis* grows vegetatively. Cell division occurs precisely at mid cell giving rise to similar sized daughter cells. However, *B. subtilis* responds to nutrient deprivation and/or cell density signals by entering an alternative growth mode allowing differentiation of the cells into highly resistant dormant spores that can withstand starvation and harsh conditions. Sporulating cells divide asymmetrically giving rise to specialised cell types (Figure 1.5.1). The smaller cellular compartment resulting from the asymmetric cell division is the prospective spore (termed the forespore) which later matures into the dormant spore. The larger compartment is referred to as the mother cell

which provides a nursery for maturation of the spore. Almost immediately after separation of the two compartments distinct genetic programs are activated in the forespore and the mother cell which stipulate their different cell fate. The forespore is then engulfed by the mother cell in a process reminiscent of eukaryotic phagocytosis. In the following hours the forespore undergoes significant morphological changes including desiccation, mineralisation and coating in multiple protective layers. The final step of sporulation is completed by lysis of the mother cell which releases the stress resistant spore into the environment (reviewed by Errington 2003).

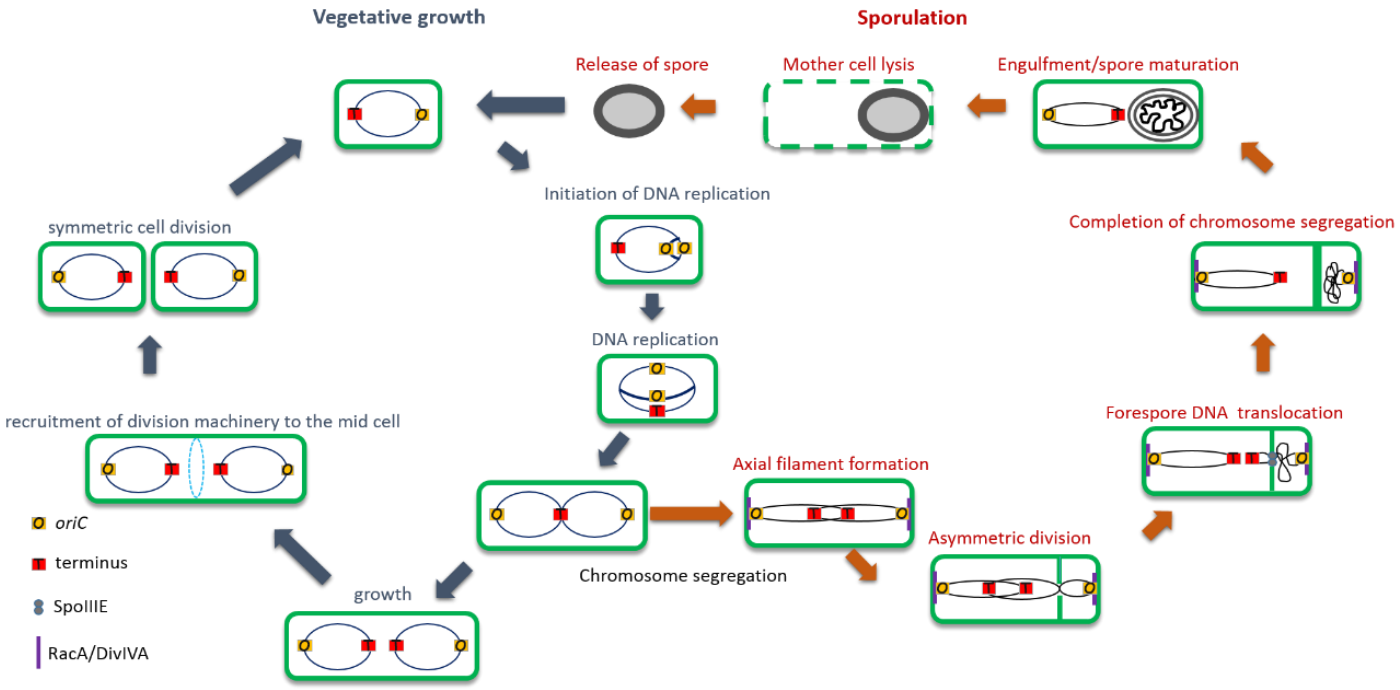


Figure 1.5.1. Simplified schematic overview of *B. subtilis* life cycle. During vegetative growth (highligted by blue arrows on left) cells divide symetrically giving rise to identical daugther cells. Nutrient deprivation and cell density signals triger spore development in *B. subtilis* (highligted by red arrows on the right). Asymetric cell division yields cellular compartments of uneven size with distinct cell fate, the smaller compartment; forespore will differentiate into a stress resistant dormant spore while the larger compartment; mother cell prepares the spore for dormancy. Figure adapted from Errington 2003, Wang et al., 2014a.

1.5.1 Initiation of sporulation

A wide range of environmental and internal signals are taken into account in an intricate decision making process before the sporulation pathway is entered. Starvation plays a prominent role in stimulating sporulation, and a high cell density is also required. This ensures that spore development is only triggered when vegetative growth is no longer favourable (Errington, 2003).

1.5.2 Spo0A the master regulator of sporulation

The entry to the sporulation pathway is a function of master developmental regulator Spo0A. Spo0A is a member of the response regulator family of transcription factors which directly or indirectly governs expression of over 500 genes. Spo0A consists of an N-terminus phosphoacceptor regulatory domain and a C-terminal DNA-binding domain (Strauch et al., 1990, Molle, 2003).

1.5.3 Spo0A activation is governed by a multi component phosphorelay

The activity and availability (expression levels) of Spo0A is governed by phosphorylation (Figure 1.5.2). Spo0A is activated by phosphorylation of a conserved aspartic acid residue at the N-terminal. The phosphorylated Spo0A (Spo0A~P) forms homodimers which stimulate DNA-binding capability in the C-terminal domain, driving both positive and negative gene regulation (Strauch et al., 1990, Molle, 2003, Muchová et al., 2004).

A complex multicomponent phosphorelay consisting of five histidine autokinases (KinA, KinB, KinC, KinD and KinE), six phosphatases (RapA, RapB, RapE, Spo0E, YnzD and YisI) and two phosphorelay proteins (Spo0F and Spo0B) govern the activity of the Spo0A (Burbulys et al., 1991, Jiang et al., 2000, Perego and Hoch, 1991)(Figure 1.5.2).

For sporulation to commence a threshold level of Spo0A~P is required. Multiple positive and negative signals are integrated through the opposing action of the kinases and phosphatases into the phosphorelay to ensure the accuracy of signal interpretation and eventually entry into the costly developmental pathway of sporulation.

1.5.4 KinA and KinB are required for initiation of sporulation

There are several kinases in the phosphorelay and there is evidence that each might respond to distinct stimuli and serve different purposes, though the biochemical signals that promotes autophosphorylation of the kinases remain unresolved (Jiang et al., 2000). KinA and KinB are the major kinases in the phosphorelay, accounting for all sporulation observed under laboratory conditions. Absence of KinA leads to reduction of sporulation frequency to 5% of the wild-type value, suggesting that KinA is the most active kinase in the phosphorelay (Perego et al., 1989). Disruption of both KinA and KinB abolish sporulation completely (Trach and Hoch, 1993).

In the absence of KinA and KinB the threshold levels of Spo0A~P required for initiation of sporulation is not achieved, though low levels of Spo0A~P are detected (Trach and Hoch, 1993). KinC, KinD and KinE appear to be responsible for activation of Spo0A to low levels which allows regulation of genes under control of Spo0A throughout the cell cycle (such as *abrB*, a global suppressor that prevents expression of genes facilitating transition into the sporulation pathway in vegetatively growing cells) without triggering sporulation (Jiang et al., 1999, Jiang et al., 2000).

1.5.5 The phosphorelay is subjected to regulation by phosphatases

Upon receiving positive signals for triggering sporulation KinA and KinB phosphorylate Spo0F. Spo0F~P transfers the phosphoryl group to Spo0B, which in turn

phosphorylates Spo0A, promoting accumulation of Spo0A~P molecules (Burbulys et al., 1991, Strauch and Hoch, 1993). The phosphoryl group carriers and phosphotransferase reactions in the phosphorelay are subjected to regulation by phosphatases. In response to signals contrary to sporulation (such as competence development or vegetative growth conditions), phosphotases impact the outcome of phosphorelay by directly dephosphorylating Spo0A~P or targeting the intermediate protein Spo0F~P (Figure 1.5.2)(Perego and Hoch, 2002).

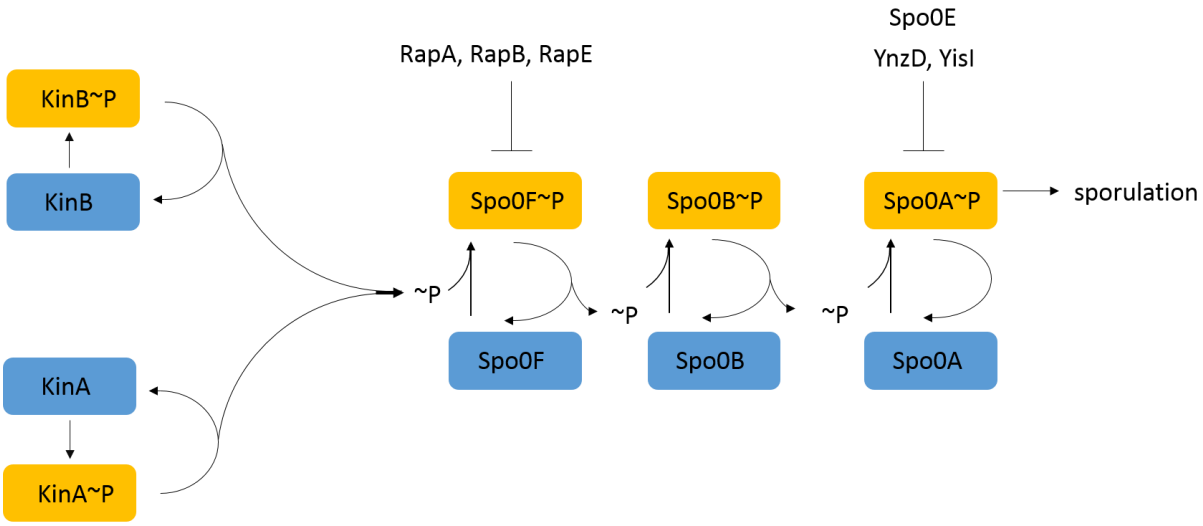


Figure 1.5.2. *B.subtilis* phosphorelay. Simplified representation of *B. subtilis* phosphorelay. KinA and to a lesser extent KinB autophosphorylation feed phosphoryl groups into the phosphorelay, the intermediate phosphorelay proteins Spo0F and then Spo0B receive and transfer the phosphoryl group consecutively leading to phosphorylation of Spo0A. The phosphorelay is subjected to regulation by phosphatases that negatively regulate activation of Spo0A. Adapted from de Jong et al., 2010.

1.5.6 Spo0A~P stimulates its own accumulation

Spo0A~P stimulates accumulation of additional Spo0A~P molecules through a number of positive feedback systems that promotes both transcriptional upregulation and phosphorylation of Spo0A. As well as interacting with its own promoter and activating further transcription of *spo0A*, Spo0A~P also represses expression of factors involved in its transcriptional repression such as *abrB* (Strauch et al., 1992, Perego et al., 1988). Repression of *abrB* relieves the repression of genes under regulation of AbrB, one of which is the *sigH* gene that encodes for a stationary sigma factor σ^H (Weir et al., 1991, Strauch, 1995, Fujita and Sadaie, 1998). σ^H promotes activation of Spo0A by directly inducing transcription of phophorelay genes *kinA* and *spo0F* and it also inducing expression of *spo0A* (Banse et al., 2008).

1.6 Induction of sporulation by Spo0A~P

1.6.1 Forespore chromosome segregation and symmetric cell division

Upon activation, Spo0A~P binds to specific DNA sequence elements with a consensus of 5'-TGTCGAA-3' called Spo0A-boxes (0A-boxes) and induces transcription of multiple sporulation specific genes required for setting the sporulation pathway into motion (Strauch et al., 1990, Molle, 2003).

Spore development proceeds in a stepwise manner (Figure 1.5.1); cells that have completed duplication of the genome and have two intact copies of the chromosomes segregate the origin regions towards the opposite cell poles, where the origins are anchored through interaction with the sporulation specific polar protein RacA. This promotes formation of a specific nucleoid structure extending from pole to pole with the termini in the middle (Ryter and Schaeffer, 1966, Wu and Errington, 2003, Ben-Yehuda et al., 2003). At this point the cell division machinery is redirected to a position near the cell poles through the action of a Spo0A induced protein SpoIIE. SpoIIE is synthesised shortly after initiation of sporulation. Subcellular localisation studies of SpoIIE protein show that before asymmetric septation SpoIIE is targeted to sites near the cell poles where it sequentially forms two bands in a bipolar manner, corresponding to circumferential rings of protein (Wu et al., 1998). This coincides with the assembly of two Z-rings near the two cell poles, only one of which proceeds to form a septum. In the study conducted by Wu et al (1998) it was shown that the asymmetric septa is formed near the pole at which the SpoIIE band first appears, dividing the cell into two cellular compartments of uneven size (Feucht et al., 1996, Ben-Yehuda and Losick, 2002a, Barák et al., 1996).

Formation of the asymmetric septa over the axial filament leads to capturing of about a quarter of one chromosome, centred around *oriC,* in the forespore compartment, leaving the remainder of the chromosome in the mother cell (Wu and Errington, 1994a, Sullivan et al., 2009). The origin-distal region of the chromosome destined for the forespore is later transported through the asymmetric septa by a DNA traslocase called SpoIIIE (chromosome segregation in sporulating cell will be discussed in more details below)(Wu and Errington, 1994b).

1.6.1.1 Chromosome translocation by SpoIIIE

As stated above the remainder of the forespore chromosome that is transiently positioned in the mother cell is translocated through the asymteric septa by action of SpoIIIE DNA translocase. In addition to its role in sporulating cells SpoIIIE is also thought to facilitate clearing of the DNA from the site of cell division jence playing a role in post-septational partitioning of chromosomes (Sharpe and Errington, 1995, Britton and Grossman, 1999, Lemon et al., 2001, Kaimer et al., 2011, Fiche et al., 2013). Homolouges of SpoIIIE are found throughout bacterial kingdom (Iyer et al., 2004). FtsK DNA translocase of *E. coli* is closely related to SpoIIIE. Like SpoIIIE, FtsK is also directed to the (symmetric) division site (septum) and facilitate chromosome dimer resolution (with 78% homology of their ATPase domain)(Bigot et al., 2007, Barre, 2007).

In the absence of a functional SpoIIIE (see section 1.10), spore chromosome segregation and therefore spore development is blocked (Wu and Errington, 1994b). SpoIIIE is targeted to the nascent division septum through its N-terminal domain that associates with membrane. Mutations at the N-terminus can lead to delocalisation of the protein from

the membrane and therefore block of DNA translocation. Interestingly, the delocalisation of SpoIIIE from the asymmetric septa (due to the mutation at the N-terminal) in sporulating cells disrupts confinement of σ^F activation to the forespore compartment. This suggests that SpoIIIE localisation at the asymentric septa might also provide a seal that selectively blocks intercompartmental transactions (Wu and Errington, 1997). The cytoplasmic C-terminal domain of SpoIIIE contains a DNA–dependent ATPase with capability of tracking on DNA in an ATP-dependant manner (Wu et al., 1995, Bath et al., 2000).

Using high resolusion microscopy techniques (photo activation localisation microscopy) it has been shown that SpoIIIE molecules are preferentially recruited to the site of future asymmetric septation prior to the cell division. From this point the SpoIIIE molecules are moved along with the leading edge of the constricting septa and gathered at the centre of the asymmetric septa. The SpoIIIE molecules are mostly assembled into hexamers that form complexes made up of 47 ± 20 molecules (Fiche et al., 2013). A number of models have been proposed for the action of SpoIIIE at the asymmetric septum (including SpoIIIE forming paired DNA translocating channels across the fused membrane) however the most recent data supports a model in which SpoIIIE assembles into complexes and forms an aquous pore at the asymmetric septum, stabilised by the division machinery, acting as a factor driving and ensuring translocation of DNA across the asymmetric septum before its fusion (Fiche et al., 2013).

1.6.2 Compartmentalisation triggers compartment specific genetic program

1.6.2.1 Compartment specific activation of σ^E and σ^F

Asymmetric cell division generates a transient genetic asymmetry (due to the absence of genetic material from the forespore) that plays a key role in activation of compartment specific genetic programs instrumental to cell type differentiation and spore maturation. Shortly after the separation of the two cellular compartments the first forespore specific sigma factor, σ^F, is activated, which in turn directs a signal across the septum to activate the first mother cell specific sigma factor σ^E (Figure 1.6.1) . Both σ^F and σ^E are synthesised prior to asymmetric cell division but are kept in an inactive state until the asymmetric septa is formed. Two distinct regulatory systems function together to ensure that σ^F activation only occurs in the forespore compartment. One of these systems includes a pathway of three proteins: SpoIIAB, SpoIIAA and SpoIIE.

SpoIIAB is an anti-sigma factor with protein kinase activity which directly interacts with σ^F, inhibiting its interaction with the RNA polymerase. SpoIIAA is an anti-anti-sigma factor with capability of interacting with the SpoIIAB-σ^F complex and the releasing of σ^F from SpoIIAB. Prior to asymmetric septa formation SpoIIAA is also held in an inactive state by SpoIIAB protein kinase activity phosphorylating SpoIIAA on a specific serine residue (Min et al., 1993, Diederich et al., 1994, Najafi et al., 1995, Magnin et al., 1997). As well as repositioning of the division machinery to a polar position, SpoIIE also has phosphoserine phosphatase activity. Upon asymmetric septa formation SpoIIE is sequestered to the forespore side of the asymmetric septa. This leads to localised activation of the SpoIIAA in the forespore compartment, which can then interact with SpoIIAB-σ^F complex and release/activate σ^F in the forespore.

The other mechanism by which σ^F activation is confined to the forespore is through SpoIIAB protein instability and the location of *spoIIAB* gene on the chromosome. SpoIIAB is subjected to proteolytic degradation in absence of σ^F or SpoIIAA. The *spoIIAB* gene is located distal to *oriC* on the circular chromosome which leads to the exclusion of *spoIIAB* from the forespore at the time of asymmetric septation. Consequently, in the period of time between the asymmetric septation and the translocation of the *spoIIAB* gene, SpoIIAB levels drop in the forespore to promote activation of the σ^F.

Following activation of σ^F in the forespore, σ^E is activated in the mother cell. The sequential activation of σ^F and σ^E is coupled through dependence of σ^E activity on a protein induced in the forespore by σ^F. σ^E is synthesised as a larger pre-protein, pro-σ^E in the pre-divisional cell and requires proteolytic cleavage by the membrane bound enzyme SpoIIGA for activation. However SpoIIGA activity itself is regulated by SpoIIR, a σ^F-induced protein that is synthesised in the forespore and secreted across the asymmetric septum in order to induce enzymatic activity in SpoIIGA, which is required for proteolytic processing and activation of σ^E in the mother cell (Karow et al., 1995, Londoño-Vallejo and Stragier, 1995).

1.6.2.2 Regulation of spore development

The timely and compartment specific activation of σ^F and σ^E remains a vital step for progress into the downstream events that determine proper spore development and cell fate. σ^F and σ^E are later replaced with σ^G and σ^K respectively whose expression and activation is dependent upon σ^F and σ^E.

Failure to activate either of σ^F and σ^E induces the mother cell to undergo a second aberrant asymmetric septation event that gives rise to anomalous disporic cells. As a consequence of the assembly of second asymmetric septa the mother cell chromosome is transferred into the second forespore, leaving the central compartment devoid of a

chromosome. However, accurate activation of σ^F and σ^E fixes the fate of the mother cell by preventing further asymmetric division to take place. This is achieved by expression of a number of σ^E-induced negative regulators of asymmetric cell division in the mother cell (such as SpoIID, SpoIIM and SpoIIP(Pogliano, 1999))(Lewis et al., 1996).

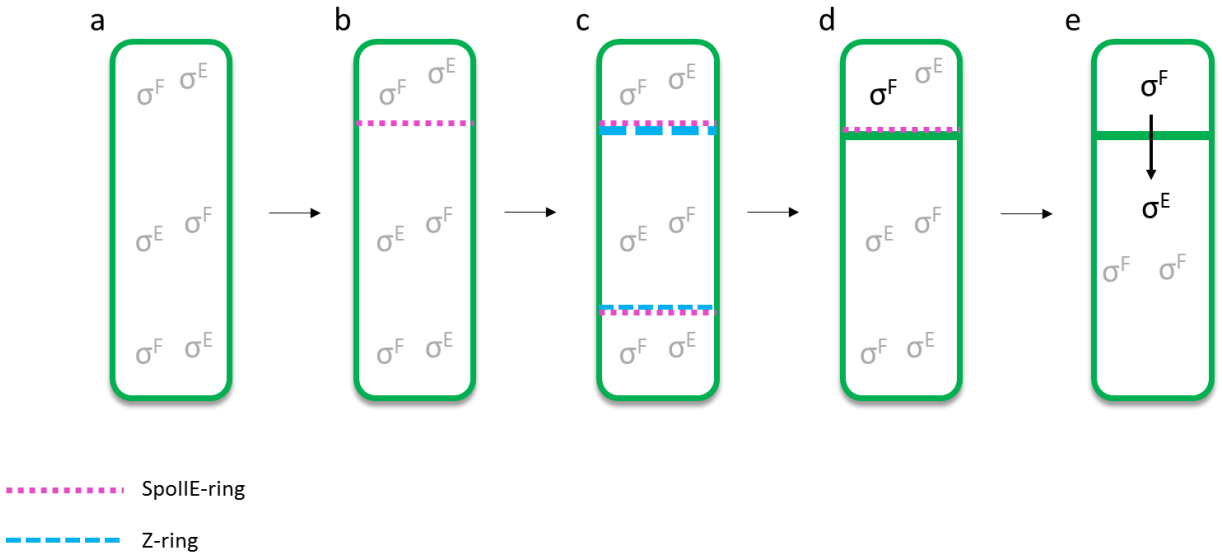


Figure 1.6.1. Asymmetric cell division and compartment specific σ-factor activation.

(a) Both σ^E and σ^F are produced and held inactive (showed in grey) in the pre-divisional cell. (b) and (c) Two SpoIIE-rings (shown as pink dotted line) are sequentially formed at a position near the cell poles through direct interaction with FtsZ that is assembled into two Z-rings (shown with blue dotted line) at the cell poles proximity (d) Only one Z-ring proceeds to form a septum. Asymmetric septation causes enrichment of SpoIIE in the forespore, this ultimately leads to activation of σ^F in the forespore (through activation SpoIIAA that releases/activates σ^F from SpoIIAB-σ^F complex). (e) Activation of σ^F in the forespores then activates σ^E in the mother cell. Figure adapted from Errington 2003.

1.7 Forespore engulfment in a phagocytosis-like process

One of the hallmarks of spore development is the engulfment of the forespore by the mother cell in a phagocytic-like process (1.7.1). This involves degradation of the cell wall material in the septum and migration of the mother cell septal membrane around the forespore, until the mother cell membranes meet and fuse together at the apex of the cell, enclosing the forespore and allowing its maturation to be completed. Several proteins are involved in driving the process of forespore engulfment including SpoIIB, SpoIID, SpoIIM, SpoIIP and SpoIIIE (Abanes-De Mello et al., 2002). SpoIID, SpoIIM and SpoIIP play a role in both degradation of wall material (this manifests as septal thinning when examined by electron microscopy) and membrane migration. The degradation of wall material is initiated at the centre of the septum and proceeds towards the edges and is thought to be catalysed by SpoIIB. SpoIIB is not essential for engulfment, although absence of SpoIIB causes a severe delay in the initiation of engulfment (Margolis et al., 1993).

SpoIID, SpoIIM and SpoIIP are initially recruited to the centre of the septum from where they spread throughout the septum, this is followed first by enrichment at, followed by movement along, the leading edge of the engulfing membrane as the forespore is enclosed within the mother cell membrane (Abanes-De Mello et al., 2002). Biochemical analysis of SpoIID revealed that SpoIID is a peptidoglycan hydrolase. This suggest that membrane migration observed during the engulfment may be driven by the activity of membrane-bound cell wall hydrolases that drag the membrane with them as they degrade peptidoglycan. For the engulfment to be completed, the migrating membranes that meet at the apex of the cell are fused together through the action of SpoIIIE.

Completion of engulfment triggers activation of σ^G in the forespore and σ^K in the mother cell which induce expression of genes involved in the final steps of the spore maturation. At this stage the forespore undergoes huge morphological changes that transform the forespore into a dormant spore that can endure a wide range of harsh environmental insults (Perez et al., 2000).

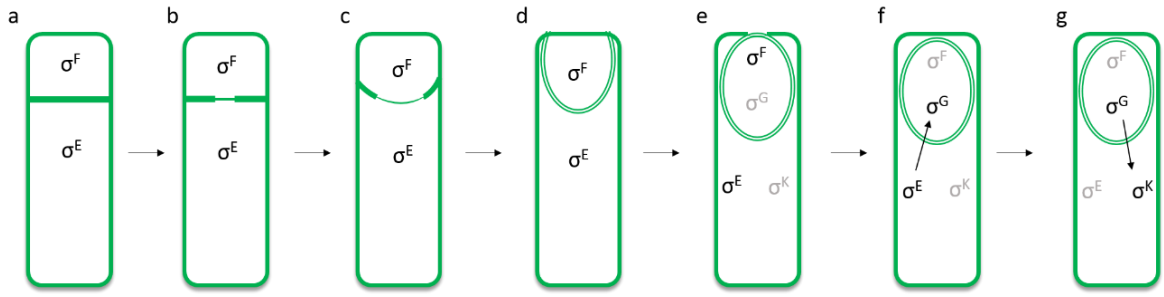


Figure 1.7.1. Engulfment of forespore by the mother cell in a phagocytic like process.

(b),(c) and (d) The engulfment of forespore involves degregation of cell wall material starting at the centre of forespore septum, SpoIID, SpoIIM and SpoIIP facilitate both degradation of wall material and membrane migration wich is catalysed by SpoIIB (e) Migrating membrane fuses together at the apex of the cell through action of SpoIIIE. (f) Completion of engulfment triger activation of σ^G in the forespore by σ^E. (g) σ^G then activates σ^K in the mother cell. Figure adapted from Errington 2003.

1.8 Post engulfment maturation

Low-molecular weight proteins are synthesised in abundance and are used for coating of prospective spore DNA, conferring protection against DNA damage as well as providing a source of amino acids that can be used at the time of germination. The prospective spore is dehydrated and mineralised by uptake of a large quantity of dipicolinic acid and divalent cations (usually Ca^{2+}) produced in the mother cell. A modified cell wall is synthesised over the spore membrane that forms the cortex, which is further covered by multiple layers of proteinous coating. When the spore maturation is completed the mother cell is lysed, releasing the dormant spore into the environment so that it can germinate and resume vegetative growth in response to germination signals (Figure 1.5.1) (see reviews by Stragier and Losick 1996, Errington 2003).

1.9 Regulation of DNA replication initiation in sporulating cells

As stated above (in section 1.6.1), upon reception of appropriate signals sporulation is triggered in cells that have completed duplication of their genome. At least two intact copies of the chromosome is required for spore development. There are several mechanisms in place to ensure the accurate onset and timely completion of DNA replication so that a diploid chromosome copy number is achieved in sporulating cells (Murray and Errington, 2008, Eldar et al., 2009, Veening et al., 2009, Xenopoulos and Piggot, 2011, Boonstra et al., 2013).

To ensure that sporulation is not triggered in cells that have not yet completed genome duplication, the Spo0A activating kinases (KinA and KinB) are negatively regulated through action of the Sda checkpoint (Rowland et al., 2004, Burkholder et al., 2001, Whitten

et al., 2007, Cunningham and Burkholder, 2008, Veening et al., 2009). Moreover, to prevent reinitiation of DNA replication in sporulating cells that have duplicated their genome, Spo0A antagonises *oriC* unwinding and induces expression of a developmental inhibitor of DNA replication initiation SirA (Rahn-Lee et al., 2009). Almost all of the systems involved in monitoring the chromosome copy number in sporulating cells regulate DNA replication at the initiation step. This is achieved through controlling availability and activity of the DNA replication initiator protein DnaA.

1.9.1 DnaA- Transcriptional regulation of Sda

It has long been appreciated that initiation of sporulation is limited to a short window of time during cell cycle. Starved cells cannot initiate sporulation without going through another cell cycle if they have recently initiated a round of DNA replication (Hauser and Errington, 1995). This has been shown to be due to action of Sda protein whose genetic regulation and biochemical properties provides a regulatory system that coordinates DNA replication and sporulation. Sda is an inhibitor of the sporulation sensor kinases, KinA and KinB. Sda thus serves to delay sporulation by limiting the phosphorylation of the master sporulation response regulator, Spo0A. The replication competent form of DnaA activates expression of the *sda* gene, causing Sda to be expressed in a pulsatile manner (with a burst of expression at the initiation of DNA replication). Sda is an intrinsically unstable protein that is degraded by the Clp protease, therefore its levels fluctuate with the cell cycle reaching a minimum immediately prior to the initiation of a new round of DNA replication (Burkholder *et al.*, 2001; Veening *et al.*, 2009). This creates a small 'window of opportunity' for a threshold concentration of Spo0A~P to be achieved and for sporulation to commence.

In addition to a number of DnaA-boxes, the *sda* promoter region contains two Spo0A-boxes (as well as a DNA damage protein LexA binding site) (Goranov et al., 2005, Burkholder et al., 2001, Ishikawa et al., 2007). Intriguingly, Spo0A~P was shown to directly activate expression of Sda. Although the modulation of an inhibitor of sporulation (Sda) by the global activator of sporulation (Spo0A) might seem puzzling, this adds another layer of regulation by inhibiting overabundance of Spo0A~P and triggering sporulation at unfavourable times (Veening et al., 2009).

1.9.2 Negative regulation of *oriC* by Spo0A

As stated before (see sections 1.5.2 and 1.6.1), Spo0A is a global regulator of sporulation genes that specifically recognizes a sequence motif termed a 0A-box. In addition Spo0A~P binds directly to multiple OA-boxes within *oriC*. The 0A-box consensus sequence (5'-TGTCGAA-3') shares homology with a complementary strand of a perfect DnaA-box (5'-TGTGGATAA-3'), therefore Spo0A~P is capable of interacting with 0A-boxes that overlap DnaA-boxes. DNase I footprinting assays have confirmed that regions within the *oriC* coinciding with DnaA-binding sites can be bound by Spo0A~P. The length of this protected region was increased in correlation with levels of Spo0A~P. Within this region three 0A-boxes (in the *incB* region of the *oriC*) were identified to overlap with three functional DnaA-binding sites. Upon activation of Spo0A, Spo0A~P is recruited to the 0A-boxes that overlap with DnaA-boxes preventing DnaA recruitment to these sites and preventing DnaA-mediated open complex formation (Castilla-Llorente et al., 2006, Boonstra et al., 2013).

1.9.3 SirA inhibits reinitiation of DNA replication in sporulating cells

SirA is a developmental inhibitor of DnaA that is induced by Spo0A~P at the early stages of sporulation. SirA was first identified as a factor responsible for regulation of

chromosome copy number in sporulating cells. Analysis of chromosomal content in vegetatively growing cells that were artificially induced to sporulate (by induction of KinA under fast growth conditions), revealed that in the absence of SirA cells were incapable of reducing their chromosome copy number; while induction of sporulation led to a drop in chromosome copy number from 4 to 2 in the wild-type, the number of chromosomes remained the same (4 chromosomes per sporulating cell) in the Δ*sirA* mutant. This implies that SirA plays a role in regulation of chromosome copy number in sporulating cells (Rahn-Lee et al., 2009). Evidence for SirA functioning as a negative regulator of DnaA was obtained in a study conducted by Wagner and colleagues. They reported that SirA overexpression in vegetatively growing cells results in disruption of DNA replication at the initiation step causing growth inhibition. However, cells that were replicating independently of DnaA (*oriN* strain) are immune to the lethal effects of SirA overexpression. Furthermore, the SirA binding determinants of DnaA have been mapped to its N-terminal domain, $DnaA^{DI}$ (Rahn-Lee *et al.*, 2011).

Curiously, disruption of SirA does not have a significant effect on the overall process of sporulation. The sporulation frequency of the Δ*sirA* mutant is similar to wild-type and apart from a mild increase in the number of chromosomes per cell and a rare formation of disporic cells, no significant morphological abnormalities are observed in the Δ*sirA* mutant. However, when combined with an *sda* deletion, the resulting Δ*sirA* Δ*sda* double mutant is significantly defective in regulation of chromosome copy number and also exhibits an increased frequency of disporic cells (Veening *et al.*,2009).

Although SirA is a sporulation specific regulator of DnaA, the majority of our knowledge on its function is obtained from experiments performed under growth

promoting conditions (such as fast growth and in rich medium that does not support sporulation) when both sporulation and SirA expression are artificially induced (Rahn-Lee et al., 2009, Wagner et al., 2009, Rahn-Lee et al., 2011). Therefore, little is known about SirA function and the mechanism of its action under biologically relevant sporulation promoting conditions such as nutrient deprivation.

1.9.3.1 Structural homologs of SirA the sole domain I interacting regulator of DnaA in B. subtilis

SirA is the only regulator of DnaA in *B. subtilis* that so far has been identified to interact with domain I of DnaA. However, a number of DnaA regulators have been discovered to interact with $DnaA^{DI}$ in other bacterial species.

Recently the crystal structure of SirA was solved in a complex with the domain I of DnaA (Jameson et al., 2014), providing the first structure of a DnaA domain in an inhibitory complex. Structural characterisation of SirA identified two structural orthologs for SirA; HobA of *Helicobacter pylori* and DiaA of *E. coli*, both of which interact with $DnaA^{DI}$ but share no sequence homology with SirA. Intriguingly, unlike SirA, HobA and DiaA are both activators of DNA replication initiation (Natrajan et al., 2009, Zawilak-Pawlik et al., 2011, Jameson et al., 2014).

HobA is an essential regulator of DnaA in *H. pylori* that is required for DnaA oligomerisation at the *oriC* and therefore initiation of DNA replication. Biochemical analysis of HobA-DnaA complex revealed that HobA forms a tetramer that interacts with four DnaA molecules (through domain I), providing support for stabilising DnaA oligomers formed at *oriC* (Zawilak-Pawlik et al., 2011). The co-crystal structure of SirA-$DnaA^{DI}$ revealed that SirA

interacts with the same structural site of DnaA that HobA interacts with, burying equivalent surface residues (Jameson et al., 2014).

DiaA promotes initiation of DNA replication in *E. coli* by stimulating assembly of DnaA-ATP molecules at the low affinity DnaA-interaction sites. Like HobA, DiaA also forms a tetramer simultaneously interacting with multiple DnaA molecules that aids the cooperative binding of DnaA at *oriC* (Keyamura et al., 2009).

In Chapter 3, using the information obtained from the first crystal structure of SirA in a complex with domain I of DnaA, I present evidence of SirA localisation in respect to cellular components including the replication origins and the replisome in cells naturally induced to sporulate (by nutritional downshift).

1.10 Chromosome organisation in sporulating cells

Examination of the genetic material in sporulating cells has indicated an orderly organisation of the chromosomes. Co-localisation studies using fluorescent labelling of *oriC* and terminus regions revealed that in sporulating cells the origin regions are positioned at the extreme cell poles while the termini are confined to the mid cell (Wu and Errington, 1994b). Moreover, forespore-confined activation of σ^F and dependence of the expression of the σ^F-induced genes on their location in the forespore compartment suggests polarity in chromosome organisation in sporulating cells (Wu and Errington, 1994b, Wu et al., 1995).

However, the first direct evidence confirming that the DNA captured in the forespores contained regions surrounding the *oriC* was produced by the study performed by Wu and Errington (1998). They used SpoIIIE36, a variant of SpoIIIE DNA translocase that could engage DNA at the time of asymmetric cell division but was blocked in translocating

the DNA across the septum. This provided a snapshot of genetic content of the forespore at the time of septation, allowing Wu and Errington (1998) to lock the DNA captured in the forespores. A σ^F-dependent *lacZ* reporter inserted at different chromosomal positions surrounding the origin was used to determine the frequency at which these loci appeared in the forespore compartment. A region of ~1 Mb centred near *oriC* was identified to be most frequently trapped in the forespore compartment (Wu et al., 1998).

A recent study by Sullivan et al., in which a sensitive single cell assay was utilised to identify the regions of chromosome captured in the forespore compartment, confirmed that about 1 Mb of the chromosome from -53° to +38° to be most frequently captured in the forespores (Sullivan et al., 2009).

1.10.1 Axial filament formation- RacA tethers origin regions to the opposite cell poles

At the early stages of sporulation origin regions of the pre-divisional cell are segregated to the extreme poles where they are anchored through interaction with polar proteins, forming a specific structure called axial filament, which is instrumental in faithful segregation of chromosome in sporulating cells (Ryter and Schaeffer, 1966).

RacA is an early sporulation protein that facilitates remodelling of the nucleoid into an axial filament. RacA binds to its recognition elements, termed *ram* sites (25 of which are found within 612 kb surrounding the origin region) and anchors them to the cell poles (through interaction with DivIVA, see below). Artificial expression of RacA in vegetatively growing cells alone induces formation of an axial filament-like structures. During sporulation in the absence of RacA axial filament formation is impaired and instead the nucleoid forms a stubby structure that often is not connected to the cell poles. This leads to increased production of DNA-less forespores (Wu and Errington, 2003, Ben-Yehuda et al., 2003).

1.10.2 DivIVA plays a key role in chromosome segregation during spore development

The Min system of *B. subtilis* facilitates division site selection in vegetatively growing cells. DivIVA is targeted to the cell poles where it recruits the MinCD complex to prevent anomalous polar divisions and formation of minicells (de Boer et al., 1989, Marston and Errington, 1999).

However, during sporulation DivIVA plays a distinct role from its division-site selection function and that is by providing a polar anchor for RacA (Wu and Errington, 2003, Ben-Yehuda et al., 2003). Absence of DivIVA significantly reduces sporulation frequency (by 15 to 20-fold) and also leads to development of an increased number of cells with DNA-free forespores. Moreover, an allele of *divIVA* (*divIVA13*) was isolated that, despite being able to localise at the cell poles and regulate MinCD complex, was specifically impaired in spore formation (<5% of wild-type). In this mutant axial filament formation was defective and most cells captured little or no DNA in the forespores, suggesting that DivIVA is required for axial filament formation (Thomaides et al., 2001). Later it was shown that DivIVA is required for polar localisation of RacA and accurate formation of axial filament(Wu and Errington, 2003, Ben-Yehuda et al., 2003).

1.10.3 Factors involved in the delivery of the origin regions to proximity of cell poles

1.10.3.1 Role of Soj and Spo0J in chromosomal origin segregation in sporulating cells

Soj and Spo0J are required for accurate chromosome orientation in sporulating cells. In the Δ*spo0J* Δ*soj* double mutant, aberrant trapping of chromosomal regions normally excluded from the forespores is observed (Sharpe and Errington, 1996). Further characterisation of chromosomal loci trapped by the Δ*spo0J* Δ*soj* mutant strain revealed

that while the specificity of regions capture in forespores were generally reduced, the absence of Soj and Spo0J affected capturing of sequences to the right of the *oriC* more than regions to left of *oriC.* Furthermore, sporulating cells lacking Soj are defective in capturing of regions surrounding the *oriC* in the forespore compartment which could be due to perturbed chromosome organisation function of Par system in absence of Soj (Sullivan et al., 2009, Wang et al., 2014a, Wang et al., 2014b).

1.10.3.2 RacA and Soj play a partially redundant role in chromosomal origin trapping in sporulating cells

As mentioned earlier (see section 1.10.1), a Δ*racA* mutant exhibits severe defects in axial filament formation. However, this defect is not reflected in sporulation frequency and Δ*racA* exhibits a similar sporulation frequency to the wild-type. However, population studies of mutants lacking both Soj and RacA revealed a significant (10-fold) decrease in the sporulation frequency of the Δ*soj* Δ*racA* mutant (Wu and Errington, 2003). Since a negligible sporulation defect is also seen in the Δ*soj* mutant, it was suggested that Soj and RacA play a redundant role in delivery of the origin regions to the cell poles, and Soj function, in chromosomal origin segregation is only required for proper spore development in the absence of RacA (Wu and Errington, 2003). However, direct evidence for Soj being involved in origin trapping in sporulating cells was provided by Sullivan *et al.* using a sensitive single cell cytological assay. It was demonstrated that in a strain lacking Soj there is an increased number of cells that failed to trap the *oriC* region within the forespore. In contrast, the other chromosomal regions surrounding the origin were not significantly affected by the absence of Soj, suggesting that this phenotype is not caused by a defect in

overall chromosome organisation but was caused by defects in positioning of the origin region specifically (Sullivan et al., 2009).

Thesis aims:

In this thesis I have aimed to:

- Determine the subcellular localisation of SirA (using GFP-SirA) in cells that have been induced to sporulate
- Determine SirA site of action/accumulation (candidates of which are *oriC* and replisome)
- Identifing SirA interaction/action partners (a candidate of which would be YabA)
- Validate the authenticity of the SirA-DnaA^{DI} interaction interface identified in the co-crystal structure SirA-DnaA^{DI} *in vivo*
- Test the effects of misregulation of DnaA (due to distruption of *sirA*, *yabA* and *soj*) on the closely linked downstream process of chromosome segregation in sporulating cells
- To test whether absence of DnaA regulators affect the sporulation frequency
- Determine the role of Soj in chromosomal origin segregation during sporulation and the property of Soj important to do so
- Determine whether there is any defect in spore development in the Δ*soj* mutant

Chapter 2. Materials and Methods

2.1 Solutions and media

Chemical composition of the solutions and the growth media used in this study are given in appendices 1 and 2.

2.2 Bacterial strains and plasmids

Strains and plasmids used in this study are described in Table 1 below. *E. coli* strain DH5a (Invitrogen) was used for the construction of all plasmids.

Table 2.2.1. Strain list

Strain	**Genotype**	**Reference**
168ed	*trpC2*	(Kobayashi et al., 2003)
168ca	*trpC2*	(Kunst et al., 1997)
AK47	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*)	Murray and Koh 2014
HM503	Δ*sirA::tet*	Rahn-Lee et al., 2009
HM519	*trpC2 thrC::*(P_{spanc}-*sirA spec)*	Rahn-Lee et al., 2011
HM550	*trpC2 thrC::*(P_{spanc}-*sirA spec) dnaA*^{A50V}*::cat*	Murray unpublished
HM552	*trpC2 thrC::*(P_{spanc}-*sirA spec) dnaA*^{N47H}*::cat*	Murray unpublished
HM610	*trpC2* Δ*sda::tet*	Veening et al., 2009

HM748	*trpC2 Δsoj::neo*	Murray and Errington 2008
HM797	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *dnaA*^{L294R}*::cat*	Scholefield et al.,2012
HM798	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *dnaA*^{V323D}*::cat*	Scholefield et al.,2012
HM799	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *dnaA*^{L337P}*::cat*	Scholefield et al.,2012
HM906	*trpC2 ΔracA::spec*	Wu and Errington 2003
HM908	*trpC2 Δsda::tet*	Veening et al., 2009 168ca (background)
IDJ056	*trpC2 oriC* $^{spo0Aboxes\_mut}$*::cat*	Boonstra et al., 2013
JWV160	*sacA::neo*(P_{sda}-*gfp*) *dnaN'*Ω(*dnaN-mCherry spec*)	Veening et al., 2009
MS104	*trpC2, gfp-dnaN::cat*	(Su'etsugu and Errington, 2011)
NR3	*trpC2 gfp-sirA::spec*	This work. pHM349 transformed into 168ed. (Jameson et al.,2014)

NR5	*trpC2 gfp-sirA::spec dnaA^{A50V}::cat*	This work. NR3 transformed with pHM328 (Jameson et al.,2014)
NR11	*trpC2 ΔsirA::tet*	This work. HM503 transformed into 168ed
NR12	*trpC2 gfp-sirA::spec ΔyabA::phleo*	This work. HM243 transformed into NR3
NR16	*trpC2 yycR::* (*tetO$_{25}$ erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) Δ*soj::neo*	This work. HM748 transformed into AK47
NR19	*trpC2 ΔyabA::phleo*	Noirot-Gros et al. (2002)
NR34	*trpC2 gfp-dnaN::cat ΔsirA::tet*	This work. NR11 transformed into MS104
NR36	*trpC2 gfp-dnaN::cat ΔyabA::phleo*	This work. NR19 transformed into MS104

NR40	*trpC2 thrC::*(P_{spanc}-*sirA spec*) *ΔyabA::phleo*	This work. NR29 transformed into HM519
NR42	*trpC2 gfp-dnaN::cat Δsoj::neo*	This work. MS104 transformed into
NR47	*trpC2 yycR::* (*$tetO_{25}$ erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *Δsda::tet*	This work. AK47 transformed with HM610
NR48	*trpC2 yycR::* (*$tetO_{25}$ erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *Δsoj::neo*	This work. HM748 transformed into AK47
NR49	*trpC2 yycR::* (*$tetO_{25}$ erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *soj^{G12V}::neo*	This work. HM750 transformed into AK47
NR50	*trpC2 soj^{D40A}::neo* Δ*sda::tet*	This work. HM752 transformed into HM610
NR52	*trpC2* Δ*racA::tet*	This work. SB180 transformed into 168ca
NR55	*trpC2 yycR::* (*$tetO_{25}$ erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *soj^{D40A}::neo* Δ*sda::tet*	This work. NR50 transformed into AK47

NR57	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *soj*^{K16A}*::neo*	This work. HM751 transformed into AK47
NR58	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *soj*^{R189A}*::neo*	This work. HM753 transformed into AK47
NR61	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *aprE::* (P_{spac}-*spo0J cat*)	This work. HM941 transformed into AK47
NR62	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *aprE::* (P_{spac}-*spo0J cat*) *Δsoj::neo*	This work. HM941 transformed into NR48
NR64	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) Δ*sirA::tet*	This work. NR11 transformed into AK47
NR66	*trpC2* Δ*spo0J::neo aprE::* (P_{spac}-*spo0J cat*)	This work. HM941 transformed into HM754
NR89	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *dnaA*^{A50V}*::cat*	This work. pHM328 transformed into AK47
NR91	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *dnaA*^{A50T}*::cat*	This work. pHM329 transformed into AK47
NR93	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *dnaA*^{N47H}*::cat*	This work. pHM330 transformed into AK47

NR97	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *dnaA*^{M161I}*::cat*	This work. pNR5 transformed into AK47
NR100	*trpC2* Δ*yabA::phleo* Δ*racA::spec*	This work. NR19 transformed into HM906
NR109	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *spoIIIE36::cat*	This work. TK1 transformed into AK47
NR110	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *spoIIIE36::neo*	This work. TK2 transformed into AK47
NR111	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *dnaA*^{Y325H}*::cat*	This work. pNR6 transformed into AK47
NR113	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *spoIIIE36::cat* Δ*sirA::tet*	This work. NR11 transformed into NR109
NR115	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *spoIIIE36::cat* Δ*soj::neo*	This work. HM748 transformed into NR109
NR117	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *spoIIIE36::neo dnaA*^{A50V}*::cat*	This work. pHM328 transformed into NR110

NR118	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *spoIIIE36::neo dnaA*^{N47H}*::cat*	This work. pHM330 transformed into NR110
NR119	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *spoIIIE36::kan dnaA*^{M161I}*::cat*	This work. pNR5 transformed into NR109
NR123	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *spoIIIE36::kan dnaA*^{L294R}*::cat*	This work. HM797 transformed into NR110
NR124	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *spoIIIE36::kan dnaA*^{V323D}*::cat*	This work. HM798 transformed into NR110
NR125	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *spoIIIE36::kan dnaA*^{L337P}*::cat*	This work. HM799 transformed into NR110
NR126	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *spoIIIE36::kan dnaA*^{A163V}*::cat*	This work. HM800 transformed into NR110
NR128	*trpC2 yycR::* (*tetO*$_{25}$ *erm*) *amyE::* ($P_{spac(c)}$-*tetR-gfp spec*) *spoIIIE36::kan dnaA*^{H162Y}*::cat*	This work. sGJS124 transformed into NR110

NR130	*trpC2 gfp-sirA^{F14A}::spec*	This work. pNR8 transformed into 168ed (Jameson et al.,2014)
NR131	*trpC2 gfp-sirA^{Q48A}::spec*	This work. pNR9 transformed into 168ed (Jameson et al.,2014)
NR132	*trpC2 ΔsirA::tet Δsoj::neo*	This work. NR11 transformed into HM748
NR133	*trpC2 ΔsirA::tet Δsoj::neo Δspo0A^{Boxes}::cat*	This work. IDJ056 transformed into NR132
NR134	*trpC2 ΔyabA::phleo Δsda::tet*	This work. NR19 transformed into HM610
NR136	*trpC2 soj^{G12V}::neo ΔracA::tet*	This work. HM750 transformed into NR52
NR138	*trpC2 soj^{K16A}::neo ΔracA::tet*	This work. HM751 transformed into NR52

NR140	*trpC2 soj^{R189A}::neo* Δ*racA::tet*	This work. HM753 transformed into NR52
NR142	*trpC2 soj^{D40A}::neo* Δ*sda::tet*	This work. HM752 transformed into HM610
NR143	*trpC2* Δ*soj::neo* Δ*racA::tet*	This work. HM748 transformed into NR52
NR154	*trpC2 gfp-sirA::spec dnaA^{N47H}::cat*	This work. pHM330 transformed into NR3
NR156	*trpC2 gfp-sirA^{Y18A}::spec*	This work. pNR10 transformed into 168ed (Jameson et al.,2014)
NR163	*trpC2 gfp-sirA*::*spec spo0J*::(*tetO$_{150}$ neo*)	This work. HM112 transformed into NR3
NR164	*trpC2 gfp-sirA*::*spec aprE::* (P_{xyl}-*tetR-mCherry cat*) *spo0J*::(*tetO$_{150}$ neo*)	This work. pAK168 transformed into NR163
NR166	*trpC2 gfp-sirA*::*spec aprE::cat*(P_{xyl}-*tetR-mCherry*) *spo0J*::(*tetO$_{150}$ neo*) Δ*yabA::phleo*	This work. NR19 transformed into NR164

		(Jameson et al.,2014)
NR168	*trpC2 gfp-sirA*::*spec dnaN'*Ω(*dnaN-mCherry spec*)	This work. JWV160 transformed into NR3 (Jameson et al.,2014)
NR171	*trpC2 amyE*::*spec*($P_{hyperspank}$-*sirA*)	This work. pNR11 transformed into 168ed (Jameson et al.,2014)
NR172	*trpC2 amyE*::*spec*($P_{hyperspank}$-$sirA^{F14A}$)	This work. pNR12 transformed into 168ed (Jameson et al.,2014)
NR173	*trpC2 amyE*::*spec*($P_{hyperspank}$-$sirA^{Q48A}$)	This work. pNR13 transformed into 168ed (Jameson et al.,2014)
NR174	*trpC2 amyE*::*spec*($P_{hyperspank}$-$sirA^{Y18A}$)	This work. pNR14 transformed into 168ed (Jameson et al.,2014)
SB180	Δ*racA::tet*	Ben-Yehuda

		et al.,2003
sGJS119	*trpC2 amyE::spec*(P_{xyl}-*yabA*) *ΔyabA::phleo dnaA*^{A163V}*::cat*	Scholefield and Murray 2013
sGJS124	*trpC2 amyE::spec*(P_{xyl}-*yabA*) *ΔyabA::phleo dnaAH*^{162Y}*::cat*	Scholefield and Murray 2013
TK1	*trpC2 spoIIIE36::cat*	Wu and Errington 1998
TK3	*trpC2 spoIIIE36::kan, amyE::*(P_{spoIIQ}*::lacO-lacZ erm*), *yxiF::*(P_{spoIIQ}-*lacI spec*)	Tomas Kloosterman (unpublished)

Table 2.2.2. Plasmid list

Plasmid	**Genotype**	**Construction**	**Reference**
pAK168	*bla aprE::cat*(P_{xyl}-*tetR-mCherry)*		Koh (unpublished)
pNR5	*bla dnaA*^{M161I}*::cat*	*dnaA*^{M161I} (oHM177/oHM196) from blue/white screen isolated strain NR3/10 ligated to pHM327(PacI and PFLMI)	This work

pNR8	*bla gfp-sirA^{F14A}::spec*	*sirA^{F14A}* (oHM280/oHM285) from pMBP::SirA^{F14A} ligated to pHM349(NsiI and XhoI)	This work. (Jameson et al., 2014)
pNR9	*bla gfp-sirA^{Q48A}::spec*	*sirA^{Q48A}* (oHM280/oHM285) from pMBP::SirA^{Q48A} ligated to pHM349(NsiI and XhoI)	This work. (Jameson et al., 2014)
pNR10	*bla gfp-sirA^{Y18A}::spec*	*sirA^{Y18A}* (oHM280/oHM285) from pMBP::SirA^{Y18A} ligated to pHM349(NsiI and XhoI)	This work. (Jameson et al., 2014)
pNR11	P$_{hyperspank}$-*sirA::spec*	*sirA* (oJW001/oJW002) from 168ed ligated to pDR111 (HindIII and NheI)	Wagner et al.,2009
pNR12	P$_{hyperspank}$-*sirA^{F14A}::spec*	*sirA^{F14A}* (oJW001/oJW002) from NR130 ligated to pDR111 (HindIII and NheI)	This work. (Jameson et al., 2014)
pNR13	P$_{hyperspank}$-*sirA^{Q48A}::spec*	*sirA^{Q48A}* (oJW001/oJW002) from NR131 ligated to pDR111 (HindIII and NheI)	This work. (Jameson et al., 2014)

pNR14	$P_{hyperspank}$- *sirA*^{Y18A}*::spec*	*sirA*^{Y18A} (oJW001/oJW002) from NR156 ligated to pDR111 (HindIII and NheI)	This work. (Jameson et al., 2014)

Table 2.2.3. Primer list

Primer	**Sequence**
oHM177	5’- CCAAGCCCTTGCTCAAATCGAA -3’
oHM189	5’- ATGTGCTTTTGATAGATGATATTC-3’
oHM190	5’- GCCGGTCACTTGAAATGACG -3’
oHM196	5’- GGTTGGATCCCTATTTAAGCTGTTCTTTAATTTC -3’
oHM254	5’- GCTGCGTTTACGGTTATTCG -3’
oHM255	5’- CGTGGTTCTGGATGAAACTG -3’
oHM280	5’- CCCCAAGCTTTTAGACAAAATTTCTTTCTTTCACC -3’
oHM285	5’- AAAACCCTCGAGATGGAACGTCACTACTATAC -3’
oJW001	5’-CGCAAGCTTACATAAGGAGGAACTACTATGGAACGTCACTACTATACG-3’
oJW002	5’-GCCGCTAGCGGATCCGGTTTTAGACAAAATTTCTTTC-3’

2.3 Oligonucleotides

All oligonucleotides were purchased from Eurogentec. Stock solutions were diluted to a final concentration of 10µM and stored at -20°C. The list of the oligonucleotides used in this study is given in Table 2.2.3.

2.4 Media supplements and antibiotics

When required the growth media was supplemented with; amino acids, inducers and/or antibiotics listed in Table 2.4.1, the final concentrations are indicated.

Table2.4.1. Antibiotics and inducers.

Antibiotics	**Stock solution**	**Final concentration**
Ampicillin	50 mg/ml	100 µg/ml
Chloramphenicol	10 mg/ml	5 µg/ml
Erythromycin	20 mg/ml	0.5 µg/ml
Kanamycin	25 mg/ml	2 µg/ml
Phleomycin	2 mg/ml	1 µg/ml
Spectinomycin	100 mg/ml	50 µg/ml
Tetracycline	10 mg/ml	10 µg/ml
Inducers		
IPTG	1 M	up to 3 mM
Xylose	25%	up to 0.1%

2.5 Growth and maintenance of bacterial strains

Depending on the requirements of each experiment, cells were grown at 30°C or 37°C with rigorous shaking. Luria-Bertani (LB) medium was used for growth to extract plasmids (*E. coli*) or genomic DNA (*B. subtilis*). Casein hydrolysate (CH) media (Sterlini and Mandelstam, 1969) and starvation media (Sterlini and Mandelstam, 1969) as modified by (Partridge and Errington, 1993) were utilised for induction of sporulation *B. subtilis* cells. Competence medium (Hamoen et al., 2002) was used for induction of competence in *B. subtilis* cells. Nutrient agar (Oxoid), was utilised as solid medium for growth and short-term maintenance of both *B. subtilis* and *E. coli.*

For long term storage, glycerol stocks were prepared by scraping cell off nutrient agar plates and resuspending them in 25% glycerol. Aliquots were immediately frozen in liquid nitrogen and stored at -80°C.

2.6 General methods

2.6.1 DNA methods

2.6.1.1 Polymerase chain reaction (PCR)

Phusion High-Fidelity DNA polymerase or Q5 High-Fidelity DNA polymerase (New England BioLabs) were used for amplification of DNA used in cloning ; GoTaq Flexi DNA Polymerase (Promega) was used for colony PCR and confirming DNA fragment size (gene deletion) or double cross-over genome integrations. 50 µl PCR reactions were prepared according to the manufacturer specifications. Oligonucleotides were used to a final concentration of 0.5 µM and dNTPs to a final concentration of 0.2 mM. Reaction buffer, MgCl2 and template DNA were added according to the manufacturer's recommendations.

2.6.1.2 Isolation of plasmid DNA

Plasmid DNA was purified from 5 ml of *E. coli* overnight cultures using QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions.

2.6.1.3 Agarose gel electrophoresis of DNA fragments

DNA was mixed with DNA loading buffer and loaded on 1% agarose gels made with 0.5X TBE buffer. Gels were run in 0.5X TBE buffer at a constant voltage of 90 V for 60 minutes. Ethidium bromide bath was used to stain DNA for visualisation with a UV transilluminator, images were taken using Lumenera USB 2.0 camera.

2.6.1.4 Isolation of DNA fragments from agarose gel

When required, DNA fragments were excised out of an ethidium bromide stained agarose gel and the DNA was purified with the QIAquick (Qiagen) Gel Extraction Kit, according to the manufacturer's recommendations.

2.6.1.5 Restriction endonuclease digestion

DNA was usually digested overnight at 30°C (unless a lower temperature was recommended by the manufacturers). Restriction enzymes were purchased from Roche, New England BioLabs or Promega, and the reactions were assembled using the reaction buffer provided by the manufacturer and the conditions recommended by the manufacturers.

2.6.1.6 Dephosphorylation reaction

Linearised plasmid DNA was generally dephosphorylated by adding 1 µl Alkaline Phosphatase (Roche) directly to the restriction endonuclease reactions and incubating for 60 minutes at 37°C.

2.6.1.7 Purification of PCR or digestion products

QIAquick (Qiagen) PCR Purification Kit was used for purification of PCR or digestion products.

2.6.1.8 Ligation of DNA fragments

DNA fragments with compatible ends were ligated using T4 DNA ligase (Roche). Ligation reactions with total volume of 10 µl were carried out overnight at 15°C in PCR machines.

2.6.1.9 DNA sequencing

The genetic content of plasmids and strains were confirmed by sequencing performed by the sequencing service of the University of Dundee, Scotland.

2.7 Protein methods

2.7.1 Sample preparation for western blot

In order to test protein levels in sporulating cells, cells were induced to sporulate (resuspension method), 150 minutes after induction of sporulation, cells were collected from 1 ml of culture by centrifugation and the pellet was frozen in liquid nitrogen and stored at -20°C. Cells were suspended in 104 µl of PBS containing protease inhibitor (Complete mini, Roche) and 1 mM EDTA. In order to break cells down, samples were sonicated twice, for 10 seconds using very low amplitudes. After addition of NuPAGE LDS Sample Buffer (Invitrogen) and NuPAGE Sample Reducing Agent, samples were incubated at 90°C for 10 minutes before centrifugation for 2 minutes at 14K rmp. The supernatant was loaded on the gel.

2.7.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated by electrophoresis using a NuPAGE 4-12% Bis-Tris gradient gel run in 1X MES buffer (Life Technologies) at 200V (constant voltage) for 40 minutes and transferred to a Hybond-P PVDF membrane (GE Healthcare) using a semi-dry apparatus (Hoefer Scientific Instruments).

2.7.3 Western blotting

Proteins of interest were probed with polyclonal primary antibodies and then detected with an anti-rabbit horseradish peroxidase-linked secondary antibody using an ImageQuant LAS 4000 mini digital imaging system (GE Healthcare).

2.8 *E. coli* methods

2.8.1 Transformation of *E. coli* DH5α competent cells

E. coli strain DH5α, was made competent using $CaCl_2$ treatment. Plasmids were then transformed into 500µl of competent DH5α cells and incubated on ice for 30 minutes. Then cells were Heat-shocked at 42˚C for 90 seconds and returned to incubation on ice for 2 minutes. Cells were then incubated in 1ml of LB at 37˚C for 1hr to allow plasmid uptake and antibiotic expression. Finally cells were plated onto NA plates supplemented with appropriate antibiotic.

2.8.2 Plasmid purification

Cells were grown in 5ml of LB (supplemented with the appropriate antibiotic) overnight at 37˚C. Plasmids were isolated from 2-5 ml of cultures using the QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's protocol. Plasmid DNA was then stored at -20˚C.

2.9 *B. subtilis* methods

2.9.1 Competent cell preparation and transformation

The competence was induced in *B. subtilis* by the method of Anagnostopoulos and Spizizen (Anagnostopoulos and Spizizen, 1961) as modified by Hamoen (2002). Cells were inoculated in 5 ml of minimal media and were grown overnight at 37°C. The overnight culture was diluted 1:20 in 5 ml of fresh minimal media and grown for 3 hours at 37°C. 5 ml of starvation media was added to the culture and grown for an additional 2 hours. DNA (genomic DNA or plasmid) was then added to 400-600 µl of the competent cells, and growth was continued for an hour to allow the up-take of the DNA and expression of the antibiotic marker. Transformants were plated on nutrient agar plates containing the appropriate antibiotic.

2.9.2 Chromosomal DNA extraction (Salt)

Chromosomal DNA used in transformation was usually prepared using the method described by (Ward and Zahler, 1973). Strains were inoculated in 5ml of LB media and grown overnight at 37°C with vigorous shaking. The next day the cells from 1 ml of the culture were washed and resuspended in 1 ml of SSC buffer (consisting of 0.01mM sodium citrate, 0.15 M NaCl and deionised water), and incubated with lysozyme for 20 minutes at 37°C. 1 ml of NaCl (4M) was added to lyse the cells. Genomic DNA was isolated by filtering lysate using a 0.45 µm filter and stored at -20°C.

2.9.3 Chromosomal DNA extraction (DNeasy kit)

Chromosomal DNA used as template in PCR reactions was extracted with DNeasy Blood and Tissue kit (Qiagen) from 1 ml of overnight culture grown in LB at 30°C.

2.9.4 Induction of sporulation (resuspension method)

To induce sporulation in *B. subtilis* the resuspension method of (Sterlini and Mandelstam, 1969) was used as modified by (Partridge and Errington, 1993). 5 ml of culture was grown overnight in casein hydrolysate (CH) media at 30˚C with. The following day, cells were diluted to the A_{600} of 0.1 in 5 ml of fresh CH media and were grown at 37˚C until reached the A_{600} of 1.0. Then cells were collected by centrifugation (9000 RMP for 10 minutes) and were resuspended in 5 ml of sporulation media (pre-warmed to 37 ˚C) and were grown at 37˚C.

2.9.5 Origin trapping assay (blue/white screening)

To introduce point mutations in *dnaA*, a DNA library was generated by performing error-prone PCR (using Phire polymerase enzyme) to amplify the *dnaA* gene. Chromosomal DNA from a strain in which a chloramphenicol marker was inserted downstream of the *dnaAN* operon was used as substrate. This DNA library was used to transform a screen strain that allowed selection of cells with an origin trapping defect (blue/white selection).

The screen strain that allowed distinction of clones with an origin trapping defect based on the expression and activity of β-galactosidase enzyme was made competent (10 ml culture) and was transformed with the DnaA library (10 µl). The transformants were plated on nutrient agar plates containing 5µg/ml chloramphenicol + 0.008% X-gal and incubated 37°C for at least 48 hours. Blue colonies were picked and restreaked on nutrient agar plates containing 0.008% X-gal as well as the wild-type. The genomic DNA was prepared from the colonies that appeared darker blue compared to the wild-type (using Qiagen DNeasy Blood and Tissue kit) and was backcrossed to the parent strain to ensure that the mutation causing the trapping defect was linked to the DnaA. Genomic DNA from the clones with linked

mutation to DnaA was used as a template in PCR to amplify *dnaA* gene with oHM254 and oHM455 and were sent for sequencing with oHM189 and oHM190 primers. The mutations identified in the sequencing were mapped to the DnaA structure using PyMOL program.

2.9.6 Sporulation frequency assay

Cells were induced to sporulate by resuspension method, after 24 hours of incubation at 37°C the sporulation frequency was determined as the ratio of heat-resistant colony forming units (heat-treatment of the samples to 80°C for 20 minutes) to total colony forming units.

2.10 Microscope imaging

After induction of sporulation, samples were taken every 30 minutes and visualised using fluorescence microscopy. Microscopy was performed using glass slides covered with a ~1.5% agarose pad containing sporulation media. A glass coverslip (0.17 mm VWR) covered cells immobilised on the agarose pad. The dye FM5-95 was added to the agar pads to visualise the membrane (2.9 µg/ml final). To visualise the nucleoid the DNA was stained with 4'-6-diamidino-2-phenylindole (DAPI 2.5 µg/ml final). Microscopy was performed on an inverted epifluorescence microscope (Zeiss Axiovert 200M) fitted with a Plan-Neofluar objective (Zeiss 100x/1.30 Oil Ph3). Light was transmitted from a 300 Watt xenon arc-lamp through a liquid light guide (Sutter Instruments) and images were collected using a Sony CoolSnap HQ cooled CCD camera (Roper Scientific). All filters were Modified Magnetron ET Sets from Chroma and details are available upon request. Digital images were acquired and analysed using METAMORPH software (version V.6.2r6).

Chapter 3: Structure and interactions of the Bacillus subtilis sporulation inhibitor of DNA replication, SirA, with domain I of DnaA

Introduction

Across the kingdoms of life, DNA replication is tightly regulated to ensure co-ordination with cell growth and development. Failure to maintain and control chromosome copy number is frequently associated with disease or cell death. Regulation of DNA replication is mainly exerted at the initiation step when an initiator protein binds to the origin of replication, and promotes the assembly of a nucleoprotein complex from which replication forks diverge.

The prokaryotic DNA replication initiatior protein is DnaA. DnaA is a multi-domain protein with four distinct domains (Kaguni, 2006). Domain I is known to have a number of interaction partners, including replication regulators and DNA helicase (Seitz et al., 2000, Abe et al., 2007). Domain II is a flexible linker, that may allow nuances in regulatory control (Molt et al., 2009). Domain III binds and hydrolyses ATP, mediates DnaA oligomerisation(Erzberger et al., 2006) and binds single stranded DNA thus aiding duplex unwinding (Duderstadt et al., 2011). Domain IV binds double stranded DNA, interacting with the DnaA-box motifs (Fujikawa et al., 2003). The binding of a threshold level of DnaA-ATP at *oriC* leads to duplex unwinding and the recruitment of other initiation proteins (Leonard and Grimwade, 2011) (DnaA and DNA replication initiation is discussed in more details in chapter1).

In bacteria the number of replication initiation events that occurs per cell cycle is highly dependent of nutritional availability. In rapid-growing bacteria like *B. subtilis* and *E. coli*, the time required for complete duplication of the genome (50 to 60 minutes) exceeds the mass doubling time (20 to 30 minutes). During rapid growth in order to co-ordinate the

DNA replication and cell division, DNA replication is reinitiated before completion of the previous cell cycle leading to assembly of multiple replication forks. Consequently daughter cells inherit chromosomes that are already undergoing replication, giving rise to partial diploidy and tetraploidy within the population.

For sporulation to proceed, *B. subtilis* requires two intact copies of the chromosome, one to be retained in the mother cell and the other to be packed into the dormant spore. Proper spore development and viability is dependent upon achievement of diploidy at the onset of sporulation in the pre-divisional cell (Veening et al., 2009). DNA replication is therefore regulated and monitored at the commencement of sporulation.

SirA, a protein produced under Spo0A~P regulation, has been identified as an inhibitor of DNA replication that plays a specific role in preventing replication reinitiation in cells committed to sporulation (Rahn-Lee et al., 2009). Although single deletion mutants of s*irA,* like those of *sda* (see chapter 1)*,* display only mild phenotypes, under conditions of nutrient depletion leading to sporulation a *sda/sirA* double mutant is severely impaired in chromosome copy number control, indicating a shared role in controlling DNA replication (Veening et al., 2009). Artificial induction of expression of *sirA* in vegetatively growing *B. subtilis* blocks replication and causes cell death in a DnaA-dependent manner (Wagner et al., 2009). Cells artificially induced to sporulate under conditions of rapid growth undergo a marked decrease in chromosome copy number which is partially relieved by deletion of *sirA* (Rahn-Lee et al., 2009). These experiments imply that SirA is an inhibitor of DNA replication during sporulation that acts by binding to DnaA. Furthermore, amino acid substitutions in the N-terminal domain of DnaA ($DnaA^{DI}$) relieve SirA inhibition (Rahn-Lee et al., 2011).

In this chapter the localisation of GFP-SirA in sporulating cells is determined. Using fluorescent markers for the origin of replication and also the replication machinery it was established that SirA accumulates away from the origins at the replisome, likely through a direct interaction with DnaA. Additionally, utilisation of a GFP-SirA localisation assay and a SirA overexpression assay confirms that the SirA-DnaA^{DI} determinants (as revealed from the co-crystal structure solved by Katie Jameson and Tony Wilkinson) are required for the formation of SirA-DnaA^{DI} complex *in vivo*.

Results

3.1 GFP-SirA co-localises with the replisome in sporulating cells

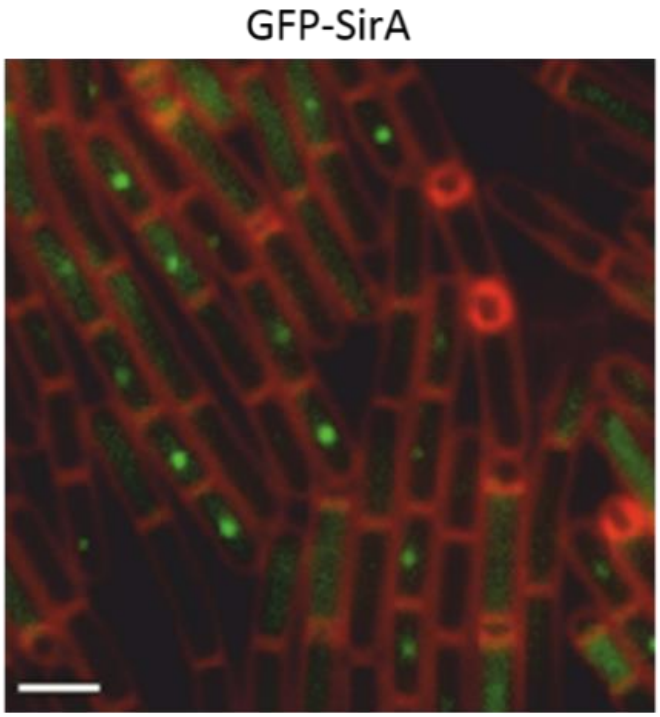
Localisation of SirA was achieved by replacing the endogenous gene with *gfp-sirA* (expressed from its native transcriptional and translational regulatory sequences; Figure 3.1.1A), inducing cells to sporulate by nutrient deprivation, and detecting the localisation of GFP-SirA using epifluorescence microscopy (Figure 3.1.1B). A time course experiment showed that GFP-SirA foci began to appear approximately 90 minutes after cells were resuspended in starvation medium. By 150 minutes, the number of cells containing a GFP-SirA focus reached its maximum (~20%; Figure 3.1.1C). In the majority of cases (>80%) GFP-SirA foci were located near mid-cell.

Previous studies suggested that SirA inhibits new rounds of DNA replication by inhibiting the binding of DnaA to the origin of replication (Wagner *et al.*, 2009). However, the mid-cell localisation of GFP-SirA foci is contrary to origin positioning in sporulating cells where the two origins are positioned towards the cell poles, suggesting that SirA was not accumulating at *oriC*.

A


P sirA
tkt
spec
gfp
sirA
yneF

B


GFP-SirA

C

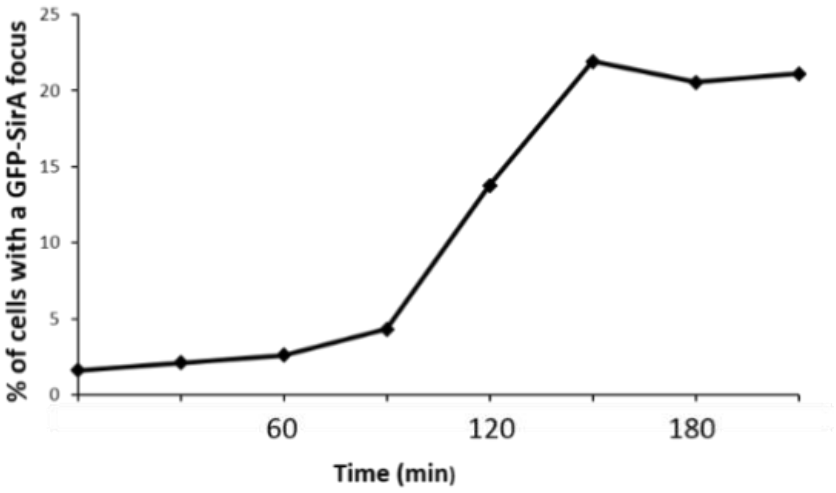

% of cells with a GFP-SirA focus
25
20
15
10
5
0
60
120
180
Time (min)

Figure 3.1.1. Localisation of GFP-SirA *in vivo*. **A.** Schematic diagram showing the modified *sirA* locus used for localisation studies. Chromosomal *sirA* was replaced with *gfp-sirA* under the control of its native expression system. **B.** GFP-SirA localisation during sporulation of *B. subtilis* 150 minutes post resuspension in starvation media. Membrane dye FM5-95 was used to highlight the outlines of the cells. Scale bar = 3 µm. **C.** Temporal analysis of GFP-SirA foci formation during sporulation. At least 500 cells were analysed at each time-point. *gfp-sirA* (NR3).

In order to further investigate GFP-SirA localisation, a strain was constructed that allowed visualisation of both *oriC* and SirA. An array of *tet* operators was inserted near *oriC* and the Tet repressor was fused to a red fluorescent protein (TetR-mCherry); interaction of TetR-mCherry with the *tetO* array produces a fluorescent focus near each origin of replication. Cells were induced to sporulate by nutrient deprivation and the localisation of GFP-SirA was determined in respect to the origin regions. In the majority of cells with a GFP-SirA focus there was no co-localisation of SirA with *oriC* (78% displayed non-overlapping signals; Figure 3.1.2). Only 8% of the GFP-SirA foci appeared to co-localise with the origin regions, with the remaining 14% of cells containing a GFP-SirA focus that partially overlapped with the origin marker. These results show that during sporulation GFP-SirA mainly accumulates away from the replication origin.

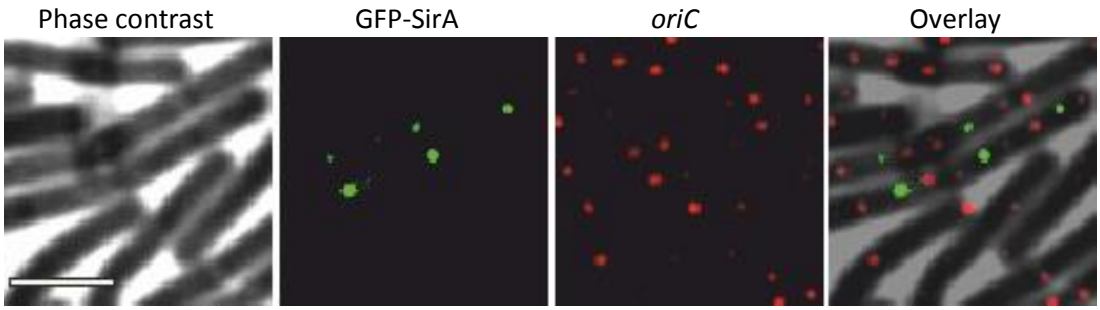


Figure 3.1.2**. GFP-SirA localises away from the origin regions in cells induced to sporulate.** Colocalisation of GFP-SirA with *oriC* during sporulation (150 minutes post resuspension in starvation media). More than 200 cells were analysed and a representative image is shown. Scale bar = 3 µm. *gfp-sirA oriC*$^{tetO/\text{TetR-mcherry}}$ (NR164).

A previous study in *B. subtilis* reported that DnaA co-localises with the replisome at mid-cell via the YabA-DnaN complex (Soufo *et al.*, 2008). I hypothesised that SirA might be interacting with DnaA when it is bound to the replication machinery. To begin testing this model GFP-SirA localisation was examined in cells that contained a fusion of a red fluorescent protein to the sliding clamp of the replisome (DnaN-mCherry). Cells were induced to sporulate by nutrient deprivation and the localisation of GFP-SirA was determined in respect to the replisome. Strikingly, in cells containing a GFP-SirA focus the vast majority co-localised with DnaN-mCherry (88%; Figure 3.1.3). This result indicates that GFP-SirA mainly accumulates at the replisome.

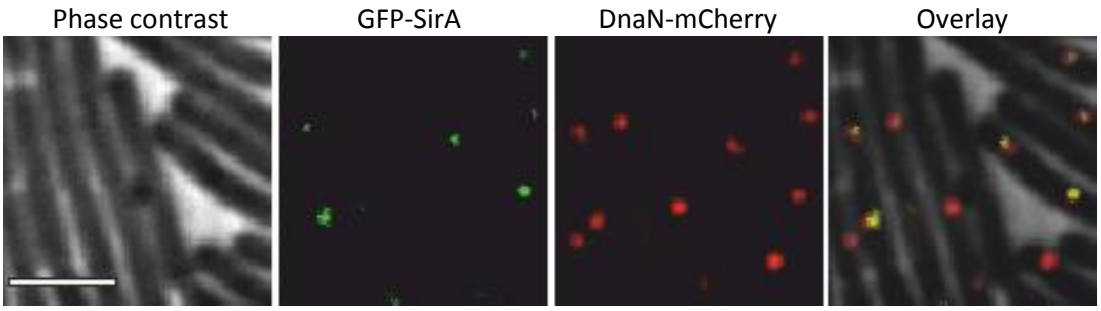


Figure 3.1.3. GFP-SirA colocalises with the replisome in cells induced to sporulate. Co-localisation of GFP-SirA with the replisome during sporulation (150 minutes post resuspension in starvation media). More than 100 cells were analysed and a representative image is shown. Scale bar = 3 µm. *gfp-sirA dnaN-mCherry* (NR168).

3.2 SirA localisation and activity in a Δ*yabA* mutant

Since DnaA was reported to be bound to the replisome through interaction with YabA during vegetative growth (Soufo et al., 2008), I set out to determine whether SirA localisation to the replisome was via YabA-bound DnaA. I hypothesised that if the interaction of SirA with the replisome is dependent of YabA, GFP-SirA foci formation would be lost or altered in absence of YabA. To examine this model the localisation of GFP-SirA was determined in a Δ*yabA* mutant. The results showed that a similar frequency of GFP-SirA foci formed in the Δ*yabA* mutant compared to the parent strain (17% of cells appeared to have a GFP-SirA focus in the Δ*yabA* mutant compared to the parent strain that exhibited~20% GFP-SirA foci formation). However, in the majority of cells GFP-SirA localisation was shifted towards the poles.

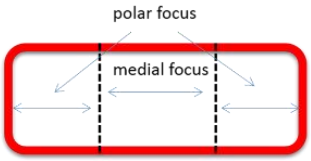
In order to quantitatively analyse the positioning of the GFP-SirA focus within the cells and determine potential alterations in GFP-SirA localisation in the Δ*yabA* mutant, an image processing software (ObjectJ) was used to divide the cells into three equal sections along the cell length and determine the GFP-SirA focus localisation in respect to the section it resided in (Figure 3.2.1A) . The GFP-SirA foci that was positioned within the middle section was counted as a medial focus and the foci that resided in the outer sections were counted as polar foci.

The results indicated that in fact in the Δ*yabA* mutant the majority of the cells with a GFP-SirA focus (85%) contained a polar focus compared to the wild-type strain in which most cells contained a GFP-SirA focus at the midcell (>80% of cells contained a medial GFP-SirA focus in the wild-type)(Figure 3.2.1B).

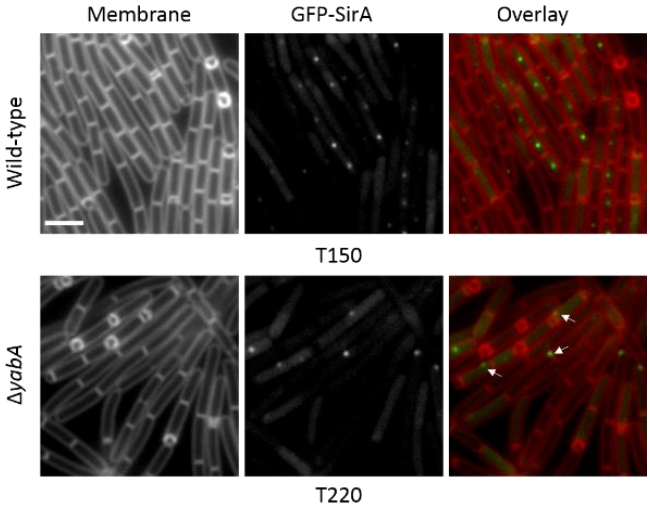
I speculated that this change in GFP-SirA localisation (from medial to polar) in the Δ*yabA* mutant could be caused by DnaA release from the replisome and subsequent enrichment at replication origins. To test whether SirA colocalises with *oriC* in a Δ*yabA* mutant, GFP-SirA localisation was examined in a strain background that contained the *tetO*/TetR-mCherry origin marker system (Figure 3.2.1C). The results showed that in the majority of cases GFP-SirA does not colocalise with the origin regions in absence of YabA (in 70% of cells GFP-SirA foci appeared away from the origin markers however in 18% of the cells with a GFP-SirA focus a partial overlap of GFP-SirA foci and the origin markers was detected leaving only about 12% of cell with a GFP-SirA focus colocalising with the origin markers). We attempted to determine GFP-SirA localisation in respect to the replisome in the Δ*yabA* mutant using DnaN-mCherry as a replisome marker, however, for unknown reasons GFP-SirA foci formation was not observed in this background.

In addition to its localisation, the regulatory activity of SirA was determined in the absence of YabA. Overexpression of SirA was reported to inhibit cell growth by blocking DNA replication (Wagner et al., 2009), therefore I overexpressed SirA from an IPTG inducible promoter ($P_{hyperspank}$) and monitored cell growth on the solid medium in the presence and absence of YabA. The results showed that overexpression of SirA leads to growth inhibition independently of YabA (Figure 3.2.1D), indicating that SirA does not require YabA to inhibit DNA replication initiation.

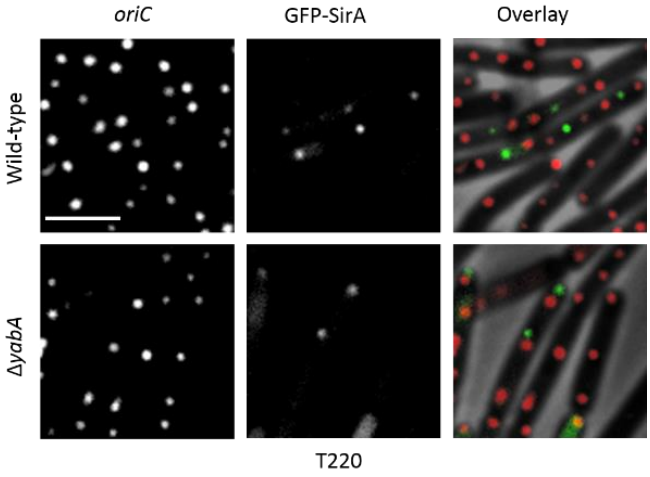
A


polar focus
medial focus

B


Membrane
GFP-SirA
Overlay
Wild-type
T150
ΔyabA
T220

C


oriC
GFP-SirA
Overlay
Wild-type
ΔyabA
T220

D

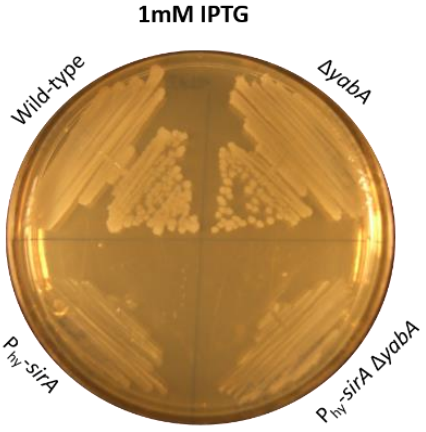

1mM IPTG
Wild-type
ΔyabA
Phy-sirA
Phy-sirA ΔyabA

Figure 3.2.1. **GFP-SirA localises towards the cell poles in absence of YabA. A.** Using ObjectJ image analysis software cells were divided into three equal sections along the long axis, and the localisation of the GFP-SirA focus was determined depending on the section that the focus laid in. **B.** GFP-SirA localisation is shown in the wild-type and the Δ*yabA* mutant. GFP-SirA foci are formed in similar proportion of cells in the Δ*yabA* compared to the wild-type, however the in the Δ*yabA* mutant the GFP-SirA foci are predominantly localised towards the cell poles. Images of the Δ*yabA* mutant were taken 220 minutes after resuspension in sporulation media due to the Δ*yabA* mutant exhibiting a delay in initiation of sporulation (see chapter 4, figure 4.3.1 and Table 4.3.1). *gfp-sirA* (NR3); *gfp-sirA* Δ*yabA* (NR12) **C.** GFP-SirA foci do not colocalise with the replication origins in the Δ*yabA* mutant. *gfp-sirA oriC*$^{tetO/TetR\text{-}mcherry}$ (NR164)*; gfp-sirA oriC*$^{tetO/TetR\text{-}mcherry}$ Δ*yabA* (NR166) **D.** *yabA* deletion does not restore the growth inhibition caused by SirA overexpression. Cells were grown on nutrient agar plates containing 1mM IPTG for 48 hours. Wild-type (168ed); Δ*yabA* (NR19); P$_{spanc}$-*sirA* (HM519); P$_{spanc}$-*sirA* Δ*yabA* (NR40).

3.3 Crystal structure of SirA-DnaA^{DI}complex

The crystal structure of SirA in complex with domain I of DnaA was solved to 1.7 Å resolution by Katie Jameson in the laboratory of Professor Anthony Wilkinson (University of York;(Jameson et al., 2014)). The co-crystal structure revealed that SirA forms a heterodimer with DnaA^{DI}. The interacting surface of SirA-DnaA^{DI} is extensively α-helical in nature, dominated by side chain-side chain contacts Figure 3.3.1)). (Several residues were identified

on SirA and DnaA^{DI} to contribute to the core of the interface; Thr26, Trp27 and Phe49 on SirA binding surface of DnaA^{DI} (Figure 3.3.1C) and Phe14, Tyr18 and Gln48 on corresponding DnaA^{DI} binding surface of SirA (Figure 3.3.1B). Mapping of previously identified SirA binding determinants of DnaA (Asn47, Phe49 and Ala50 reported by Rahn-Lee 2011); to the SirA-DnaA^{DI} structure revealed that Asn47 and Phe49 residues of DnaA make a direct contact with SirA. Asn47 forms a pair of hydrogen bonds with Gln48 on SirA and the side chain of Phe49 projects into a hydrophobic pocket created by α-helices on SirA surface (Figure 3.3.1D). In contrast, Ala50 is buried within DnaA^{DI} and does not form direct contacts with SirA within the complex; however, the positioning of Ala50 determines the structure of the interface, hence substitution of this residue with amino acids with bulkier side chain alters/disrupts the SirA interaction site (Figure 3.3.1D).

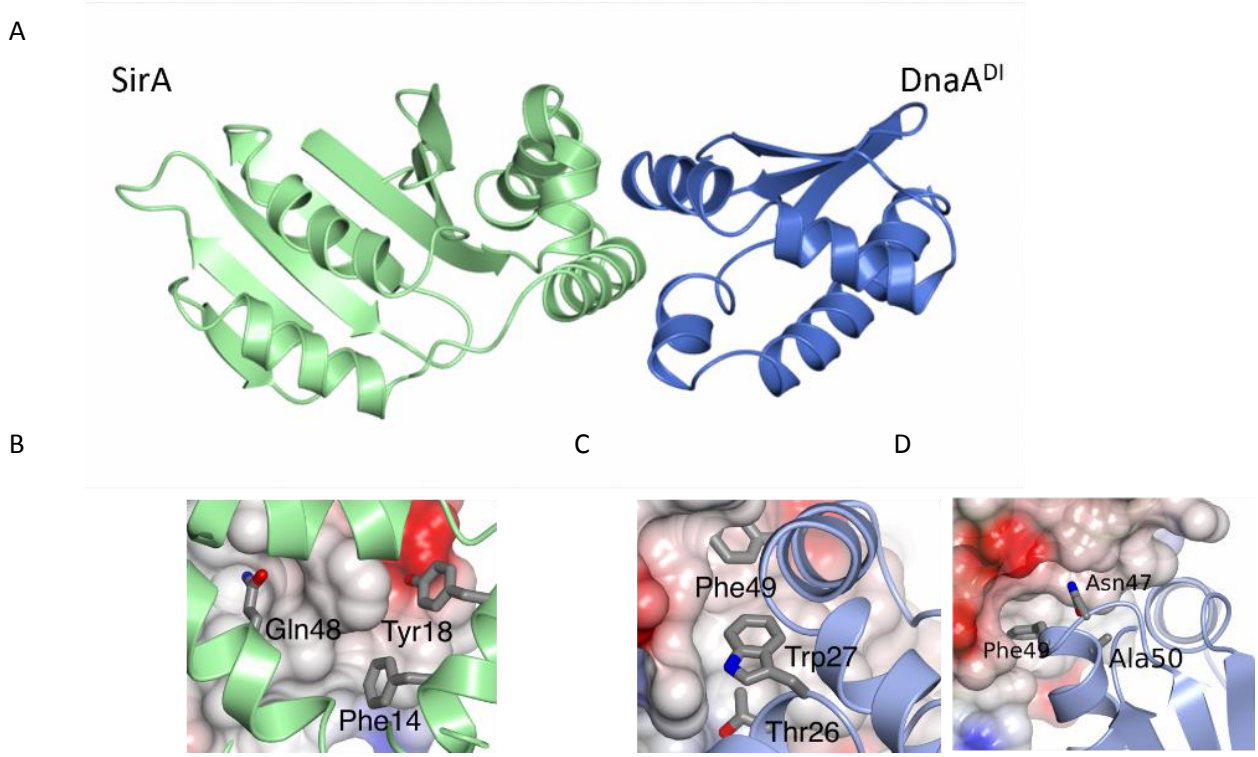


Figure 3.3.1. **Structure of SirA-DnaA^{DI} complex. A**. Ribbon diagram of SirA-DnaA complex with SirA shown in light green and DnaA^{DI} in blue B). **B** and **C.** Core residues from the DnaA ^{DI}-interacting surface of SirA. **B**. and the SirA interacting surface of DnaA^{DI}. **B**. DnaA^{DI} is shown as an electrostatic surface with SirA represented as a green ribbon with the side chains of F14, Y18 and Q48 displayed as cylinders. **C**. SirA is shown as an electrostatic surface with DnaA^{DI} represented as a blue ribbon with the side chains of T26, W27 and F49 displayed as cylinders. D) Mapping onto the structure of DnaA^{DI} the sites corresponding to mutations in *dnaA* that allow growth of *B. subtilis* when *sirA* is being overexpressed (Rahn-lee 2011). SirA is shown as a partially transparent electrostatic surface and DnaA^{DI} as a ribbon with the side chains of residues Asn47, Phe49 and Ala50 in cylinder format (taken from Jameson *et al*., 2014).

3.4 Substitutions at the SirA-DnaA^{DI} interface affect GFP-SirA localisation in vivo

SirA activity was examined *in vivo* to determine the physiological relevance of the proposed SirA-DnaA interface observed in the crystal structure. First, to investigate whether the residence of GFP-SirA at the replisome was dependent upon an interaction with DnaA, the wild-type *sirA* (in the context of the *gfp-sirA* chimera) was replaced with *sirA* mutants altering the residues identified in the SirA-DnaA^{DI} structure implicated in complex formation (*gfp-sirA^{F14A}, gfp-sirA^{Y18A}* or *gfp-sirA^{Q48A}*) and the localisation GFP-SirA proteins was determined during sporulation. All of the *sirA* mutants caused a significant decrease in the number of cells containing a fluorescent focus (Figure 3.4.1A). Both *gfp-sirA^{F14A}* and *gfp-sirA^{Y18A}* mutants reduced the number of GFP foci to background levels (i.e. – in the absence of a GFP fusion), while the *gfp-sirA^{Q48A}* mutant decreased foci formation 2.5-fold. These results indicate that the amino acid residues in SirA identified in the structure at the interface with DnaA^{DI} are required for GFP-SirA localisation at the replisome.

In a complementary approach I attempted to replace *dnaA* with *dnaA^{T26A}*, *dnaA^{W27A}*, and *dnaA^{F49A}*, however, I was unable to isolate any of these mutants (see Discussion). In contrast, mutations in *dnaA* at locations that were previously shown to inhibit SirA activity *in vivo* (*dnaA^{A50V}* and *dnaA^{N47H}*) could be readily generated; therefore GFP-SirA localisation was determined using these *dnaA* alleles. Figure 3.4.1B shows that both DnaA variants inhibited GFP-SirA foci formation, with DnaA^{A50V} reducing foci formation to background levels and DnaA^{N47H} decreasing foci formation 2.4-fold. Immunoblot analysis confirmed that the reduced GFP-SirA foci formation observed in SirA mutants and DnaA interaction-mutants is not due to proteolytic release of GFP (Figure 3.4.1C). Taken together with the

analysis of the *sirA* mutants, these results show that the SirA-DnaA^{DI} interface identified in the crystal structure is critical for GFP-SirA localisation *in vivo* and they suggest that GFP-SirA localisation is mediated through a direct interaction with replisome-bound DnaA. I attempted to directly determine whether GFP-DnaA colocalised with the replisome during sporulation, but unfortunately the previously published *gfp-dnaA* construct caused a severe sporulation defect (Soufo et al., 2008).

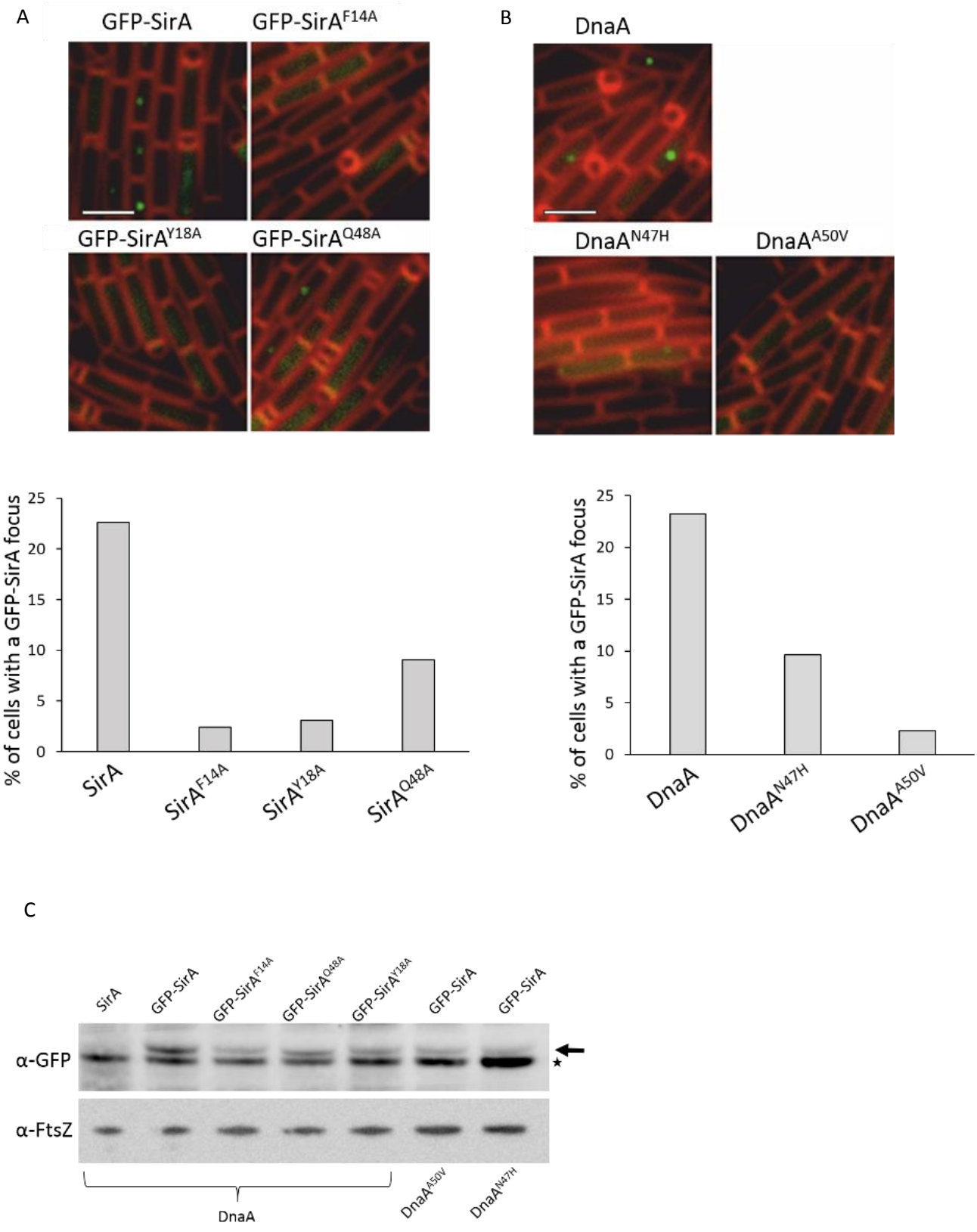
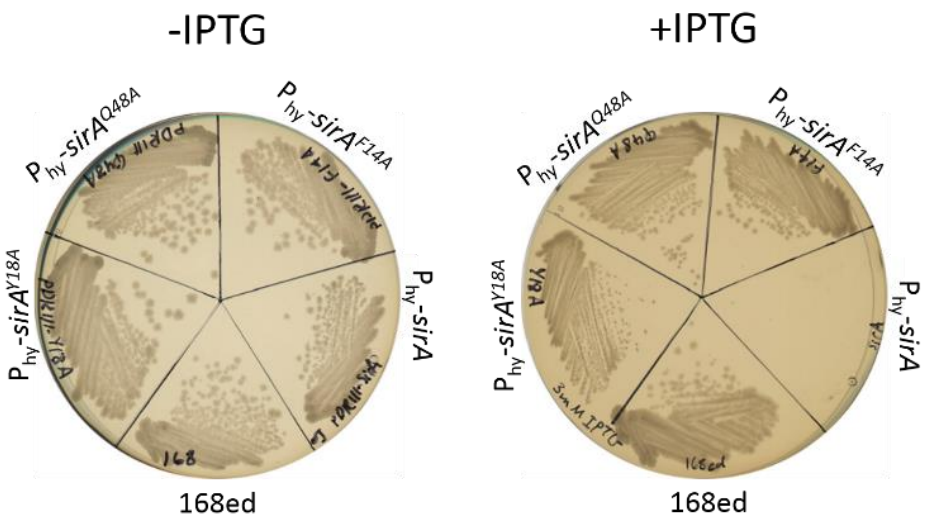

A
GFP-SirA
GFP-SirA^{F14A}
GFP-SirA^{Y18A}
GFP-SirA^{Q48A}
B
DnaA
DnaA^{N47H}
DnaA^{A50V}
% of cells with a GFP-SirA focus
0
5
10
15
20
25
SirA
SirA^{F14A}
SirA^{Y18A}
SirA^{Q48A}
% of cells with a GFP-SirA focus
DnaA
DnaA^{N47H}
DnaA^{A50V}
C
SirA
GFP-SirA
GFP-SirA^{F14A}
GFP-SirA^{Q48A}
GFP-SirA^{Y18A}
GFP-SirA
GFP-SirA
α-GFP
α-FtsZ
DnaA
DnaA^{A50V}
DnaA^{N47H}

Figure 3.4.1. Examination of the SirA-DnaA interface *in vivo*. A. Amino acid substitutions in SirA inhibit GFP-SirA foci formation. For each strain over 700 cells were analysed and the experiment was repeated at least three times. Quantification of a representative dataset is shown. Scale bar = 3 µm. *gfp-sirA* (NR3)*; gfp-sirA*^{F14A} (NR130)*; gfp-sirA*^{Y18A} (NR156)*; gfp-sirA*^{Q48A} (NR131). **B.** Amino acid substitutions in DnaA inhibit GFP-SirA foci formation. For each strain over 700 cells were analysed and the experiment was repeated at least three times. Quantification of a representative dataset is shown. Scale bar = 3 µm. *gfp-sirA* (NR3)*; gfp-sirA dnaA*^{A50V} (NR5)*; gfp-sirA dnaA*^{N47H} (NR154). **C.** Immunoblot analysis showing levels of GFP-tagged SirA proteins. Cell samples were taken 150 minutes post resuspension in starvation media. The arrow points to GFP-SirA, the star highlights a contaminating band. Immunoblot of FtsZ was utilised to standardise the amount of protein from different samples. *Wild-type* (168ed)*; gfp-sirA* (NR3)*; gfp-sirA*^{F14A} (NR130)*; gfp-sirA*^{Y18A} (NR156*); gfp-sirA*^{Q48A} (NR131)*; gfp-sirA dnaA*^{A50V} (NR5)*; gfp-sirA dnaA*^{N47H} (NR154).

3.5 Substitutions at SirA-DnaA^{DI} interface render cells resistant to lethal effects of SirA overexpression in vegetatively growing cells

To test whether SirA variants that displayed decreased foci formation were also defective in DnaA regulation, SirA proteins were overexpressed. Wild-type and mutant *sirA* genes were placed under the control of an IPTG-inducible promoter ($P_{hyperspank}$) integrated at an ectopic locus. Induction of wild-type SirA inhibited cell growth on solid media, in contrast to the SirA variants (SirA^{F14A}, SirA^{Y18A}, SirA^{Q48A}; Figure 3.5.1A). Furthermore, induction of wild-type SirA during vegetative growth in liquid media produced elongated cells that contained a single nucleoid, consistent with inhibition of DNA replication initiation (Figure 3.5.1B). Induction of SirA variants did not affect DNA distribution or cell morphology, and these cells were indistinguishable from a control strain lacking the ectopic *sirA* construct (Figure 3.5.1B). These results show that amino acid residue substitutions in SirA that impair protein localisation also affect the ability of SirA to inhibit DnaA activity.

A



B

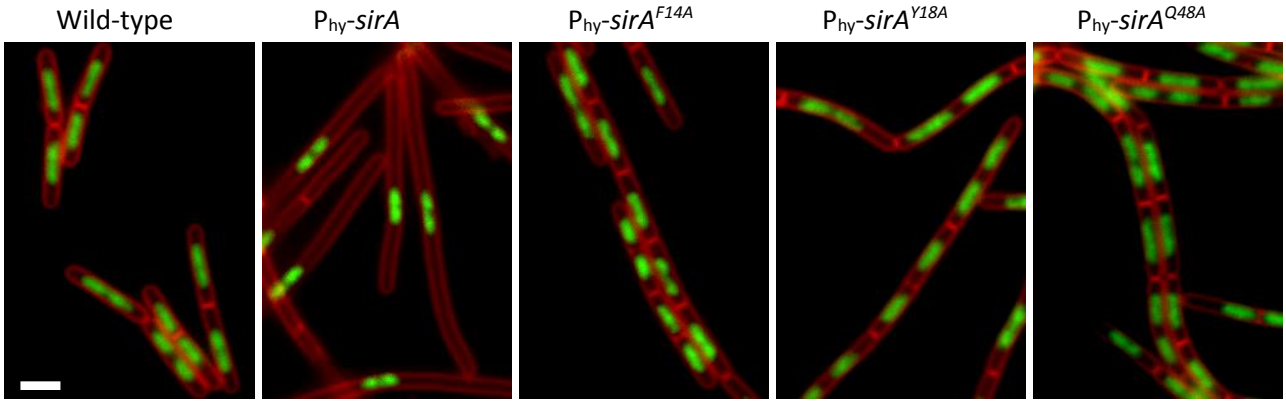


Figure 3.5.1. **Effect of overexpression of wild-type SirA and SirA variants on growth. A.** Wild-type and mutant *sirA* were placed under control of an IPTG-inducible promoter and streaked on nutrient agar plates in the presence and absence of IPTG (3 mM). Wild-type (168ed); $P_{hyperspank}$-*sirA* (NR171); $P_{hyperspank}$-$sirA^{F14A}$ (NR172); $P_{hyperspank}$-$sirA^{Q48A}$ (NR173); $P_{hyperspank}$-$sirA^{Y18A}$ (NR174). **B.** The effects of overexpressing various SirA proteins at the single cell level was studied by growing cells in liquid CH medium. The images were taken

180 minutes after induction of gene expression with IPTG (3 mM). Membrane dye FM5-95 was used to highlight the outlines of the cells, DAPI was used to stain the DNA. Scale bar = 3 µm. Wild-type (168ed); $P_{hyperspank}$-*sirA* (NR171); $P_{hyperspank}$-$sirA^{F14A}$ (NR172); $P_{hyperspank}$-$sirA^{Q48A}$ (NR173); $P_{hyperspank}$-$sirA^{Y18A}$ (NR174).

Discussion

The initiator of bacterial DNA replication, DnaA, is stringently regulated so that DNA replication is coordinated with cell growth and differentiation. Five negative regulators of DNA replication have been identified in *B. subtilis:* SirA (Rahn-Lee et al., 2011), YabA (Noirot-Gros et al., 2006), Spo0A (Castilla‐Llorente et al., 2006) Soj (Murray and Errington, 2008) and DnaD (Bonilla and Grossman, 2012). YabA, Soj and DnaD bind to domain III of DnaA and are thought to block the assembly of helical DnaA filaments at *oriC* (Cho et al., 2008, Scholefield et al., 2012, Scholefield and Murray, 2013). Phosphorylated Spo0A binds to a set of Spo0A-boxes at *oriC* which overlap with DnaA-boxes, suggesting that Spo0A~P occludes DnaA from the replication origin (Boonstra et al., 2013).

SirA is distinct and represents the first, and so far only, *B. subtilis* regulator that interacts with domain I of DnaA (Rahn-Lee et al., 2011). In other organisms however, regulators have been identified which interact with DnaA^{DI} namely, *E. coli* DiaA (Keyamura *et al.*, 2009) and Hda (Su'etsugu *et al.*, 2013), and *H. pylori* HobA (Natrajan et al., 2009). For *E. coli*, DnaA^{DI} has also been shown to interact with the DNA helicase, DnaB (Sutton et al., 1998, Seitz et al., 2000), and to play a role in the oligomerisation of DnaA (Weigel et al., 1999, Felczak et al., 2005), forming dimers *in vitro* (Abe et al., 2007). Here we took advantage of the structure of the SirA-DnaA^{DI} complex from *B. subtilis* elucidated by Jameson *et al*., revealing DnaA^{DI} bound in an inhibitory complex. This structure complements that of HobA-DnaA^{DI} from *H. pylori* in which DnaA^{DI} is bound in a complex that leads to activation(Natrajan et al., 2009).

SirA binds to a site on DnaA^{DI} that corresponds closely to that bound by the regulators HobA from *H. pylori* (Natrajan *et al.*, 2009) and DiaA from *E. coli* (Keyamura et al.,

2009). HobA and DiaA are structural homologs which form tetramers that promote DnaA oligomerisation and activate the initiation of DNA replication (Zawilak-Pawlik et al., 2011). Each HobA/DiaA tetramer binds to four $DnaA^{DI}$ molecules in a way that is thought to facilitate DnaA-binding to the array of DnaA-boxes distributed at *oriC* (Natrajan et al., 2009). In marked contrast, monomeric SirA appears to bind a single molecule of $DnaA^{DI}$ to inhibit DNA replication initiation. Although SirA and HobA/DiaA have quite different three dimensional structures, each buries a structurally equivalent site on $DnaA^{DI}$. It is intriguing therefore that this elicits different regulatory outcomes.

It has been previously proposed that SirA inhibits DnaA binding to *oriC*, based on the observations that SirA disrupts DnaA-GFP localisation at *oriC* during vegetative growth, and that there is a SirA-dependent decrease in the amount of DnaA at *oriC* following artificial induction of sporulation (Rahn-Lee et al., 2011, Wagner et al., 2009). At first glance, SirA may achieve this by inhibiting DnaA-oligomerisation at *oriC*, since domain I fragments of *E. coli* DnaA form dimers *in vitro,* and the dimerisation surface has been identified (Felczak et al., 2005). However, the corresponding surface in *B. subtilis* $DnaA^{DI}$ is located on the opposite side of $DnaA^{DI}$ to the SirA binding surface so that SirA binding would not be expected to prevent dimer formation. Furthermore, the biochemical analysis of $DnaA^{DI}$ did not indicate dimers or oligomers formation by *B. subtilis* $DnaA^{DI}$ *in vitro* (Jameson et al., 2014). Thus, it seems unlikely that SirA influences DnaA assembly simply by inhibiting $DnaA^{DI}$-$DnaA^{DI}$ interactions.

My localisation studies indicate that SirA accumulates away from *oriC* and with the replisome near mid-cell during sporulation. However, a negligible frequency of SirA localisation with the *oriC* (in 8% of total cells with a GFP-SirA foci) in the population. This

could represent the transient window of time during cell cycle at which DnaA is enriched at the *oriC* as reported by Soufo et al., (2008).

I hypothesise that SirA could interact with replisome-bound DnaA proteins to stabilise replisome-DnaA complexes, thereby inhibiting DnaA rebinding at the origin. This is reminiscent of a DnaA-tethering model proposed for YabA(Soufo et al., 2008). However, my data shows that the frequency of GFP-SirA foci formation and SirA regulatory activity is not alleviated in absence of YabA and that the Δ*yabA* mutant exhibits a shift in the GFP-SirA foci from midcell to a polar position (Figure 3.2.1C). Interestingly, this shift in the position of GFP-SirA foci appearance in the Δ*yabA* mutant does not lead to an increase in colocalisation of GFP-SirA foci with the *oriC*. These findings and the absence of GFP-SirA foci formation in DnaN-mCherry Δ*yabA* background (which could be due to additive alleviation of DnaA accumulation at the replisome due to tagging of DnaN at C-terminal and the absence of YabA) collectively indicate that further experiments are required to understand the basis for SirA localisation and its dependence upon DnaA.

My finding that mutations directing alanine substitutions at the SirA binding surface of DnaA could not be introduced *in vivo* suggests that SirA could act by inhibiting a critical interaction of DnaA with other components of the initiation complex. In *E. coli*, $DnaA^{DI}$ is implicated directly in the recruitment of the helicase to the nascent replicative complex(Abe et al., 2007). There is no evidence for a DnaA-helicase interaction in *B. subtilis* however, in which helicase recruitment involves additional DNA remodelling proteins (Zhang et al., 2005). DnaD forms multimeric scaffolds on the DNA and recruits DnaB, which in turn is thought to bridge an interaction with the helicase-helicase loader(Zhang et al., 2008, Smits

et al., 2010). Thus, I speculate that SirA may inhibit a DnaA-DnaD interaction arresting assembly of the initiation complex.

In summary, this work has demonstrated that SirA localises at the replisome in a DnaA-dependent manner in sporulating *B. subtilis* cells and has provided important information to help elucidate the mechanism of action of SirA, the understanding of which is currently limited by imprecise knowledge of the function of domain I of DnaA in this organism.

Chapter 4: Regulation of DNA replication initiation is required for proper chromosome segregation during spore development

Introduction

The regulation of DNA replication initiation is important for proper coordination of DNA synthesis with cell cycle events during spore development in *B. subtilis*, as timely inhibition of replication initiation is required for maximal sporulation frequency and spore fitness (Veening et al., 2009). It is thought that both vegetatively expressed regulatory proteins (Soj, YabA and DnaD) and developmental specific factors (SirA and Spo0A) inhibit DnaA activity to prevent reinitiation in sporulating cells (Rahn-Lee et al., 2009, Castilla-Llorente et al., 2006, Boonstra et al., 2013, Noirot-Gros et al., 2002, Murray and Errington, 2008, Bonilla and Grossman, 2012).

In bacteria DNA replication and segregation occur simultaneously, such that following the initiation of DNA synthesis the newly replicated origin regions are actively segregated away from one another. In *B. subtilis* segregated chromosomes adopt an *ori-ter* configuration where the replication origin regions are located near opposite cell poles (Wang 2014). DNA replication initiates at these polar sites, followed by the condensin-dependent movement of the duplicated origins towards mid-cell. The origin regions are then actively segregated away from each other towards the cell poles in a reaction facilitated by the Soj-Spo0J-*parS* partitioning system.

Sporulation in *B. subtilis* requires a diploid intermediate, as each cell type (mothercell and forespore) requires a copy of the chromosome to drive the developmental process. Prior to asymmetric cell division the replication origin regions are tethered to the cell pole by the RacA-DivIVA complex in an exacerbated *ori-ter* conformation. This

chromosomal arrangement helps to ensure that the origin region specifically becomes trapped in the forespore, a prerequisite for σ^F-driven gene expression.

It has been suggested that Soj plays a role in origin trapping during sporulation. First, a population based reporter assay indicated that a Δ*soj* Δ*racA* double mutant created a broad synthetic origin trapping defect (Wu 2003). Second, a single cell reporter assay indicated that a Δ*soj* mutant alone displayed a specific defect in trapping the *oriC* proximal region (Sullivan 2009). Because Soj has been shown to affect both DNA replication (via DnaA) and origin segregation (via the Spo0J-*parS* system), and because DNA replication is intimately connected with chromosome segregation, it was unclear whether the origin trapping defect observed in a Δ*soj* mutant was caused by misregulation of DNA replication, DNA segregation, or both.

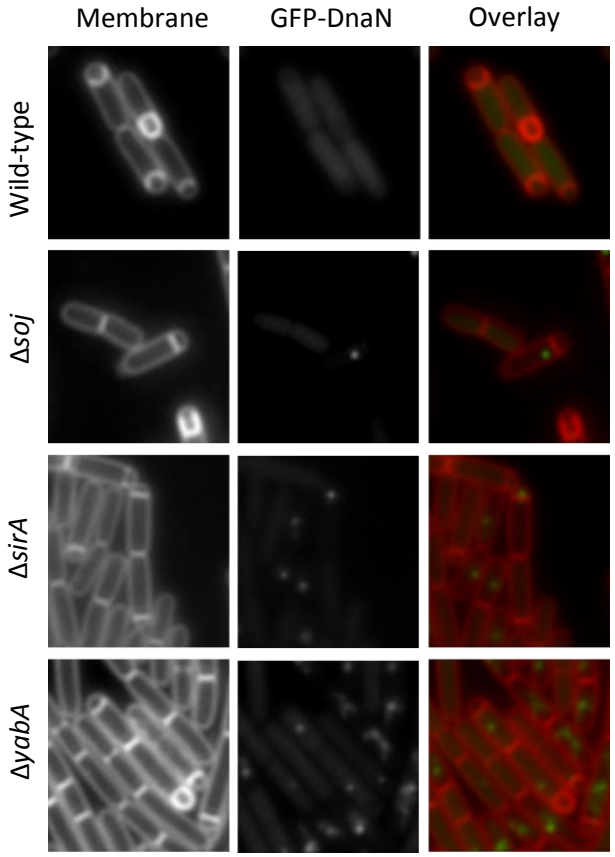
The aim of this chapter is to shed light on the importance of regulation of DNA replication initiation for *oriC* trapping during sporulation of *B. subtilis*. The results indicate that timely control of DNA replication initiation is required for accurate chromosome segregation during spore development. In addition the results strongly suggest that Soj likely influences *oriC* trapping through its roles in both DNA replication and segregation.

Results

4.1 Ongoing DNA replication is observed in cells that have initiated sporulation in absence of DnaA regulators

To begin testing whether ongoing DNA replication reinitiation in cells committed to sporulate would affect chromosome origin trapping, the presence of DNA replication in cells lacking DnaA regulatory proteins (SirA, Soj, or YabA) was analysed utilising a strain that contained a fusion of a green fluorescent protein to the sliding clamp component of the replisome (GFP-DnaN). In cells with ongoing DNA replication, GFP-DnaN forms a focus at the replisome. Cells harbouring GFP-DnaN and replication regulator mutants were induced to sporulate and ongoing DNA replication was determined in sporulating cells containing an asymmetric septa.

In the wild-type strain only 1.4% of cells with an asymmetric septa contained a GFP-DnaN focus (Figure 4.1.1). In contrast the percentage of sporulating cells with ongoing DNA replication was increased in Δ*soj* and Δ*sirA* 3- to 4-fold (Figure 4.1.1), while the Δ*yabA* mutant showed a significantly greater 15-fold increase in ongoing DNA replication. While in Δ*soj* and Δ*sirA* mutants almost all cells with ongoing replication had a single GFP-DnaN focus, multiple GFP-DnaN foci were observed in the Δ*yabA* mutant, suggesting that in the absence of YabA DNA replication is reinitiated at a high frequently.



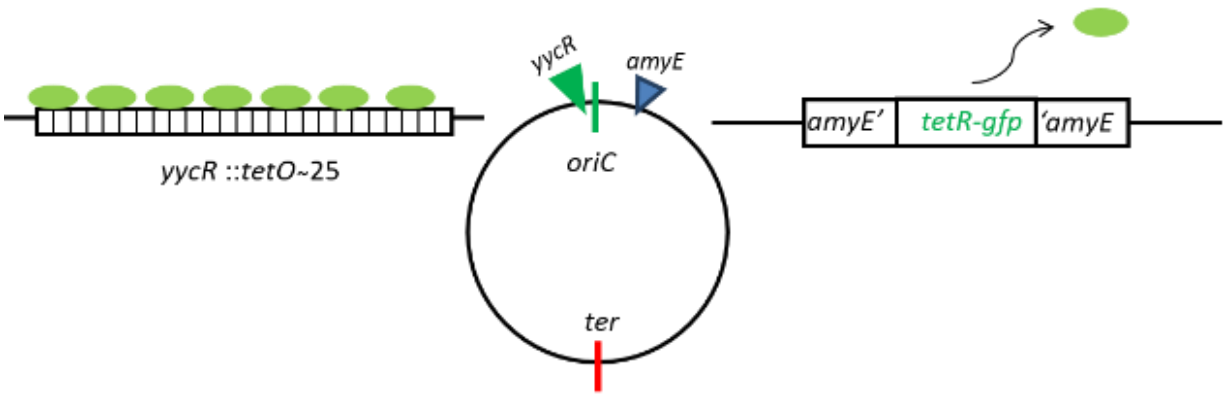
% of sporulating cells with GFP-DnaN foci		
Exp 1	Exp 2	Exp 3
2	1.1	1.1
4.3	5.3	5.1
6	5.7	5.2
17.4	21	26

***Figure 4.1.1*. Ongoing DNA replication wild-type and cells lacking regulators of DnaA.** Cells were induced to sporulate by resuspension in nutrient poor media, microscope images were taken 180 minutes after induction of sporulation. The experiment was repeated at least three times and each time >300 cells were analysed. Representative images are shown in the left pane, the results for each experiment are shown to the right. The average the percentage of sporulating cells with an ongoing DNA replication for each strain are as follows: wild-type 1.4 ±0.5%; Δsoj 4.9±0.5%; ΔsirA 5.6±0.4%; ΔyabA 21.3±4.5 %. FM5-95 membrane stain was used to highlight the outline of the cells. gfp-dnaN (MS104); gfp-dnaN Δsoj (NR42); gfp-dnaN ΔsirA (NR34); gfp-dnaN ΔyabA (NR34).

4.2 Mutants that lack regulators of DnaA exhibit origin trapping defect

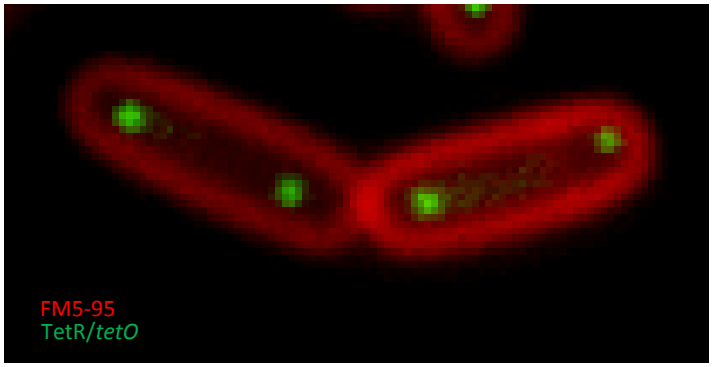
Having shown that inactivation of SirA, Soj or YabA leads to increased DNA replication in sporulating cells (this was independently confirmed with counting number of *oriC* marker per cell in these mutants; data not shown), next chromosome origin segregation was analysed at the single cell level. To do this a strain was constructed that allowed visualisation of replication origin regions in cells undergoing sporulation. This strain contained an array of ~25 *tet* operators (*tetO*$_{25}$) inserted near *oriC* (~80 kb to the left) and a fusion of *tet* repressor with *gfp* (TetR-GFP) inserted at the *amyE* locus (Figure 4.2.1A). The interaction of TetR-GFP with the *tetO* array creates a fluorescent point signal near the replication origin. In vegetatively growing cells, the *oriC* proximal TetR-GFP foci were typically well separated and localised towards the cell poles (Figure 4.2.1B)(Glaser, 1997). In sporulating cells (determined by visualisation of the cell membrane by staining with a hydrophobic dye; FM5-95) proper chromosomal origin segregation and tethering of the origin regions to the cell poles by RacA prior to asymmetric septation leads to localisation of one TetR-GFP origin marker in the forespore compartment and the other in the mother cell (Figure 4.2.1C). Under these conditions the absence of a TetR-GFP focus within the forespore is classified as an origin trapping defect.

A



B

Vegetative



C

Sporulating

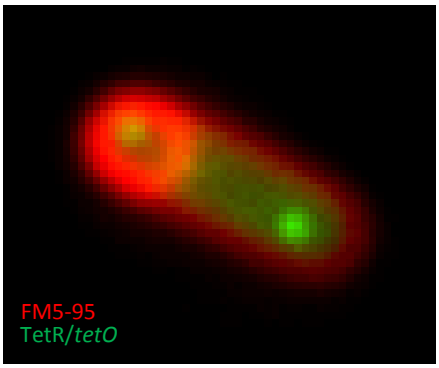
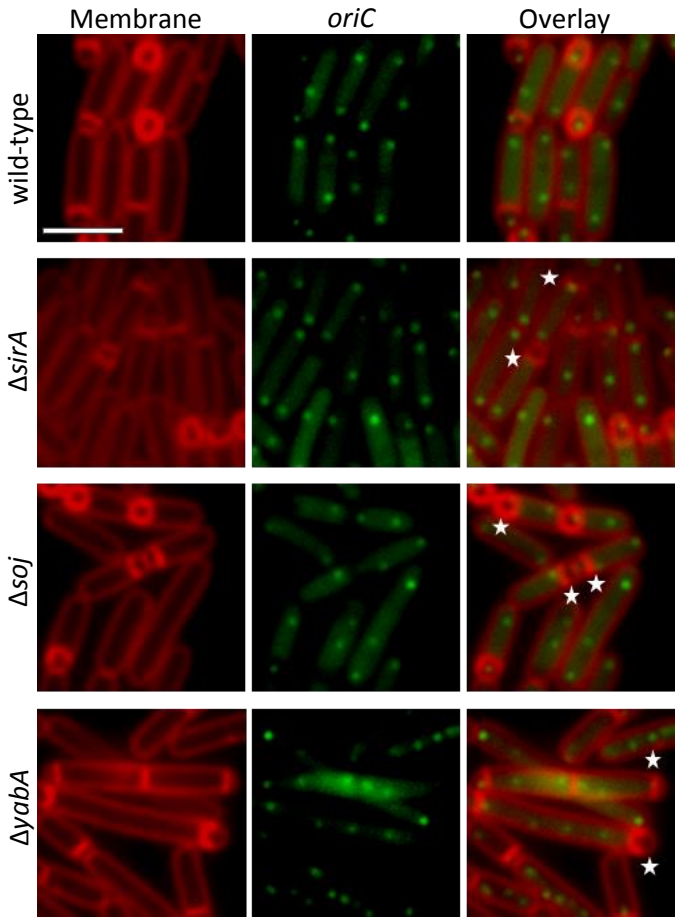


Figure 4.2.1. *tetO*/TetR-GFP origin marker system. A tandem array of 25 *tet* operators (*tetO*) was inserted to an origin proximal region next to *yycR* gene. TetR-GFP fusion was expressed from the *amyE* locus. Binding of TetR-GFP to the *tetO* array allowed visualization of the origin regions in the GFP channel. B) Cells shown during vegetative growth with the single GFP focus formed at the *oriC* per nucleoid, the outline of the cells are highlighted using FM5-95 membrane stain. C) A sporulating cell is shown, the asymmetric septa has divided the cell into a forespore (smaller compartment) and a mother cell (the larger compartment). A GFP focus marking the *oriC* is captured in the forespore compartment and another is visible in the mother cell. *oriC*$^{tetO25/TetR\text{-}GFP}$ (AK47).

Sporulation was induced by nutrient deprivation (resuspension method, as described in methods section) and the origin trapping status of DNA replication regulator mutants was determined and compared to the wild-type at 180 minutes after induction of sporulation (Figure 4.2.2). At this time point the number of cells that developed a forespore peaks, with approximately a 25% of cells forming asymmetric septa.

This analysis showed that the absence of any of the DnaA regulatory proteins causes an origin trapping defect. The average origin trapping results obtained from two independent experiments (in each experiment >250 sporulating cells were analysed) indicated that the Δ*soj* mutant consistently exhibits the highest defect in origin trapping with more than 5-fold the number of cells lacking an origin marker in the forespore compartment in comparison to the wild-type. In comparison the Δ*yabA* and Δ*sirA* mutants produced milder changes, with 3.6-fold and 2-fold increases in forespores missing a TetR-GFP focus, respectively. These results suggest that the regulation of DnaA is required for proper origin trapping within the forespore of sporulating cells.

A



	% of sporulating cells with an origin trapping defect	
	Exp 1	Exp 2
wild-type	2.9	3.5
Δ*sirA*	6.7	7.1
Δ*soj*	18.1	17.2
Δ*yabA*	11	11.9

B

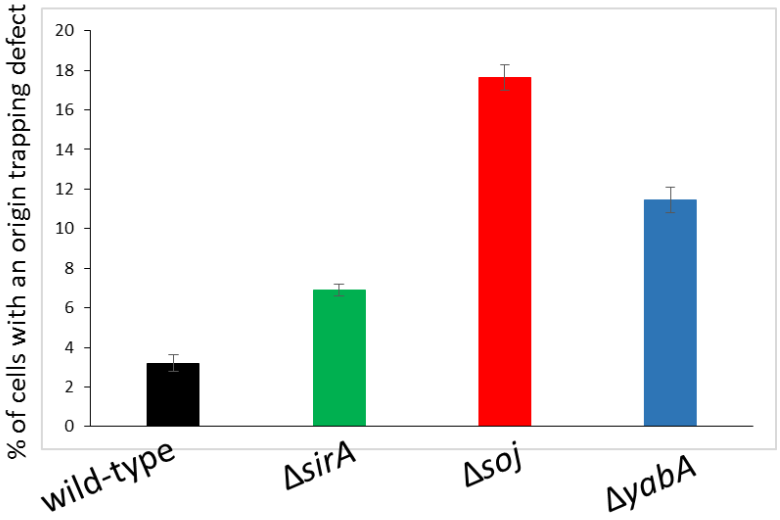


Figure 4.2.2. Cells in which a regulator of DnaA has been deleted exhibit increased origin trapping defect. A. Images of sporulating cells; wild-type, Δ*sirA*, Δ*soj* and Δ*yabA*. Asterisks highlight the forespores with an origin trapping defect. Cells were induced to sporulate using resuspension method. Images of cells were taken 180 minutes after induction of sporulation. The experiment was repeated twice and for each strain over >250 cells were analysed. The results obtained from two independent experiments are shown next to image of the respective strain. Scale bar = 3 µm. **B.** A graph of representative dataset illustrating the origin trapping defect in wild-type, Δ*sirA*, Δ*soj* and Δ*yabA*. *oriC*$^{tetO25/TetR\text{-}GFP}$ (wild-type/ parent AK47); Δ*sirA oriC*$^{tetO25/TetR\text{-}GFP}$ (NR64); Δ*soj oriC*$^{tetO25/TetR\text{-}GFP}$ (NR48); Δ*yabA oriC*$^{tetO25/TetR\text{-}GFP}$ (NR57).

4.3 Sporulation initiation is delayed in the Δ*yabA* mutant

It was reproducibly observed that the Δ*yabA* mutant produced fewer asymmetric septa compared to the wild-type strain and other mutants (either Δ*soj* or Δ*sirA*). Two hours after induction of sporulation the number of cells that had initiated sporulation (as judged by asymmetric septation) in the Δ*yabA* mutant was about 5-fold lower than the wild-type strain (Table 4.3.1). As noted earlier the absence of YabA during sporulation leads to a significant increase in cells with an asymmetric septa that also exhibit ongoing DNA replication (4.1.1). Previous work has shown that pulsatile activation of the Sda checkpoint inhibits sporulation in response to DNA replication initiation (Veening et al., 2009).

To determine whether the failure to regulate DNA replication initiation in the Δ*yabA* mutant leads to activation of Sda checkpoint and thereby delays initiation of spore development, the frequency of asymmetric septation was determined in a Δ*yabA* Δ*sda* double mutant (Figure 4.3.1). The results showed that deletion of *sda* in the Δ*yabA* mutant restored the normal timing of asymmetric septa to be near that of the wild-type. Furthermore, sporulation frequency was modestly decreased in a Δ*yabA* mutant and this effect was reversed in the absence of Sda (Table 4.3.1). These results are consistent with the model that the Sda checkpoint is activated in the Δ*yabA* mutant.

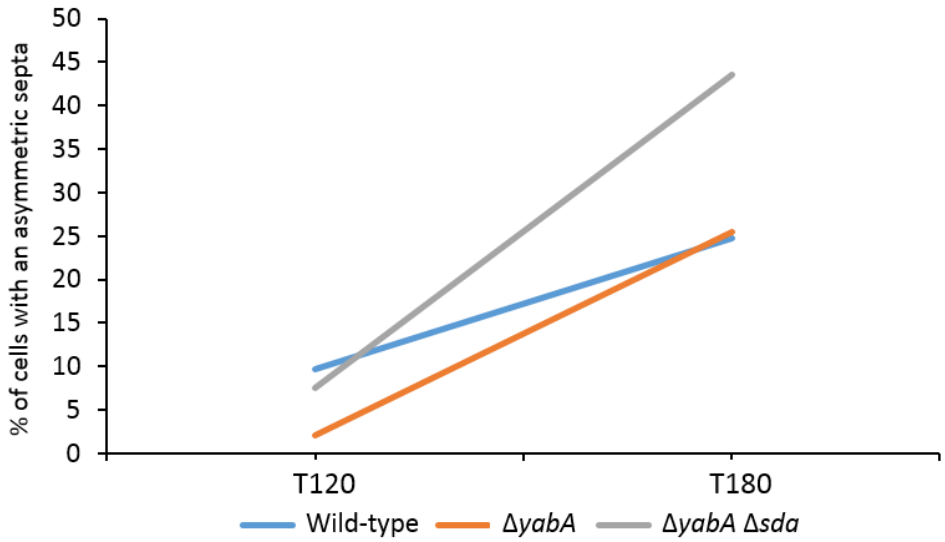


Figure 4.3.1. **Δ*yabA* mutant exhibits a delay in asymmetric septa formation compared to the wild-type**. Cells were induced to sporulate by resuspension in starvation medium, microscope images were taken 120 and 180 minutes after induction of sporulation and the number cells with an asymmetric septa was determined for each time point. To visualise the membrane, FM5-95 membrane stain was used. >300 cells were analysed for each sample. Representative data from two experiments are shown. 168ca (wild-type); Δ*yabA* (NR19); Δ*yabA* Δ*sdaA* (NR134).

Table 4.3.1. The *ΔyabA* mutant exhibits reduced sporulation frequency

strain	Sporulation frequency Experiment 1	Sporulation frequency Experiment 2	Average sporulation frequency	Standard Deviation
wild-type	63%	62%	62.5%	1
Δ*sdaA*	44%	52%	48%	6
Δ*yabA*	16%	25%	20.5%	6
Δ*yabA* Δ*sda*	56%	68%	62%	8

Cells were induced to sporulate by resuspension in starvation media, 24 hours after induction of sporulation the number of total colony forming units and spores (heat resistance was used to determine the number of spores per mL) were determined for one mL of cell culture. Representative data from two experiments is shown. Wild-type (168ca); Δ*sda* (HM908); Δ*yabA* (NR19); Δ*yabA* Δ*sda* (NR134).

4.4 Chromosomal origin trapping in DnaA variants resistant to regulation

4.4.1 DnaA variants that do not respond to regulatory factors are defective in replication origin capturing in the forespore

To test whether the origin trapping defects exhibited in the cells lacking regulators of DnaA were due to a direct result of DnaA over activity and not additional roles that DnaA regulators play in chromosomal origin segregation (particularly Soj), a genetic screen was employed to identify mutations in *dnaA* that render cells defective in faithful trapping of the origin region in the forespores. A sensitive genetic screening system (developed by Wu and Errington 1998 and modified by Tomas Kloosterman, unpublished Figure 4.4.1) allows detection of clones displaying an origin trapping defect as judged by blue/white colony selection. The strain used for the screen contains a σ^F-inducible *lacI* inserted near *oriC* (*yxiF* at -16°) and a σ^F-inducible/LacI-repressible *lacZ* at a genomic site away from the *oriC* (at *amyE*; +28°) but within the region normally trapped in the forespore. Both of these loci are normally trapped in the forespore compartment upon formation of the asymmetric septum, which leads to repression of *lacZ* and white colonies (Wu et al., 1998, Wu and Errington, 2003, Sullivan et al., 2009). The reporter strain also contains the *spoIIIE36* allele that cannot translocate the remainder of chromosome into the forespore (Wu and Errington, 1994b), thus allowing assessment of the origin trapping at the time of asymmetric cell division. Cells that specifically fail to trap the *lacI* gene in the forespore appear blue on nutrient agar plates containing the indicator X-gal.

In order to create mutations in *dnaA*, a library was generated using error-prone PCR with a template that harboured a chloramphenicol resistance cassette downstream of the *dnaAN* operon (Scholefield et al., 2012). PCR products were directly transformed into the

reporter strain and colonies that turned blue in the presence of the LacZ indicator X-gal were restreaked onto indicator plates side-by-side with the parent strain (Figure 4.4.2A). The genomic DNA from the blue colonies was backcrossed into the parent strain to confirm that the mutations causing the origin trapping defect were linked to *dnaA*. Sequencing of *dnaA* identified several distinct mutations that caused single amino acid substitutions in DnaA (Figure 4.4.2A).

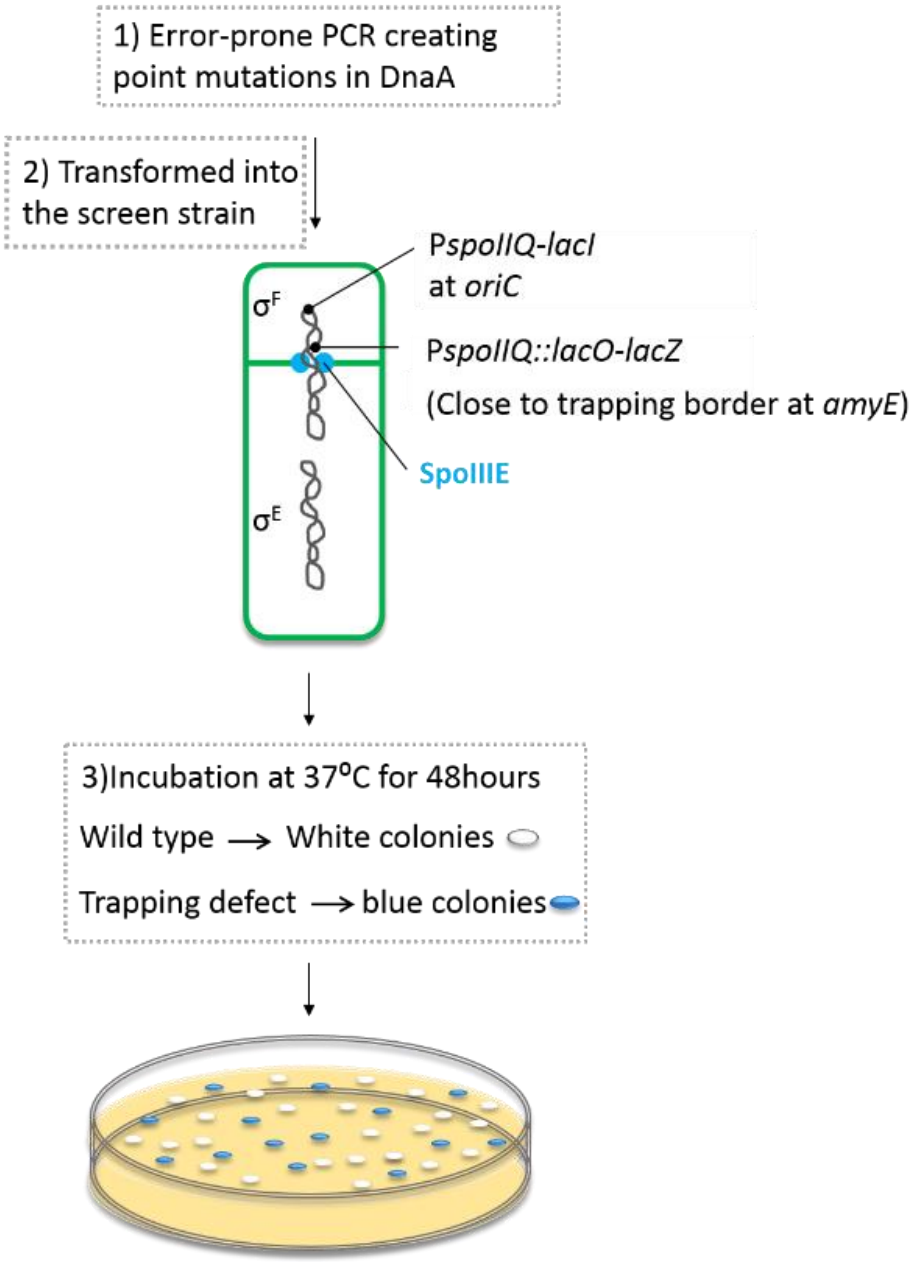


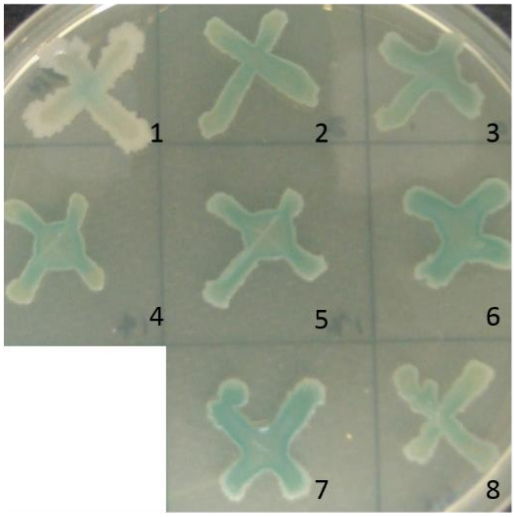
Figure 4.4.1. **Schematic diagram showing the origin trapping screen, blue white selection.** A *dnaA* library was generated by amplification of *dnaA* in an error-prone PCR. This dnaA library was used to transform a screen strain that contained σ^F-inducible *lacI* inserted near *oriC* (at -16°) and σ^F-inducible/*LacI*-repressible *lacZ* near the trapping border at *amyE* locus(at +28°).The transformants were plated on nutrient agar plates containing X-gal (final concentration 0.008%) and were incubated at 37°C for 48 hours. Cells that have correctly

captured the regions surrounding *oriC* appear white/very light blue and cells that have failed to do so appear dark blue.

Intriguingly, mapping of the mutations identified in the screen to the structure of DnaA revealed that the amino acid substitutions conferring to origin trapping defects were located in regions of DnaA previously identified to contribute to interactions with DnaA regulatory proteins SirA, Soj or YabA (figure4.4.2A and B)(Scholefield et al., 2012, Scholefield and Murray, 2013, Rahn-Lee et al., 2011).

The amino acid substitutions identified in the screen that were located in domain I of DnaA (DnaA^{A50V}, DnaA^{A50T} and DnaA^{N47D}) are near the interface where SirA binds (Jameson 2014; Rahn-Lee et al., (2011)). Amino acid substitutions were also identified in domain III of DnaA that clustered near the proposed binding site for Soj (DnaA^{L294H} and DnaA^{Y325H} (Scholefield et al., 2012) and YabA (DnaA^{M161I} (Scholefield and Murray, 2013)).

A



Strain number	Nucleotide change	Amino acic substitution
#1	.....	Wild-type
#2	T881A	L294H
#3	G483A	M161I
#4	C711G	H237L
#5	C149T	A50V
#6	A139G	N47D
#7	T973C	Y325H
#8	G148A	A50T

B

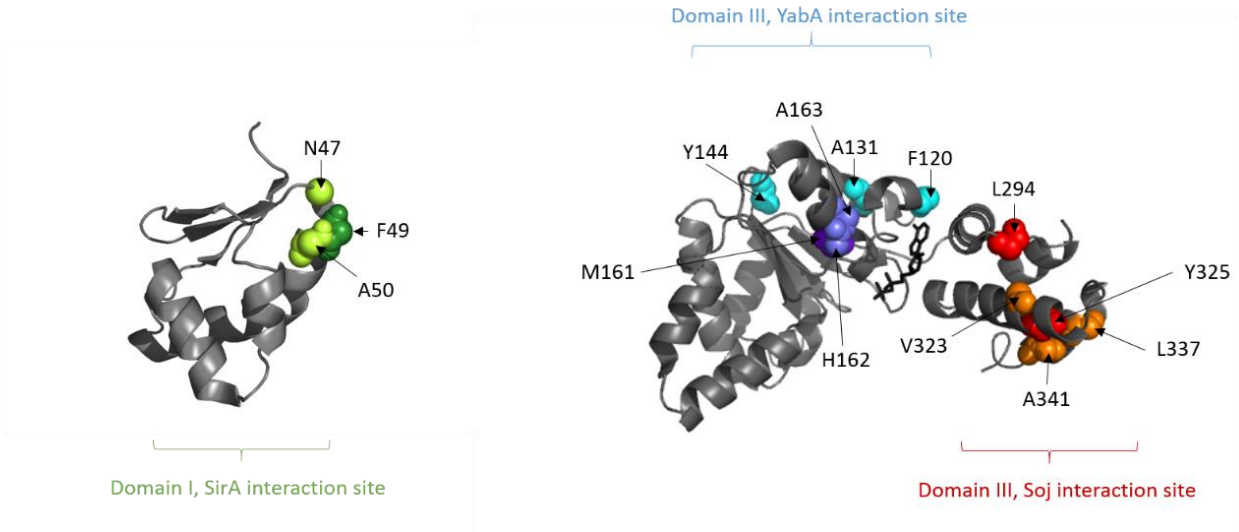


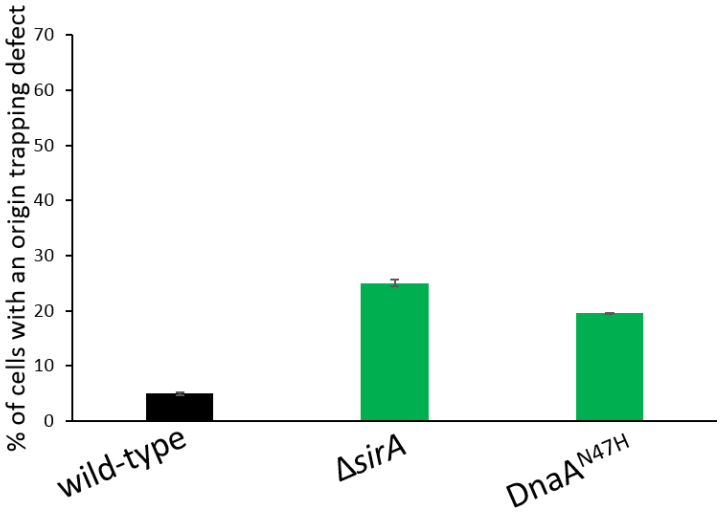
Figure 4.4.2. **Strains isolated in the origin trapping screen harbour mutations at/near regions of DnaA previously identified as regulator interaction determinants. A.** strains isolated in blue /white trapping assay were streaked on X-gal containing plates and incubated at 37°C for at least 48 hours. The table indicates the nucleotide changes identified in the DnaA gene sequence and the resulting amino acid substitution for each strain isolated. **B.** A ribbon diagram DnaA domain I (on left) (PDB ID: *E. coli* domain I – 2E0G (Abe et al., 2007) and DnaA domain III (on right *A. aeolicus* domain III PDB ID:2HCB (Erzberger et al., 2006)) are shown, ADP is indicated as black stick. SirA interacts with DnaA domain I, on DnaA domain I diagram, a cluster of interaction determinants of SirA are highligted with

green sphears. N47, F49 and A50 residues were previously identified by Rahn Lee *et al*., (2011), N47 and A50 residues (shown in light green) were also detected in the blue/white origin trapping assay. YabA and Soj both interact with Domain III of DnaA. On the DnaA domain III, the interaction determinants Soj and YabA are highlighted.YabA interaction determinants shown as cyan spheres were identified in a study conducted by Cho *et al*., (2008), and the slate blue spheres represent YabA binding determinants identified by Scholefield et al .,(2013), residue M161 shown in purple was identified by origin trapping screen to be in a cluster with the residues reported by Scholefield *et al*.,(2013). Soj interaction determinants identified by Scholefield et al., (2012) are shown in orange and red, the red sphears represent; Y325 and L295 residues that were also detected in the origin trapping screen.

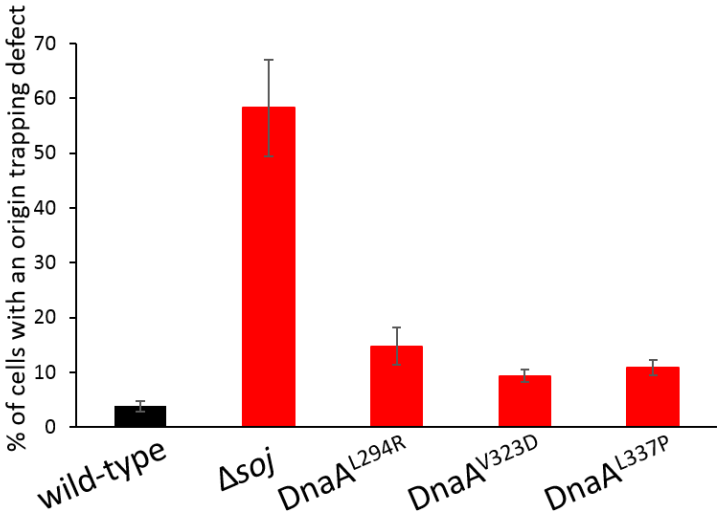
In order to quantitatively determine the origin trapping defect exhibited by DnaA variants that disrupt interactions with known regulatory proteins (Cho et al., 2008, Rahn-Lee et al., 2011, Scholefield et al., 2012, Scholefield and Murray, 2013), *dnaA* mutants were transformed into a reporter strain containing the TetR-GFP/*tetO* origin marker system and *spoIIIE36*. Cultures were induced to sporulate by nutrient deprivation and origin trapping status was determined with respect to the parent strain at 180 minutes after induction of sporulation (figure 4.4.3). Strikingly, all of the DnaA variants exhibited a small origin trapping defect, indicating that regulation of DNA replication initiation is required for proper chromosomal origin trapping in sporulating cells. It is worth noting that in this reporter strain containing the *spoIIIE36* allele the origin trapping defects of the Δ*sirA* and Δ*soj* mutants was more profound then previously observed (figure 4.4.3A and B), suggesting that origin trapping defects are compensated for/alleviated by the efficacy of the SpoIIIE DNA translocase. Unfortunately, the trapping defect of the Δ*yabA* mutant could not be determined in the context of *spoIIIE36* because the cells grew poorly upon induction of sporulation (figure 4.4.3C).

Interestingly, a significant difference was observed between the origin trapping defect of the Δ*soj* mutant and the DnaA variants that perturb the interaction with Soj (Scholefield et al., 2012). While the *dnaA* mutants produced 3-fold more forespores without a TetR-GFP focus, the Δ*soj* mutant caused a 13-fold increase. This suggests that the effect of Soj on faithful origin trapping in forespores is not solely through regulation of DNA replication initiation, consistent with data indicating that Soj plays an important role in chromosomal origin segregation.

A


% of cells with an origin trapping defect
70
60
50
40
30
20
10
0
wild-type
ΔsirA
DnaA^N47H

B


% of cells with an origin trapping defect
70
60
50
40
30
20
10
0
wild-type
Δsoj
DnaA^L294R
DnaA^V323D
DnaA^L337P

C

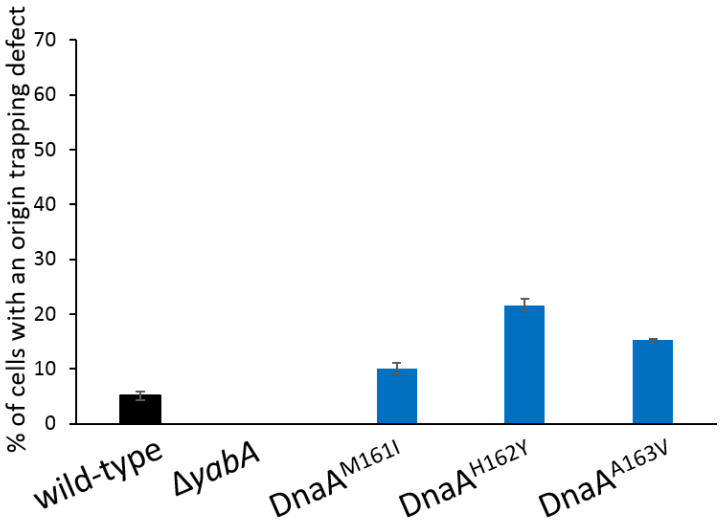

% of cells with an origin trapping defect
70
60
50
40
30
20
10
0
wild-type
ΔyabA
DnaA^M161I
DnaA^H162Y
DnaA^A163V

Figure 4.4.3. **Δ*sirA* and *Δsoj* null mutants and DnaA variants resistant to regulation by SirA, Soj or YabA exhibit reduced constancy in chromosomal origin trapping in the forespores.** **A.** *sirA* deletion or mutations in domain I of DnaA disrupting the regulation of DnaA by SirA causes origin trapping defect. Cells were induced to sporulate by resuspension method, 180 minutes after induction of sporulation images were taken and analysed. The experiment was repeated at least twice, analysing >200 cells each time. A graph showing representative dataset is shown on the left pane and results from two independent experiments are shown on the right. *spoIIIE36 oriC*$^{tetO/TetR\text{-}GFP}$ (wild-type/ parent NR110); Δ*sirA spoIIIE36 oriC*$^{tetO/TetR\text{-}GFP}$ (NR113); *dnaA*^{A50V} *spoIIIE36 oriC*$^{tetO/TetR\text{-}GFP}$ (NR117); *dnaA*^{N47H} *spoIIIE36 oriC*$^{tetO/TetR\text{-}GFP}$ (NR118). **B.** *soj* deletion or mutations in domain III of DnaA disrupting the regulation of DnaA by Soj causes origin trapping defect, however the origin trapping defect exhibited by the Δ*soj* mutant significantly exceeds the trapping defect exhibited by DnaA variants resistant to Soj. *spoIIIE36 oriC*$^{tetO/TetR\text{-}GFP}$(wild-type/ parent NR109); Δ*soj spoIIIE36 oriC*$^{tetO/TetR\text{-}GFP}$(NR115); *dnaA*^{L294R} *spoIIIE36 oriC*$^{tetO/TetR\text{-}GFP}$ (NR123); *dnaA*^{V323D} *spoIIIE36 oriC*$^{tetO/TetR\text{-}GFP}$ (NR124); *dnaA*^{L337P}*spoIIIE36 oriC*$^{tetO/TetR\text{-}GFP}$ (NR125)**C.** DnaA variants with amino acid substitutions that confer resistance to regulation by YabA exhibit an origin trapping defect. The origin trapping defect of Δ*yabA* mutant could not be elucidated due to cells being sick in SpoIIIE36 background. *spoIIIE36 oriC*$^{tetO/TetR\text{-}GFP}$ (wild-type/ parent NR109); Δ*yabA spoIIIE36 oriC*$^{tetO/TetR\text{-}GFP}$ (NR144) *dnaA*^{M161I} *spoIIIE36 oriC*$^{tetO/TetR\text{-}GFP}$ (NR119); *dnaA*^{A163V} *spoIIIE36 oriC*$^{tetO/TetR\text{-}GFP}$ (NR126); *dnaA*^{H162Y} *spoIIIE36 oriC*$^{tetO/TetR\text{-}GFP}$ (NR128).

4.5 Sporulation frequency is affected in cells lacking multiple DnaA regulatory proteins

To examine whether sporulation frequency is effected in cells lacking multiple regulators of DnaA, I attempted to construct a strain in which *sirA*, *soj* and *yabA* deletions were combined with mutations in specific Spo0A-boxes at the *oriC* (Boonstra et al., 2013). However, the Δ*sirA* Δ*soj* Δ*spo0A*^{Boxes}Δ*yabA* quadruple mutant was very sick and rapidly gave rise to suppressor mutations. Further analysis revealed that combining Δ*yabA* with any of the other DnaA regulatory mutants caused strains to become sick. Therefore, in order to obtain information on the sporulation frequency of a strain lacking multiple regulators of DnaA, the sporulation frequency was measured in a Δ*sirA* Δ*soj* Δ*spo0A*^{Boxes} triple mutant (Table 4.5.1). The results showed a modest 3-fold decrease in the sporulation frequency of the triple mutant compared to wild-type. Unfortunately, due to a lack a compatible antibiotic markers I was unable to determine whether this effect was due to activation of the Sda checkpoint.

Table 4.5.1. Sporulation frequency of *ΔsirA Δsoj Δspo0A*^{Boxes} triple mutant is not significantly lower than wild type.

strain	Sporulation frequency Experiment 1	Sporulation frequency Experiment 2	Average Sporulation frequency	Standard deviation
wild-type	52%	64%	58	8
Δ*sirA* Δ*soj* Δ*spo0A*^{Boxes}	19%	21%	20	1

Cells were induced to sporulate by resuspension in starvation media, 24 hours after induction of sporulation the number of total colony forming units and spores were determined for one mL of cell culture. The experiment was repeated at least three times, representative data is shown. Wild-type (168ca); Δ*sirA* Δ*soj* Δ*spo0A*^{Boxes} (NR133).

Discussion

Previously it was shown that initiation of DNA replication is actively inhibited in cells that have initiated sporulation. Several systems are employed in *B. subtilis* to ensure that i) sporulation is only initiated in cells that have completed replicating their genome (Sda checkpoint) and ii) the DNA replication is not reinitiated in cells that have begun differentiating into spores (Spo0A and SirA).

Upon activation, the master regulator of sporulation Spo0A directly inhibits unwinding of *oriC* by interacting with 0A-boxes within the origin that overlap with DnaA-boxes (Boostra 2013). Amongst many other genes Spo0A also induces expression of SirA to ensure DNA replication is not reinitiated in cells undergoing sporulation. Spores that contain more than one copy of the chromosome have been shown to have reduced viability (Veening et al., 2009). Although it has been long appreciated that DnaA is tightly regulated in sporulating cells, the consequences of DnaA misregulation on the coordination of cellular processes have not been clear. Here it was demonstrated that proper regulation of DnaA is required for efficient chromosomal origin segregation in sporulating cells. Cells that lack the regulatory factors SirA, Soj or YabA, or that encode DnaA variants resistant to regulation by SirA, Soj or YabA, exhibit reduced fidelity in capturing of an origin region in the forespore during spore development.

I propose that the chromosomal origin segregation defect observed in absence of DnaA regulation could be caused by the mislocalisation of newly segregated origins towards midcell. It was recently shown that following DNA replication initiation at the outer edge of the nucleoid, duplicated origins are transported together towards midcell by the condensin complex (Wang et al., 2014a). This localisation would move the origins away from the cell

pole and therefore result in the defect in origin trapping. It is also possible that activation of the replication origins during sporulation results in sister origin cohesion, either via chromosome organisation factors (condensin, RacA, Hbsu) or recombination intermediates, thereby delaying timely origin separation.

Although chromosomal origin trapping defects were exhibited by Δ*sirA* and Δ*yabA* mutants, the origin trapping defect observed in the Δ*soj* was considerably higher, especially when considering that the strain only produced a modest number of sporulating cells with ongoing replication. Furthermore, the origin trapping defect of the Δ*soj* mutant was 10-fold higher than the DnaA mutants defective in regulation by Soj. Taken together with work indicating that Soj plays an active part in chromosome origin separation in *B. subtilis* (Lee and Grossman, 2006, Wang et al., 2014b, Wang et al., 2014a), my results suggested that Soj likely has a role in chromosome origin segregation distinct from its DnaA-regulatory activity. Chapter 5 is dedicated to understanding the role of Soj in chromosomal origin segregation in sporulating cells.

Chapter 5: Role of Soj (ParA) in chromosome segregation during sporulation

Introduction

The viability of an organism is reliant on the faithful inheritance of the genetic material from the parents to the progenies. Both eukaryotes and bacteria employ multiple systems to ensure the stable transmission of the genetic information to the following generations. Unlike the well characterised events involving chromosome segregation in eukaryotes, the mechanisms underlying accurate segregation of bacterial chromosomes remain relatively poorly understood (Reyes-Lamothe et al., 2012, Wang et al., 2013).

Here we took advantage of a highly tractable system of chromosome segregation in sporulating cells to study the role of Soj, a component of the *B. subtilis* partitioning system. During sporulation asymmetric septa formation produces two cellular compartments of uneven size, the smaller forespore compartment which will mature into a stress-resistant spore and the larger mother cell which provides the means of maturation for the spore. During the early stages of sporulation, the two sister chromosomes are segregated towards opposite cell poles where they become affixed by the origin anchoring protein RacA, thereby forming a specific structure termed the axial filament (Ryter and Schaeffer, 1966, Ben-Yehuda et al., 2003, Wu and Errington, 2003). Asymmetric cell division leads to the capture of the origin region of the chromosome destined for the spore within the forespore compartment (the remaining of the chromosome is later transferred into the forespore, see chapter 1)(Wu and Errington, 1994a, Sullivan et al., 2009). In this context we recognise the absence of an origin region from the forespore compartment as an origin trapping defect.

Soj is dynamic protein with the ability to switch its conformation from a monomer to a dimer in an ATP-dependant cycle. Biochemical and structural studies of Soj revealed amino acid substitutions that arrest the protein in different conformational states with limited activities (Figure 5.0.1A). Soj^{K16A} is deficient in ATP-binding. Soj^{G12V} is an ATP-binding proficient monomer, as the valine substitution sits at the tight dimer interface. Soj^{R189A} is an ATP_2-bound dimer that is specifically unable to bind DNA. All of these Soj variants are thought to inhibit DnaA activity and repress DNA replication initiation. In contrast, Soj^{D40A} is an ATP_2-bound dimer that interacts with DNA and stimulates DnaA to promote DNA replication initiation (Leonard et al., 2005, Murray and Errington, 2008, Scholefield et al., 2011).

Localisation studies of Soj (in which the endogenous *soj* gene was replaced with *gfp-soj* expressed from its native transcriptional and translational expression system) revealed that GFP-Soj localises at the septum and also forms punctate DnaA-dependent foci within the cytoplasm (Figure 5.0.1B). Similar localisation pattern was observed for GFP- Soj^{G12V} and GFP- Soj^{R189A}. GFP-Soj^{K16A} was mainly distributed throughout the cytoplasm, although it occasionally formed faint foci within the cytoplasm. GFP-Soj^{D40A} solely formed bright Spo0J-dependent foci within the cell (Murray and Errington, 2008).

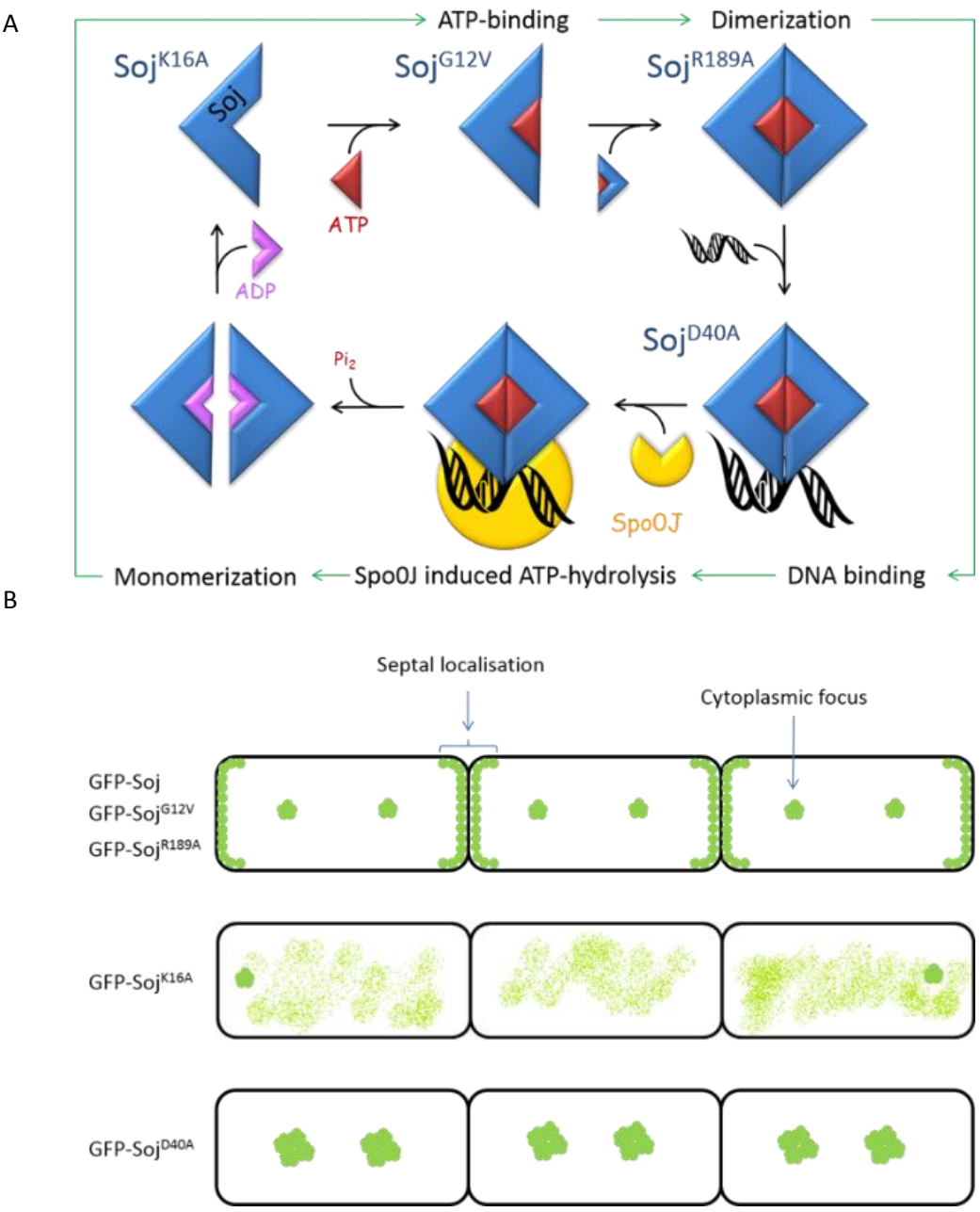


Figure 5.0.1. Soj is a dynamic protein that goes through an ATP-dependent cycle. A) Schematic diagram showing Soj variants locked in a conformation in respect to Soj cycle. *B)* Schematic diagram of localisation of wild-type Soj and Soj mutants in vegetatively growing cells. GFP-Soj localises at the septum and as faint foci throughout the cytoplasm, arrows highlight the septal localisation and cytoplasmic foci. GFP-Soj^{G12V} and GFP-Soj^{R189A} exhibit a

similar localisation pattern to the wild-type. GFP-Soj^{K16A} localised as a diffused haze throughout the cytoplasm with occasional faint foci detectable within the cells. GFP-Soj^{D40A} solely localises as bright foci within the cells. Figure adapted from Murray and Errington (2008).

Based on previous work Soj was thought to play a redundant role in chromosome segregation during spore development, as a synthetic origin trapping defect was observed in population studies using a Δ*soj* Δ*racA* double mutant (Ireton et al., 1994, Wu and Errington, 2003). However, direct evidence for Soj being involved in origin trapping in sporulating cells was subsequently provided by Sullivan et al. using a sensitive single cell cytological assay. They demonstrated that in a strain lacking Soj there is an increased number of cells that failed to trap the *oriC* region within the forespore. In contrast, the other chromosomal regions surrounding the origin were not significantly affected by the absence of Soj, suggesting that this phenotype is not caused by a defect in overall chromosome organisation but was specifically caused by defects in positioning of the origin region (Sullivan et al., 2009).

Recently it has been revealed that Soj facilitates the separation of sister origins, likely through a diffusion-ratchet mechanism involving Spo0J (Lee and Grossman, 2006, Wang et al., 2014b, Wang et al., 2014a). Based on studies of orthologous partitioning systems, dissociation of Soj dimers by Spo0J:*parS* nucleoprotein complexes creates depletion zones resulting in a Soj concentration gradient over the nucleoid that drives Spo0J*:parS* movement (Vecchiarelli et al., 2013, Vecchiarelli et al., 2014, Lim et al., 2014). However, a Δ*soj* mutant does not cause a chromosome segregation defect and replication origin positioning is

relatively unperturbed (Ireton et al., 1994, Lee and Grossman, 2006). These observations indicate that during vegetative growth Soj plays an auxiliary role in chromosome origin separation.

The focus of this chapter is to investigate the role of Soj in chromosomal origin segregation during sporulation. Towards this end I have utilised a sensitive single-cell cytological assay to detect proper localisation of the *B. subtilis* chromosome origin region during spore development. To shed light on which aspects of Soj activity is required for the faithful origin trapping, I combined the chromosome origin trapping assay with a range of *soj* alleles that allowed distinct properties of Soj to be examined. Using this assay I show that either removal of Soj, or rendering Soj incapable to localise at the septum, leads to reduced chromosome origin trapping in the forespores.

Results

5.1 Soj null mutant exhibits an increased number of anucleated forespores

As discussed in chapter 4, deletion of *soj* led to a pronounced defect in chromosome origin trapping that could not be solely attributed to loss of DnaA regulation. Therefore, I wanted to investigate the role of Soj in chromosome origin trapping in greater molecular detail. To begin I asked whether, in a Δ*soj* mutant, the cells that failed to trap the origin regions in the forespore contained any DNA. In this experiment the DNA was visualised using DAPI and the *tetO*$_{25}$/TetR-GFP origin marker system was used to detect origin trapping.

As described in Chapter 4, the Δ*soj* mutant consistently showed a 5-fold increase in the number cells that failed to trap an origin marker in the forespore compartment compared to the wild-type (average origin trapping defect of 24.3±1.3% and 4.7±1.1% for the Δ*soj* mutant and wild-type, respectively). Of the cells that displayed an origin trapping defect, only 1.1±0.2% of forespores lacked DNA in the wild-type strain, whereas 4.9±0.4% of forespores lacked DNA in the *Δsoj* mutant. The results show that there was 4.5-fold increase in the number of cells that failed to trap any DNA in the forespore compartment in the Δ*soj* mutant compared to the wild-type (Figure 5.1.1). This suggests that during sporulation the faithful Soj-dependent segregation of origin regions facilitates a more general chromosome localisation/trapping activity.

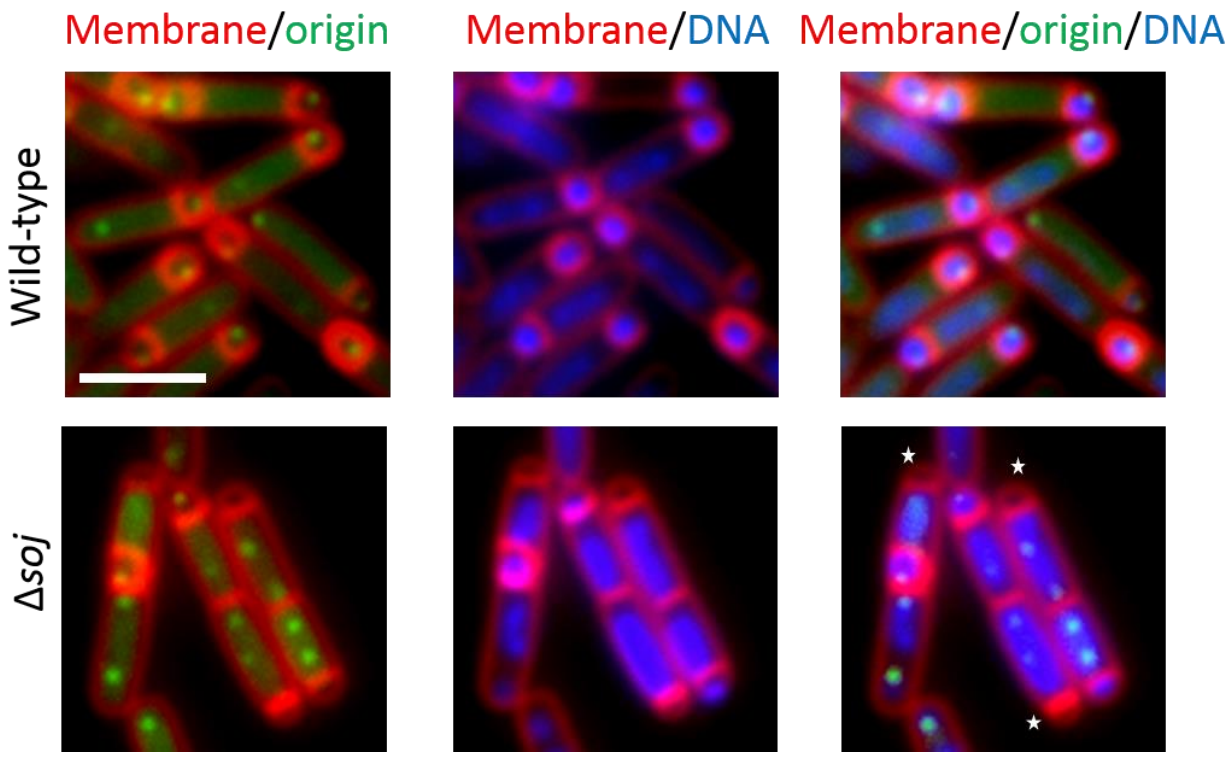


Figure 5.1.1. An increased number of anucleated forespores are observed in the Δ*soj* mutant compared to the wild-type. Cells were induced to sporulate using resuspension method. Images of cells were taken 180 minutes after induction of sporulation. DAPI staining was used to visualise the DNA and FM5-95 was utilised to stain the membrane. Asterisks highlight anucleated forespores. The experiment was repeated at least 3 times and each time 500≤ cells were analysed and the origin trapping and presence of DNA in the forespores were determined, representative data is shown here. *Δsoj oriC*$^{tetO25/\text{TetR-GFP}}$ (NR48).

5.2 Soj plays a role in chromosomal origin trapping independently of the Spo0J/SMC system

Previous studies showed that Spo0J plays a pivotal role in chromosome segregation in *B. subtilis* by recruiting the structural maintenance of chromosomes complexes (SMC) to the origin of replication (Ireton et al., 1994, Gruber and Errington, 2009, Sullivan et al., 2009, Wang et al., 2014b, Bürmann et al., 2013, Wang et al., 2013). To investigate whether the origin trapping defect exhibited by the Δ*soj* mutant is indirectly caused by perturbation of the Spo0J/SMC system, I examined the effects of Spo0J overexpression on chromosome origin trapping in the Δ*soj* mutant. If Spo0J activity was partially diminished in the absence of Soj, then I hypothesised that overexpression of Spo0J would reverse this effect.

To overexpress Spo0J I constructed a strain that contained an additional copy of the gene under control of an IPTG inducible promoter (P_{spac}) at the *aprE* locus (Figure 5.2.1A). Spo0J was induced by addition of IPTG four hours prior to resuspension in starvation media and origin trapping was observed using the *tetO*$_{25}$/TetR-GFP reporter system. The origin trapping defect observed in the Δ*soj* mutant was unaffected by Spo0J overexpression (Figure 5.2.1B), suggesting that the phenotype was not due to insufficient SMC recruitment. In order to confirm that functional Spo0J was being expressed from our construct, I backcrossed genomic DNA from this strain into a Δ*spo0J* mutant and observed that expression of Spo0J from *aprE*(P_{spac}-*spo0J*) restored the ability of the strain to sporulate (Figure 5.2.1C).

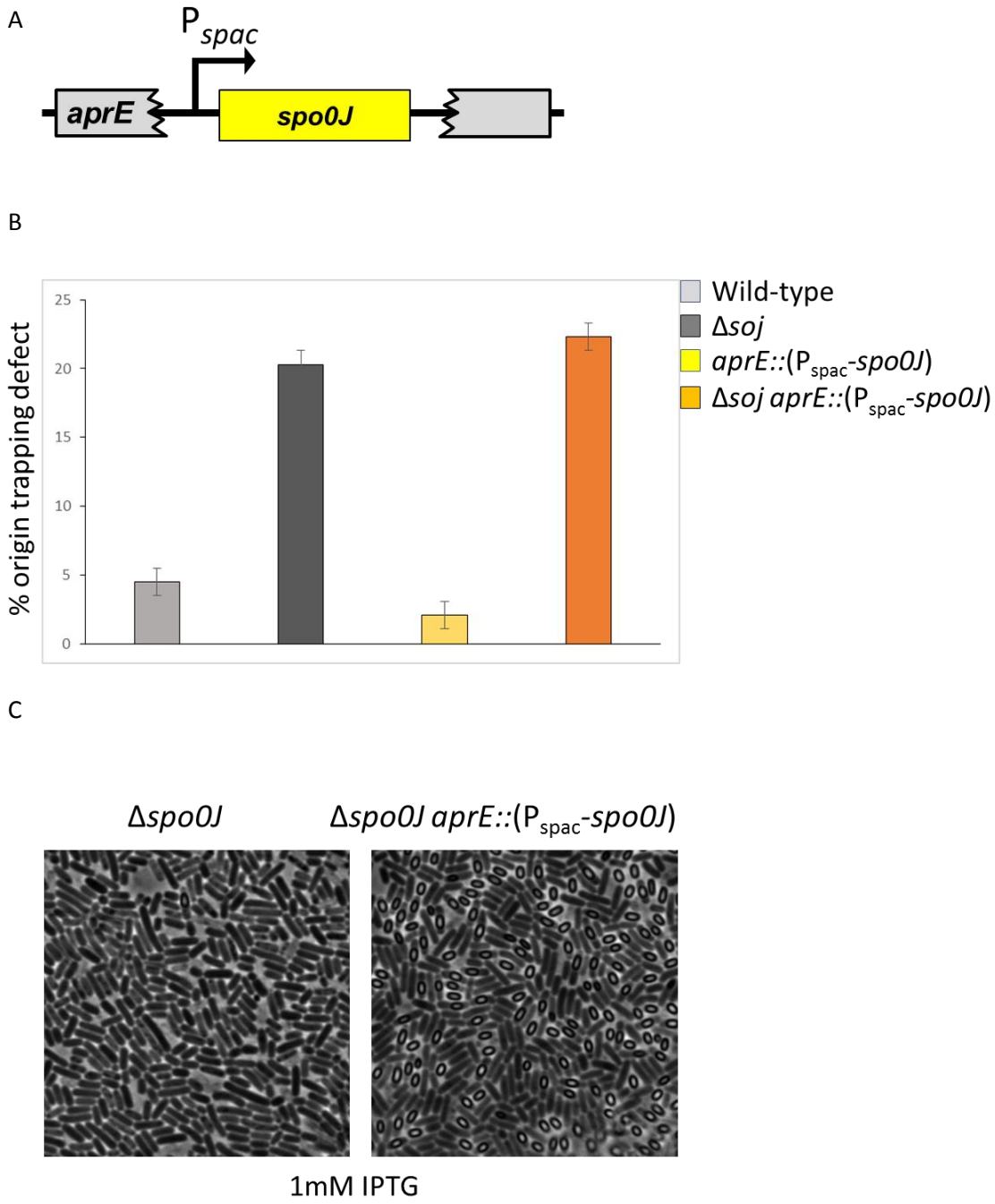


Figure 5.2.1. Spo0J overexpression does not reverse the origin trapping defect in the Δ*soj* mutant. A. Spo0J overexpression construct. *spo0J* gene was put under regulation of an IPTG inducible promoter and inserted at aprE locus. The experiment was repeated at least twice, each time ≥350 cells were analysed, and the representative data is shown. **B**. Over-expression of Spo0J does not restore faithful origin trapping in the Δ*soj* mutant. Cells were

induced to sporulate, 4 hours prior to induction of sporulation, expression of Spo0J was induced by addition of IPTG (final concentration 1mM). C. Spo0J is expressed from the P_{spac} promoter at aprE locus. Sporulation defect exhibited by Δspo0J mutant in reversed by over-expression of Spo0J. Δspo0J strain and Δspo0J *aprE* (P_{spac}-*spo0J*) strain were streaked on nutrient agar plated containing 1mM IPTG. Microscope images were taken after 72 hours of incubation at 37°C. *oriC*^{tetO25}/TetR-GFP(wild-type/ parent AK47); Δ*soj oriC*^{tetO25}/TetR-GFP (NR48); *aprE*::(P_{spac}-*spo0J*) oriC^{tetO25}/TetR-GFP (NR61); *Δsoj aprE*::(P_{spac}-*spo0J*) *oriC*^{tetO25}/TetR-GFP(NR62); Δ*spo0J aprE*::(P_{spac}-*spo0J*) (NR66); *Δspo0J* (HM754).

5.3 Variants of Soj exhibit different levels of chromosome origin trapping

To further clarify the conformation and/or function of the Soj protein that is required for the faithful segregation of *oriC* into the forespore, I tested origin trapping in a range of strains where the endogenous *soj* gene was replaced with alleles of *soj* encoding for Soj variants with distinct properties (Figure 5.3.1A).. The results showed that both Soj^{K16A} and Soj^{D40A} exhibited a significant defect in origin trapping, while Soj^{G12V} and Soj^{R189A} trapped the origin similar to wild-type (Figure 5.3.1B).

Interestingly, the origin trapping results in the Soj variants correlate with the localisation pattern exhibited by the Soj variants, where the Soj proteins that have lost the ability to localise at the septum (Soj^{K16A} and Soj^{D40A}) display an origin trapping defect compared to the Soj mutants that retain septum localisation (Soj^{G12V} and Soj^{R189A}) (see Figure 5.0.1B).

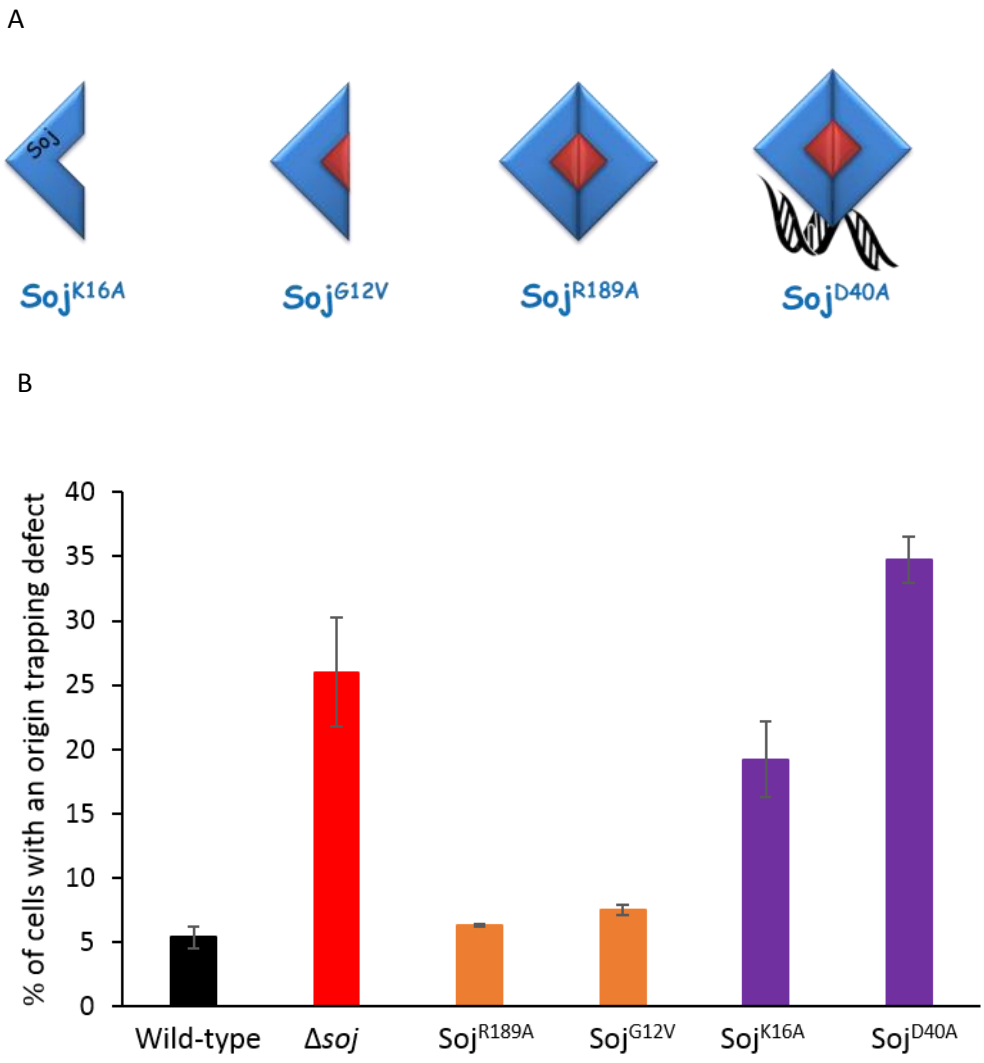


Figure 5.3.1. Chromosomal origin trapping state of Soj variants. **A**. Point mutations in Soj arrests the protein in distinct conformation with certain properties. **B.** Origin trapping defect of cells harbouring Soj mutants with distinct properties. Cells were induced to sporulate using resuspension method. Images of cells were taken 180 minutes after induction of sporulation. The experiment was repeated at least three times, each time ≥500 cells were analysed. Soj^{D40A} mutant is an activator of DnaA and therefore turns on the Sda DNA replication checkpoint and inhibits sporulation. In order to allow sporulation to proceed the sda gene was deleted in this mutant. Δsda mutant was also included in the experiment as a

control which showed very similar trapping defect to the wild type. *oriC*$^{tetO25/TetR-GFP}$ (wild-type/AK47); Δ*sda oriC*$^{tetO25/TetR-GFP}$ (NR47); Δ*soj oriC*$^{tetO25/TetR-GFP}$ (NR48); *soj*^{G12V} *oriC*$^{tetO25/TetR-GFP}$ (NR49); *soj*^{D40A} Δ*sda oriC*$^{tetO25/TetR-GFP}$ (NR55); *soj*^{K16A} *oriC*$^{tetO25/TetR-GFP}$ (NR57); *soj*^{R189A} oriC$^{tetO25/TetR-GFP}$ (NR58).

5.4 The forespore length analysis of Soj variants

The septal localisation of Soj has been shown to be dependent upon MinD (Autret and Errington, 2003, Murray and Errington, 2008). As discussed in Chapter 1, (see section 1.4.2.3) MinD serves as a negative regulator of cell-division that inhibits assembly of the division machinery at the cell poles by recruiting MinC. Disruption of *minD* gene causes aberrant cell division events leading to formation of both long filamentous cells and short minicells (Autret and Errington, 2003). I speculated the high trapping defect shown by the Soj mutants unable to localise at the septum could occur through perturbed regulation of cell-division in absence of Soj at the cell pole. Such alternation in the activity of the Min system could lead to changes in the forespore length. I measured the length of forespores as a readout of cell division regulation to test whether there is a correlation between forespore length and the trapping defect observed for Soj variants (Figure 5.4.1). The average forespore length for the wild-type strain was 800 to 900 nm, with a symmetrical distribution around the mean. The median for wild-type also laid between 800 to 900 nm (785 nm). The average forespore length for the Δ*soj* mutant was similar to the wild type however the median value (678 nm) deviated from the mean and was located in 600-700 class. The distribution was also slightly shifted towards the lower valuesin the Δ*soj* mutant. Interestingly, the length of the forespores in the strains expressing Soj^{G12V} and Soj^{R189A} variants were distributed asymmetrically with a shift towards the higher values, with average of 900-1000 (median= 959 nm) and 800-900 (median= 945 nm), respectively. The average forespore length for Soj^{K16A} and Soj^{D40A} did not deviate from the wild-type strain (with median values of 786 nm and 754 nm respectively), although there was greater variability for the Soj^{D40A} mutant. A two-tailed Student's *t*-test was performed on the data,

in all cases a high degree of confidence (greater than 99%) was observed except in Soj^{D40A} that exhibited a *p* value of 0.06 and hence stistical significance.

Taken together, the analysis of forespore length distributions of Soj variants did not indicate a correlation between the forespore length and the higher origin trapping defect observed in Soj^{K16A} and Soj^{D40A}. However, this analysis also revealed a potential relationship between Soj septum localisation and forespore size, suggesting that Soj may affect the Min system. Consistent with this notion, it was previously observed that GFP-Soj fluorescence at the septum was brighter in a Δ*minC* mutant, hinting at competition between Soj and MinC for binding MinD (Murray and Errington, 2008).

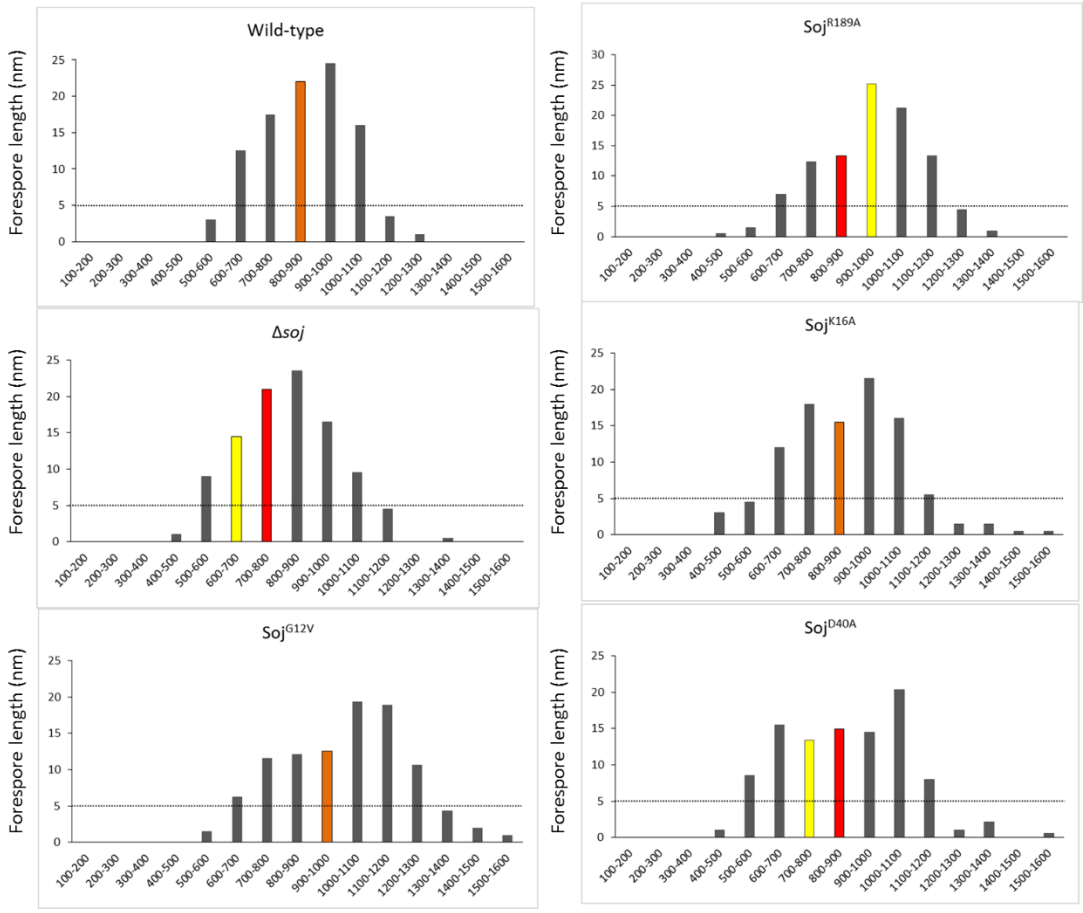


Figure 5.4.1. The forespore lengths of soj deletion strain and Soj mutants. The bar representing the median class for each mutant is shown as a yellow bar, the average class for each mutant is shown in red, if the average and the median value was located in the same class, that class was shown as an orange bar. The bars higher than the dotted line (bars with values higher than 5%) were considered as major peaks. Average forespore length was smaller than the wild-type value in the Δ*soj*, Soj^{G12V} had a higher average forespore length than the wild-type. The average forespore length did not deviate from the wild-type value for the remaining Soj mutants. The forespore length distribution shifted towards higher values in Soj^{G12V} and Soj^{R189A}. The forespore length distribution was affected

in both Soj^{G12V} and Soj^{D40A} which both had 7 major peaks. The median value for each class in respect to the wild-type are as followed: Δ*soj* < Soj^{D40A}< wild-type < Soj^{K16A} < Soj^{R189A} < Soj^{G12V}. The experiment was repeated twice, each time forespore length of >350 cells were measured for each strain. 168ca (wild-type); Δ*soj* (HM748); *soj*^{G12V} (HM750); *soj*^{K16A} (HM751); *soj*^{R189A} (HM753); *soj*^{D40A} Δ*sda* (NR50).

5.5 RacA disruption in Soj variants that exhibit a higher trapping defect leads to a greater decrease in sporulation frequency

Previous studies reported that while disruption of either Soj or RacA (a sporulation specific protein that anchors the origin region to the cell pole) did not affect sporulation frequency, deletion of both *soj* and *racA* does caused a significant decrease in the sporulation frequency. This synthetic phenotype suggested that Soj and RacA may play redundant roles in segregation of the origin regions during sporulation (Wu and Errington, 2003). Based on these findings, I set out to study whether there is a correlation between the origin trapping defect exhibited by the Soj variants and the synthetic sporulation defect observed in the presence of a Δ*racA* mutant.

The sporulation frequency was determined for strains harbouring either a *soj* deletion or *soj* alleles that exhibited the lowest (Soj^{G12V}) and the highest (Soj^{K16A}) origin trapping defects, in the presence and absence of RacA (Figure 5.5.1). Cells were induced to sporulate using the resuspension method and sporulation frequency was determined by quantifying colony forming units (CFU) before and after heat treatment (only spores will survive this stress).

A similar sporulation frequency was observed for the wild-type strain and the Δ*soj* and Δ*racA* single mutants, as previously reported (Wu and Errington, 2003). Surprisingly, in my hands the Δ*soj* Δ*racA* double mutant did not exhibit a severe sporulation defect as was previously reported (Wu and Errington, 2003), although it did display a reproducible 2-fold decrease in sporulation frequency. This difference may be due in part to the method used to measure sporulation frequency; here I calculated the CFU per ml of a culture before and

after heat-treatment, whereas Wu et al., (2003) determined sporulation efficiency using microscopy.

The sporulation frequency of either Soj^{G12V} or Soj^{K16A} alone was slightly higher than the wild-type (which could be due to negative regulatory effect of Soj^{G12V} on DnaA, and therefore Sda). When combined with Δ*racA* deletion, the only significant decrease in sporulation frequency was exhibited by Soj^{K16A}, with about a 3-fold decrease compared to the Soj^{K16A} single mutant and 2-fold decrease compared to the wild-type. In contrast the sporulation frequency of the Soj^{G12V} Δ*racA* double mutant was essentially unchanged. Although in my hands I did not observe as severe sporulation defect as previously described for Δ*soj* Δ*racA* double mutant, the results of sporulation assays combining Soj variants with a Δ*racA* mutant correlates with the origin trapping defect.

It should be noted that although the Soj^{D40A} variant exhibited the highest origin trapping defect, the sporulation frequency for a *soj*^{D40A} Δ*racA* double mutant is not shown here. The Soj^{D40A} mutant requires deletion of the checkpoint gene *sda* to sporulate, and for unknown reasons I was unable to construct *soj*^{D40A} Δ*racA* Δ*sdA* triple mutant, in spite of attempting to combine the mutations in different orders.

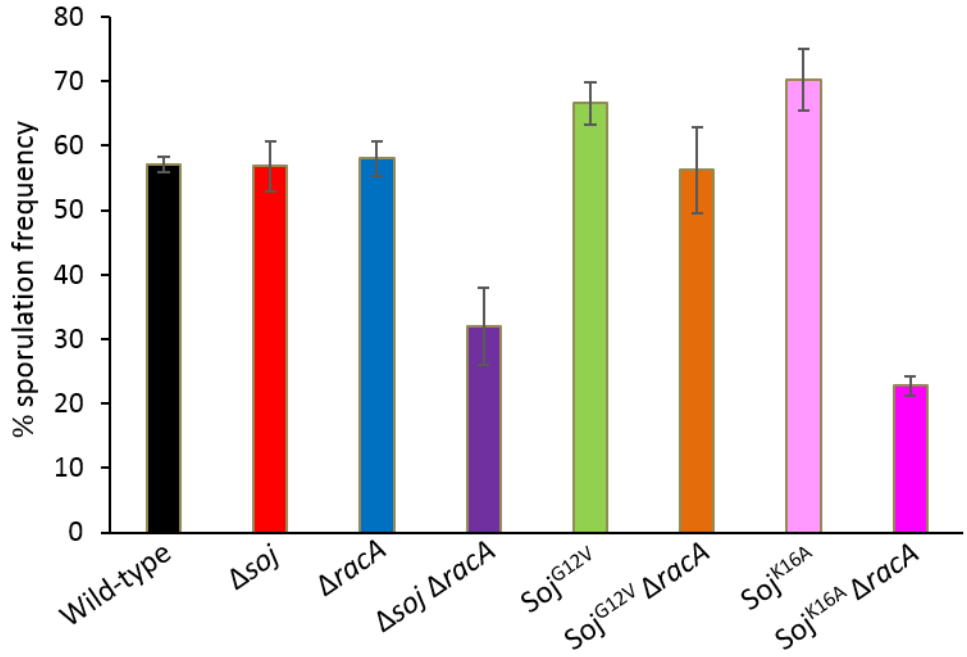


Figure 5.5.1. Percentage of sporulation frequency of Soj variants combined with racA deletion. Cells were induces to sporulate by resuspension method and CFU was determined after at least 24 hours of incubation at 37°C before and after heat-treatment. The experiment was repeated twice, the average sporulation frequency is shown. 168ca (wild-type); *Δsoj* (HM748); *soj^{G12V}* (HM750); soj^{G12V} *ΔracA* (NR136); *ΔracA* (SB180); *Δsoj ΔracA* (NR143); *soj^{K16A}* (HM751); *soj^{K16A} ΔracA* (NR138).

5.6 *soj* deletion leads to delay in process of spore maturation

In order to establish how the origin trapping defect observed in the Δ*soj* mutant might influence the overall process of sporulation, I investigated the temporal development of spores. Previous studies reported no major defects in the frequency of spore formation in a Δ*soj* mutant (Ireton et al., 1994). However, in these studies sporulation frequency was determined from the cultures that were allowed to grow for long periods of time prior to analysis. Therefore, the extended growth period might have masked more subtle defects caused by the failure to efficiently capture a chromosome origin region in the forespore. As stated previously (see the chapter1) the capturing of the origin region of the forespore chromosome is required for production of a transient genetic asymmetry between the forespore and the mother cell compartment, thereby driving the activation of compartment specific sigma factors and therefore distinct gene expression programs (reviewed by Errington 2003).

I hypothesised that the origin trapping defect exhibited by the Δ*soj* mutant might cause a delay in initiation and/or completion of the sporulation process. In order to test this possibility I first compared the percentage of cells that had initiated sporulation (judged by presence of asymmetric septa visualised by staining of the membrane) in synchronised cultures of a wild-type strain and a Δ*soj* mutant. Similar number of cells with an asymmetric septa were observed in both the wild-type and the Δ*soj* mutant 180 minutes after induction of sporulation, indicating that there is no detectable delay entering the developmental process in the Δ*soj* mutant (Figure 5.6.1A).

I then went on to perform a temporal sporulation frequency experiment to compare the efficiency of spore maturation in the two strains. Cells were induced to sporulate and 6-

7 hours after induction of sporulation the percentage of heat-resistant spores was determined (Figure 5.6.1B). The results showed that at two time points post induction of sporulation (T_6 to T_7) the number of heat-resistant spores was greater in the wild-type compared to the Δ*soj* mutant, suggesting that the origin trapping defect observed in the Δ*soj* mutant might cause a delay in spore development .

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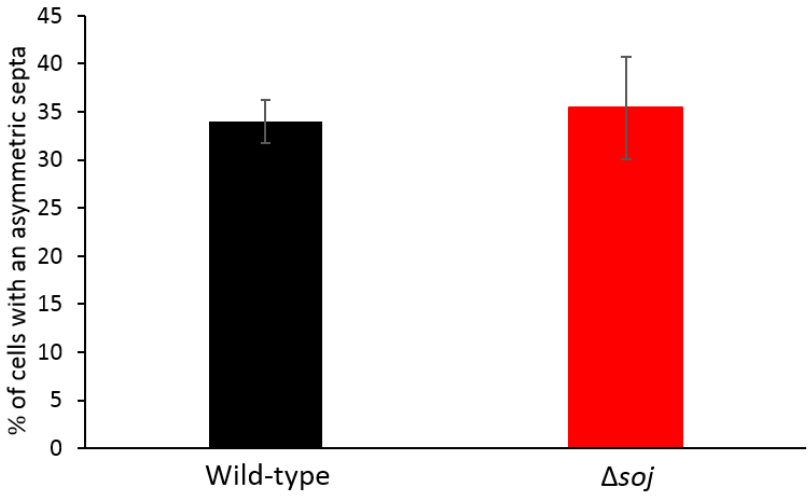


B

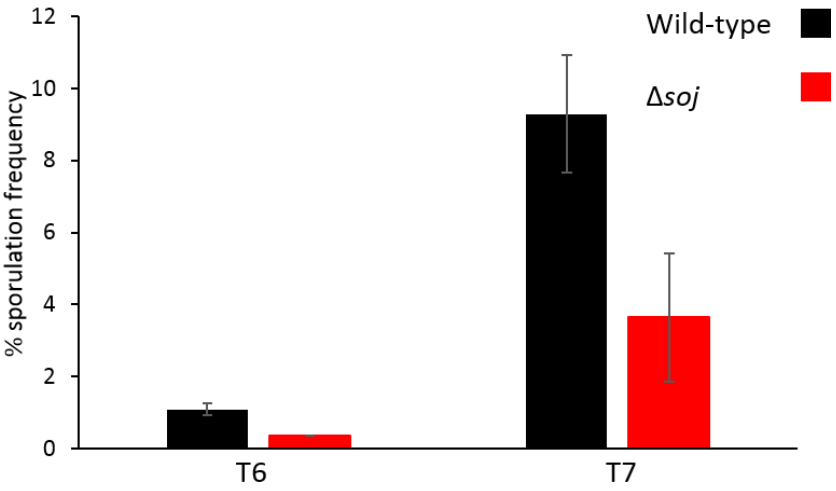


Figure 5.6.1. Temporal sporulation frequency of the Δ*soj* mutant reveals a delay in spore maturation compared to the wild-type. A. frequency of asymmetric septation in wild-type and Δ*soj* mutant 180 minutes after induction of sporulation. **B.** Sporulation frequency was determined for T6 and T7 hours after induction of sporulation. Wild-type shows a greater increase in the number of heat-resistant spores over an hour compared to the Δ*soj* mutant. The experiment was repeated twice, representative data is shown (to determine frequency of asymmetric septation 100 cells were analysed each time). 164ca (wild-type); Δ*soj* (HM748).

Discussion

Septal localisation of Soj may be required for proper chromosomal origin segregation

Soj is a dynamic protein with multiple functions and sites of action. Although recent studies have confirmed the role of Soj in chromosomal origin segregation, the property of the Soj required for and the underlying mechanism by which it fulfils origin segregation has not been stablished (Sullivan et al., 2009, Wang et al., 2014b, Wang et al., 2013). In this chapter Soj variants with distinct properties were tested to determine their efficacy in accurate and timely capture of origin regions in the forespore of sporulating cells. The results indicate that cells harbouring either of two Soj variants, Soj^{G12V} or Soj^{R189A}, exhibit a similar origin trapping fidelity to the wild-type. GFP-Soj^{G12V} and GFP-Soj^{R189A} adopt the same localisation pattern as the wild-type, namely recruitment to the septum and formation of discrete foci at the replication origin (Figure 5.0.1).

In contrast Soj^{D40A} and Soj^{K16A} exhibit a reduced fidelity of chromosomal origin trapping. Notably, neither Soj^{D40A} nor Soj^{K16A} localise at the septa (Figure 5.0.1). Furthermore, these variants may share the property of forming dimers that interact with Spo0J. Although Soj^{K16A} was previously shown to be monomeric *in vitro,* there is evidence that it may form weak dimers *in vivo*. In contrast to the N-terminal GFP-tagged Soj^{K16A} that mainly distributes throughout the cytoplasm with occasionally forming faint foci within the cytoplasm, C-terminal GFP-tagged Soj^{K16A} (Soj^{K16A}-GFP) exhibits a localisation pattern similar to GFP-Soj^{D40A} (Murray unpublished) forming Spo0J-dependent foci.

Using a temporal sporulation frequency assay, it was shown that absence of Soj, previously thought not affect sporulation, causes a delay in completion of sporulation. I

speculate that this might be caused by the delay in activation of compartment specific genetic programs due to the absence of correct regions of the chromosome (*oriC*) in the forespore at the time of septation.

Chapter 6: Final discussion

6.1 Regulation of DNA replication initiation in *B. subtilis* is exerted at DnaA helix assembly step at the *oriC*

DnaA is widely conserved in bacteria, however the mechanism by which it is regulated significantly varies between species. This is exemplary in the regulation of *B. subtilis* and *E. coli* two of the best studied model organisms. Many of the known regulators of DnaA in *B. subtilis* carry out their function by interacting with the AAA+ domain of DnaA, however, neither nucleotide-binding nor hydrolysis is the subject of the regulators' function in *B. subtilis* (as it is in *E. coli*). YabA, DnaD and Soj interact with domain III and specifically inhibit DnaA oligomerisation and helix formation at *oriC* (Scholefield et al., 2012, Cho et al., 2008, Bonilla and Grossman, 2012, Scholefield and Murray, 2013, Boonstra et al., 2013, Rahn-Lee et al., 2011).

Uniquely, SirA interacts with domain I of DnaA that mainly functions as a hub for protein-protein interactions. Previous studies suggested that SirA prevents binding of DnaA molecules to *oriC* (Rahn-Lee 2011).In Chapter 3, it was shown that SirA accumulates away from the origin regions and with the replisome in a DnaA dependent manner. These results suggest a role for SirA in sequestration/stabilisation of DnaA molecules at the replisome through interaction with an unknown partner, a candidate of which could be the sliding clamp of the polymerase (DnaN) (Jameson et al., 2014). Similarly, it has been shown that during vegetative growth YabA also tethers DnaA molecules to the replication fork, sequestering them away from *oriC* (Soufo et al., 2008). Hence, coupling DNA replication elongation with negative regulation of DnaA assembly at *oriC* appears to be a regulatory strategy widely used in bacteria (Mott and Berger, 2007).

In *E. coli* the main DnaA inhibitory system (RIDA) promotes replication coupled Hda-induced ATP-hydrolysis by DnaA in order to link DNA replication elongation with inhibition of DnaA. These distinct regulatory strategies employed by *B. subtilis* and *E. coli* to control the activity of DnaA could be due to the rate of nucleotide exchange by DnaA in these organisms. Biochemical analysis of DnaA nucleotide binding showed that *B. subtilis* DnaA (*Bs*DnaA) exhibits a relative high rate of nucleotide exchange compared to the *E. coli* DnaA (*Ec*DnaA). In absence of any other cellular factors the half-life of nucleotide exchange is 5 minutes for *Bs*DnaA-ATP(Bonilla and Grossman, 2012) compared to 45 minutes for *Ec*DnaA-ATP (Kurokawa et al., 2009). In addition, cells consistently contain a higher level of ATP (approximately 5-times) compared to ADP (Bochner and Ames, 1982). Taken together it is expected that ATP-binding, and thereby helix formation at the *oriC,* would be more stringent in *E. coli* than *B. subtilis* and hence the rate limiting process in regulation of DNA replication initiation. In contrast, in *B. subtilis* the nucleotide binding and exchange rate of DnaA and hence DnaA helix assembly seems to occur more dynamically which is consistent with requirement of regulation of assembly of DnaA into an initiation promoting helix.

6.2 Regulation of DnaA is required for proper spore development

Previously it was shown that *B. subtilis* and other spore forming bacteria (including *Myxococcus xanthus*, *Streptomyces coelicolor*, etc.) regulate the initiation of DNA replication at the onset of sporulation. In *B. subtilis* interconnected systems function together to achieve two chromosome copy number in sporulating cells. While the DNA replication checkpoint Sda, monitored by the activity of the initiator protein DnaA, ensures that DNA replication is not interrupted by sporulation in cells that are yet to complete duplication of their chromosome, sporulation induced regulators of DnaA (Spo0A~P and SirA) block

reinitiation of DNA replication in cells that have already committed to the differentiation pathway (Burkholder et al., 2001, Veening et al., 2009).

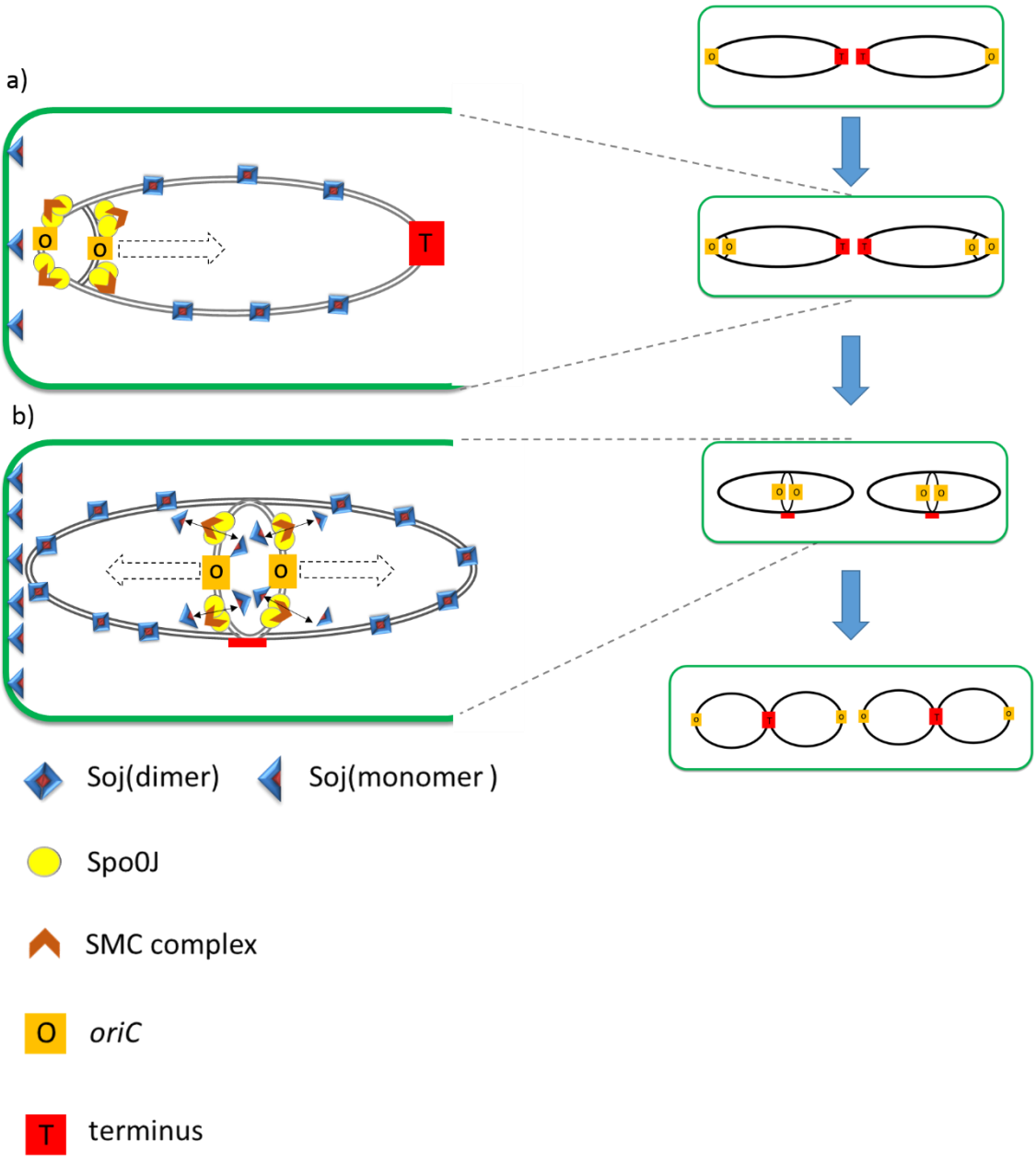
In Chapter 4, the importance of DnaA regulation for coordination of chromosome origin trapping during sporulation was studied. It was shown that the absence of DnaA regulatory proteins (SirA, Soj or YabA) leads to a decrease in the efficient inhibition of DNA replication in sporulating cells. Analysis of chromosome segregation in mutants lacking DnaA regulators also displayed a chromosome origin segregation defect. The direct role of DnaA regulation in accurate chromosomal origin trapping was confirmed using strains harbouring DnaA variants resistant to regulation by either SirA, Soj or YabA. Mutants harbouring DnaA proteins resistant to either YabA or SirA regulation exhibited a similar origin trapping defect to strains where the DnaA regulatory protein was absent, suggesting that these origin trapping defects were due to misregulation of DNA replication initiation. However, strains harbouring DnaA variants resistant to regulation by Soj displayed a significantly lower origin trapping defect compared with the Δ*soj* mutant, suggesting both DnaA-dependent and DnaA-independent roles for Soj in chromosomal origin trapping.

Recently it was reported that the *B. subtilis* nucleoid exists in two configurations: *ori*-*ter* or left-*ori*-right. Intriguingly, it was shown that the switch that caused the alternation between these two configurations was initiation of DNA replication. Following initiation of DNA replication at the nucleoid periphery a transition of chromosome configuration from *ori*-*ter* to the left-*ori*-right pattern was observed (see Chapter 1 section1.4.1) (Wang et al., 2014a). Recall that during sporulation the accurate and timely formation of an axial filament is required for proper chromosome origin trapping, in which the replication origins are bound

to the opposite cell poles and the termini are positioned at midcell in an *ori*-*ter* configuration (Wu and Errington, 2003, Ben-Yehuda et al., 2003, Wang et al., 2014a).

I speculate that the reduced fidelity in chromosomal origin trapping of sporulating cells with defective DnaA regulation could be due to movement of origin regions towards the midcell and transition of the nucleoid into the left-*ori*-right configuration following DNA replication initiation (Figure 6.2.1A). This could explain the higher origin trapping defect observed in the Δ*soj* mutant compared to the mutants lacking SirA or YabA, where not only the regulation of DNA replication is perturbed, but these mutants would also lack a driving force important for active separation of the origin regions (Figure 6.2.1B).

A


a)
O
O
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Soj(dimer)
Soj(monomer)
Spo0J
SMC complex
O
oriC
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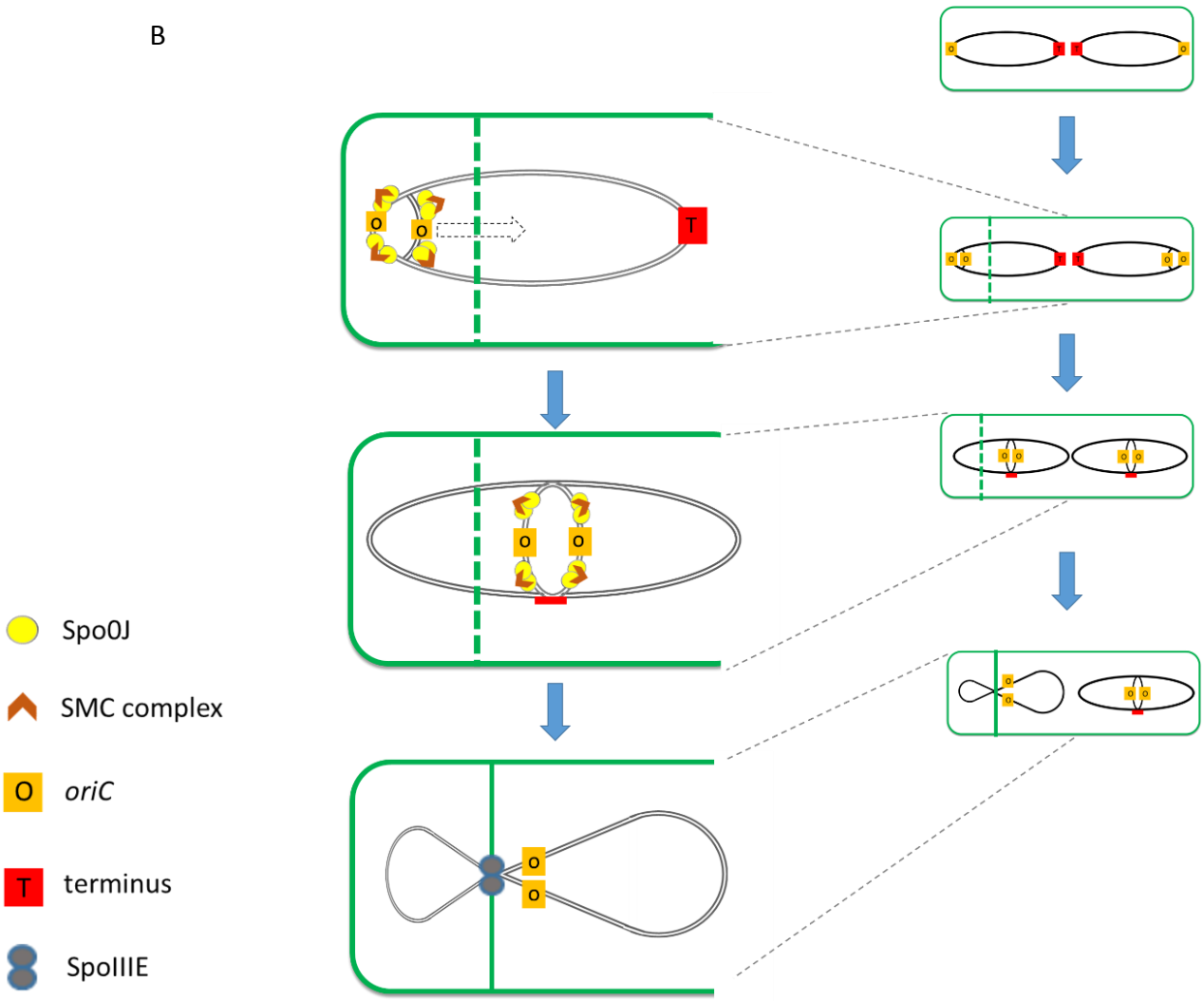

Figure 6.2.1. Chromosome organisation during DNA replication-segregation cycle of *B. subtilis*. A. Cells are born with 2 chromosomes with *ori-ter* configuration, a) initiation of DNA replication leads to movement of the sister origins as a unit to the mid cell due to action of SMC complex. b) Spo0J-mediated DNA bridging and Spo0J-mediated recruitment of SMC complex leading to further condensation at the origin regions draws the origins away from each other, Spo0J-stimulated Soj-ATP hydrolysis and monomerisation produces a depletion field around the Spo0J-bound origins which drives the origin regions away from each other and towards the nucleoid periphery. Monomeric Soj (with no ATPase activity)

accumulates at the septum stabilising the *oriC* at the periphery. Dashed arrows show the direction of the *oriC* movement Figure adapted from Wang et al., 2014a, Wang et al., 2014b and Vecchiarelli et al., 2013 and Vecchiarelli et al.,2014**. B.** In the absence of Soj, untimely initiation of DNA replication in sporulating cells can result in the migration of the origin regions towards the nucleoid centre (due to action of SMC complex) perturbing establishment of an axial filament prior to asymmetric septation (the prospective asymmetric septum is shown as a dashed green line). Moreover Soj is required for efficient delivery of the origin regions to the outer edges of the nucleoid and stabilising their polar position. Taken together, these shortcomings in the absence of Soj could cause a higher origin trapping defect.

6.3 Soj plays a role in *oriC* delivery and/or stabilisation at the cell poles in sporulating cells

In Chapter 5 characterisation of Soj in chromosomal origin trapping using previously identified variants locked in distinct conformations indicated a positive correlation between the ability of Soj to localise at the septum and proper chromosome origin trapping. Based on these results, I envisage a model where in addition to Soj and Spo0J-*parS* mediated segregation of the chromosome origins, the polar localisation of Soj also provides stability for the origin regions at the cell poles (Figure 6.3.1). Furthermore, Soj localisation at the septum was shown to require MinD (Autret and Errington, 2003, Murray and Errington, 2008). Sporulating cells lacking MinD also exhibit an origin trapping defect which could be partially caused by loss of Soj localisation at the septum in absence of MinD (Lenarcic, 2011). MinD-dependent septal recruitment of Soj monomers could be required for maintaining the Soj gradient over the nucleoid as well as producing a site-specific DnaA-helix assembly inhibition zone at the cell poles. Hence, stabilising the *oriC* tether to the cell poles by preventing initiation of DNA replication (which causes alteration in the chromosome organisation) in a pole-specific manner. Alternatively, polar Soj monomers could directly or indirectly be involved in bridging (or stabilising interaction) of DNA-binding elements bound to the *oriC* with the polar proteins. This model is consistent with the previously reported additive defect in chromosomal origin trapping and sporulation frequency of the Δ*soj* Δ*racA* double mutant and also our sporulation frequency analysis of Soj variants in combination with Δ*racA* deletion (Wu and Errington, 2003).

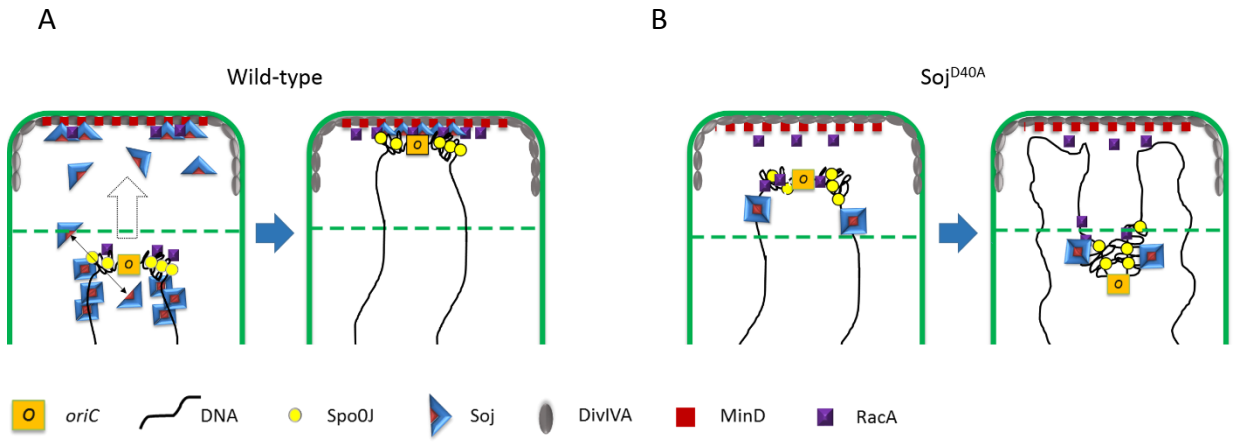

Figure 6.3.1. Soj septal localisation is required for effective *oriC* delivery and capture in the forespores. A. Monomeric Soj recruitment at the septum might provide a polar anchor for *oriC*-associated elements and/or assert inhibition of DNA replication events in cells that have achieved segregation/tethering of the origin regions to the extreme poles. **B.** Origin trapping is reduced in Soj mutants that are defective in septal localisation. Cell harbouring a variant of Soj; Soj^{D40A} incapable of Spo0J-mediated ATP-hydrolysis and septal localisation exhibit an increased origin trapping defect likely due to additive effects of the lack of segregation driving force and septal stabilisation of *oriC* anchor. The prospective asymmetric septa are shown as green dotted lines.

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Appendices

Appendix 1. Solutions and buffers

Name	**Composition**
Blocking buffer	5 % Milk powder in PBS 0.1 % Tween20
CAA (casamino acids)	20 % casamino acids
Ferric ammonium citrate	2.2 mg/ml Ferric ammonium citrate
SMM (Spizizen minimal medium)	1.4% K2HPO4 0.2% (NH4)2SO4 0.6% KH2PO4 0.1% sodium citrate 0.02% MgSO4 In 1 litre of dH2O
Solution A	1.979 g MnCl2·6H2O 0.098 g FeCl3·6H2O

	0.083 g $MgCl_2 \cdot 6H_2O$ In 100 ml of dH_2O
Solution B	53.3 g NH_4Cl 10.6 g Na_2SO_4 6.8 g KH_2PO_4 9.7 g NH_4NO_3 In 1 l of dH_2O; adjust to pH 7.0
Solution C	50 g L-glutamic acid In 1 l of dH_2O; adjust to pH 7.0
Solution D	0.1 M $CaCl_2$
Solution E	40% D-glucose
Solution F	1 M $MgSO_4$
Solution G	25 g Oxoid Casein hydrolysate 11.7 g sodium glutamate 3.125 g L-alanine 3.48 g L-asparagine 3.4 g KH_2PO_4 1.34 g NH_4Cl_2 0.27 g Na_2SO_4 0.24 g NH_4NO_3

	2.45 g FeCl3·6H2O
Solution H	50 mM MnSO4 In 1 l of dH2O
SSC	0.15 M NaCl 0.01 M sodium tricitrate adjust to pH 7.0
10X TBE buffer	1 M Tris 0.9 M boric acid 0.01 M EDTA
Transfer Buffer(for semi dry transfer)	20% methanol 0.5X MES buffer

Appendix 2. Growth medium

Name	**Composition**
CH medium	100 ml solution G 1 ml tryptophan solution 0.2 ml solution H 0.1 ml solution D 0.04 ml solution F
Competence medium	10 ml SMM 0.125 ml solution E 0.1 ml tryptophan solution

	0.06 ml solution F 0.01 ml CAA 0.025 ml ferric ammonium citrate
LB medium	10 g Tryptone 5 g Yeast extract 10 g NaCl In1 l of dH2O, adjust pH to 7, autoclave
Nutrient Agar	28 g Oxoid Nutrient Agar In1 l of dH2O, autoclave
PAB medium	17.5 g Oxoid antibiotic medium no. 3 In1 l of dH2O, autoclave
Sporulation medium	90 ml solution A+B 4 ml solution C 1 ml solution D 4 ml solution F 1 ml tryptophan solution
Starvation medium	10 ml SMM 0.125 ml solution E 0.06 ml solution F