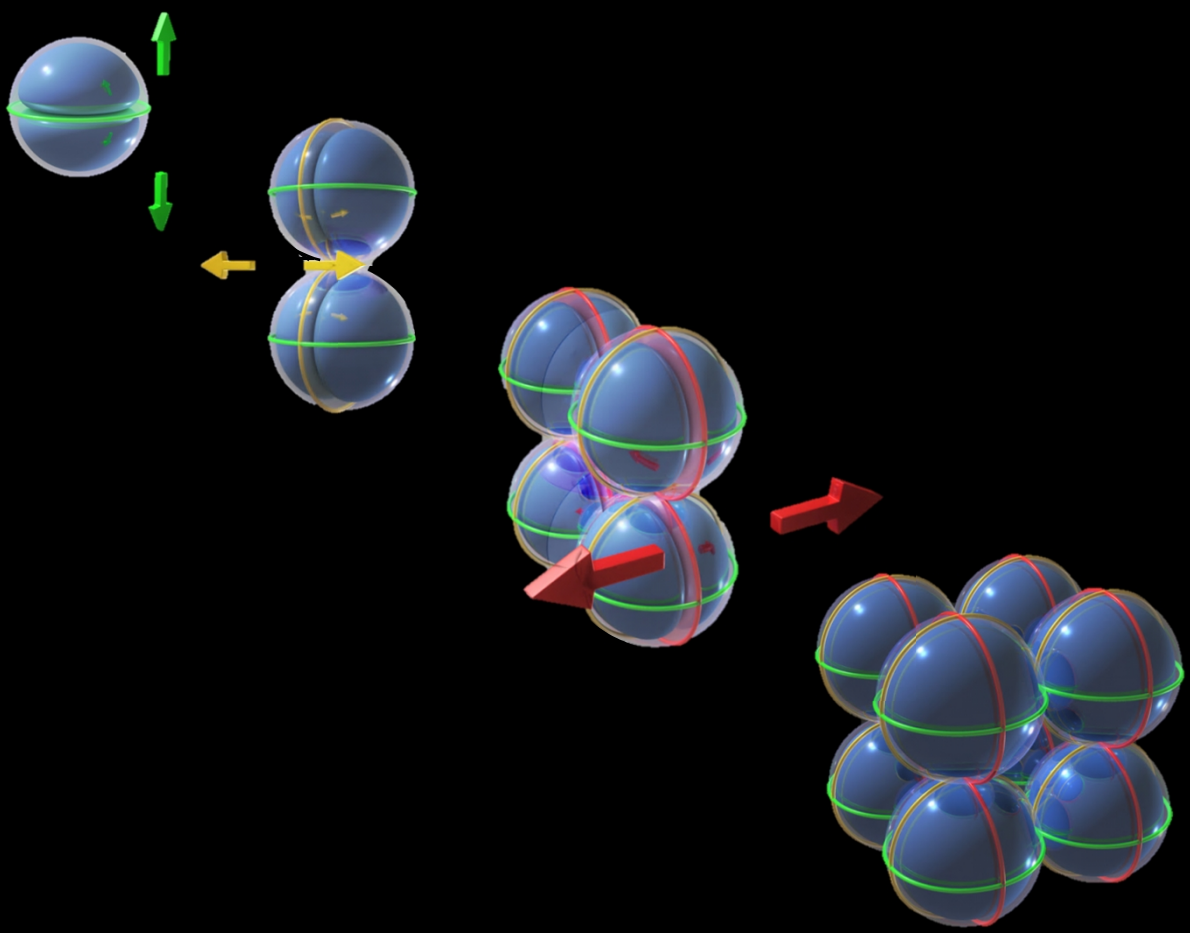
Cell division and chromosome segregation in *Staphylococcus aureus*

Helena Maria Pinto Veiga



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

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COVER IMAGE

Representation of *Staphylococcus aureus* cell division in three perpendicular planes over three successive division cycles. At the beginning of each cycle, the replicated chromosomes (blue) segregate in a specific axis (indicated by the green, yellow and red arrows) and subsequently a division septum is formed (represented by the green, yellow and red discs). Finally the mother cell splits into two identical daughter cells. All the previous division planes are represented by green, yellow and red lines around the cell.

Thesis Publications

- **International Journals**

Veiga H., Pinho M. G. (2009) Inactivation of the SauI type I restriction-modification system is not sufficient to generate *Staphylococcus aureus* strains capable of efficiently accepting foreign DNA. *Applied and Environmental Microbiology.* 75 (10): 3034-8.

Pereira P.M.*, **Veiga H.***, Jorge A.M., Pinho M.G (2010) Fluorescent reporters for studies of cellular localization of proteins in *Staphylococcus aureus*. *Applied and Environmental Microbiology.* 76 (13): 4346-53.
*P.M.P. and H.V. contributed equally to this work.

Veiga H., Jorge A.M., Pinho M.G, (2011) Absence of nucleoid occlusion effector Noc impairs formation of orthogonal FtsZ rings during *Staphylococcus aureus* cell division. *Molecular Microbiology.* Jun; 80 (5): 1366-80.

- **Portuguese Journals**

Veiga H., Pinho M.G (2012) Bacterial cell division: what it takes to divide a prokaryotic cell. *Canal BQ.* Nº9: 18-26.

Additional Publications

Atilano M.L.*, Pereira P.M.*, Yates J., Reed P., **Veiga H**., Pinho M.G.*, Filipe S.R* (2010) Teichoic acids are temporal and spatial regulators of peptidoglycan cross-linking in *Staphylococcus aureus*. *Proceedings of the National Academy of Sciences.*107(44):18991-6
*A.M.L. and P.M.P and also M.G.P. and F.S.R. contributed equally to this work.

Reed P., **Veiga H**., Jorge A.M., Terrak M., Pinho M.G, (2011) Monofunctional transglycosylases are not essential for *Staphylococcus aureus* cell wall synthesis. *Journal of Bacteriology.* 193 (10): 2549-56

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Abbreviations and Acronyms

CFP	Cyan fluorescent protein
CFU	Colony forming units
GFP	Green fluorescent protein
GlcNAc	*N*-acetylglucosamine
IPTG	Isopropyl-β-D-thiogalactopyranoside
KOPS	FtsK-orienting polarized sequence
LA	Luria-Bertani agar
LB	Luria-Bertani broth
MCS	Multiple cloning site
MIC	Minimal inhibitory concentration
MRSA	Methicillin resistant *S. aureus*
MTS	Membrane targeting sequence
MurNAc	*N*-acetylmuramic acid
NBS	Noc-binding sequence
NO	Nucleoid occlusion
PBP	Penicillin binding protein
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field gel electrophoresis
PFU	Plaque forming units
RBS	Ribosome binding site
R-M	Restriction-modification
SBS	SlmA-binding sequence
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sfGFP	Super-fast folding green fluorescent protein
SRS	SpoIIIE recognition sequence
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TUNEL	Terminal deoxynucleotidyl transferase mediated X-dUTP nick end labeling
Van	Vancomycin
VISA	Vancomycin-intermediate *S. aureus* strains
VRSA	Vancomycin-resistant *S. aureus* strains
WT	Wild type
X-Gal	5-bromo-4-chloro-3-indolyl-β-galactopyranoside
YFP	Yellow fluorescent protein

Abstract

Bacterial cell division by binary fission involves several essential steps. Initially, the mother cell duplicates in size and replicates its DNA, in preparation for division. As the new sister chromosomes are synthesized, they are progressively segregated to the future daughter cells and once the division site is cleared of the majority of chromosomal DNA, the division septum starts assembling. The complete septum then constricts and, ultimately, the mother cell splits into two identical daughters. The generation of equal, viable progeny is strictly dependent on the regulation and coordination of these processes, both in space and in time. It is particularly important that the division septum is properly positioned at midcell and that chromosome segregation and cell division are synchronized, to prevent fragmentation of the genome by septum closure over the nucleoid.

Staphylococcus aureus is a particularly interesting model in which to study cell division as the cells are spherical and they divide in alternating perpendicular planes. During three consecutive generations, the septa is sequentially placed in three orthogonal planes, a process that is preceded by the segregation of the sister chromosomes along three perpendicular axes. This mode of division is intrinsically different from that used by the better studied rod-shaped organisms *Escherichia coli* and *Bacillus subtilis,* which always segregate the chromosome in the same direction, parallel to the long axis of the cell and place the septum at the same medial plane, to generate two identical daughter cells.

This thesis focused on the study of the molecular factors and mechanisms underlying cell division and chromosome segregation in *S. aureus* spherical cells, with the aims of (i) investigating the role played by the nucleoid in the selection of *S. aureus* division planes and (ii) assessing the role of DNA translocases in the coordination between chromosome segregation and cell division. In order to perform these studies, molecular genetic tools were developed to better manipulate *S. aureus*.

One of the limitations in genetically manipulating *S. aureus* was the fact that only one strain, the highly mutagenized RN4220, could easily accept plasmid DNA isolated from *E. coli*, presumably as a result of a mutation in the *hsdR* gene of the Sau1 Type I restriction-modification system. In order to obtain transformable variants of relevant *S. aureus* strains, the *hsdR* mutation was reproduced in different backgrounds. As described in chapter two, deletion of *hsdR* gene in three different *S. aureus* strains was not sufficient to make them readily transformable, indicating that transformability of *S. aureus* RN4220 must also depend on other, unknown factor(s).

In order to increase the tool box of vectors that can be used for cell biology studies in *S. aureus*, a series of plasmids that allow the expression of fluorescent fusions to staphylococcal proteins were constructed, as described in chapter three. Additionally, a new vector was constructed to allow the insertion of genes, under the control of the IPTG-inducible P_{spac} promoter, in the ectopic *spa* locus of the *S. aureus* chromosome. This plasmid proved extremely useful for complementation studies that required a low gene dosage, as well as for the construction of strains expressing fluorescent protein derivatives at a distant place from their native locus. The latter is particularly important for proteins encoded in essential operons, where it is critical to avoid polar effects on the downstream genes. These tools were then used to study cell division and chromosome segregation in *S. aureus.*

The first question that was addressed was the role of chromosome segregation in the definition of the plane used for division. Before initiation of chromosome partioning, most of the spherical staphylococcal cell is occupied by the nucleoid. Therefore, it is not possible to define any putative division plane that divides the cell in equal halves without bisecting the nucleoid. A nucleoid-free area, suitable for assembly of the division apparatus, becomes available only upon segregation of the two newly formed chromosomes. This implies that the axis of chromosome segregation restricts the number of possible division planes to only one. Chapter four describes how the geometry of cell division was found to be regulated by the nucleoid in a process mediated by the *S. aureus* homologue of *B. subtilis* nucleoid occlusion protein Noc. Using a fluorescent derivative of

FtsZ, the first protein known to assemble at the division site, it was observed that in the absence of Noc, FtsZ forms multiple ring structures that occupy different circular planes of the spherical cells. These numerous FtsZ structures, which are not productive and do not originate a complete septum, are formed on top of the chromosome, eventually causing double-strand DNA breaks. These results show that, upon chromosome segregation, the action of Noc is required to confine the localization of FtsZ and consequently of the division septum to a single plane in the cell.

In addition to nucleoid occlusion, other regulatory mechanisms coordinate segregation of chromosomes with cell division. The conserved proteins of the FtsK/SpoIIIE family of DNA translocases for example, move DNA away from the division site before cytokinesis. In chapter five of this thesis, it is shown that the two *S. aureus* FtsK/SpoIIIE proteins, named SpoIIIE and Slp (SpoIIIE-like protein), act through independent pathways, most likely synergistically, to guarantee proper clearance of the chromosome from the division site. SpoIIIE is a membrane protein that forms foci in the center of the septum when its DNA translocase activity is required, while Slp is a multifunctional protein that assembles early at the septum. Lack of SpoIIIE causes chromosome segregation defects even during normal growth. In contrast, the C-terminal DNA translocase domain of Slp seems to be required only in situations that impair normal chromosome replication/ segregation. The second function of Slp, performed by its N-terminal and linker domains, is nevertheless critical for proper cell division, since lack of the entire Slp protein results in severe cell division and chromosome morphological defects.

Overall, this study revealed the central role of the nucleoid in establishing the division plane used for septum placement in *S. aureus*. It also highlighted the importance of regulators that couple chromosome segregation with cytokinesis, like Noc or the DNA motors SpoIIIE and Slp, in ensuring the proper progression of the division cycle.

Resumo

A divisão celular bacteriana por fissão binária envolve vários passos essenciais. Inicialmente, em preparação para a divisão, a célula mãe duplica de tamanho e replica o seu DNA. À medida que os dois cromossomas são sintetizados, são progressivamente segregados para as futuras células filhas. Quando o local de divisão deixa de estar ocupado pela maior parte do cromossoma, o septo começa a formar-se, dividindo a célula mãe ao meio. Por fim ocorre a constrição do septo e a célula mãe separa-se em duas células filhas idênticas. A regulação e coordenação destes processos, tanto no espaço como no tempo, é essencial para que seja gerada uma descendência viável e idêntica. É particularmente importante que o septo de divisão esteja perfeitamente posicionado no meio da célula e que os processos de segregação dos cromossomas e divisão celular estejam sincronizados, para evitar uma eventual fragmentação do genoma que aconteceria caso o septo se fechasse sobre o nucleoide.

Staphylococcus aureus é um modelo particularmente interessante para o estudo da divisão celular bacteriana porque tem uma morfologia esférica e divide-se alternadamente em três planos perpendiculares. Ao longo de três gerações consecutivas, o septo é sequencialmente colocado em três planos ortogonais, um processo precedido pela segregação dos cromossomas ao longo de três eixos perpendiculares. Este modo de divisão é intrinsecamente diferente do utilizado por organismos mais estudados, como os bastonetes *Escherichia coli* e *Bacillus subtilis,* que segregam os cromossomas sempre na mesma direcção, paralela ao maior eixo da célula, e formam o septo sempre no mesmo plano, para gerar duas células filhas idênticas.

Esta tese foca-se no estudo dos factores moleculares e mecanismos responsáveis pela divisão celular e segregação de cromossomas nas células esféricas de *S. aureus,* com os objectivos de (i) investigar a função desempenhada pelo nucleoide na selecção dos planos de divisão em *S. aureus* e (ii) avaliar o papel desempenhado pelas translocases do DNA na coordenação entre a segregação de cromossomas e a divisão celular. De forma a

poder realizar estes estudos, foram desenvolvidas ferramentas de genética molecular para melhor manipular *S. aureus.*

Uma das limitações da manipulação genética de *S. aureus* é o facto de apenas uma estirpe, a altamente mutagenizada RN4220, poder facilmente aceitar DNA plasmídico isolado de *E. coli*, presumidamente em resultado de uma mutação no gene *hsdR* do sistema de restrição-modificação Tipo I Sau1. De forma a obter variantes transformáveis de estirpes relevantes de *S. aureus*, a mutação no gene *hsdR* foi reproduzida em "backgrounds" diferentes. Como descrito no capítulo dois, a deleção do gene *hsdR* em três estirpes diferentes de *S. aureus* não foi suficiente para as tornar transformáveis, o que indica que a transformabilidade da estirpe RN4220 deve depender de outro(s) factor(es) desconhecido(s).

Para aumentar o número de vectores disponíveis para os estudos de biologia celular em *S. aureus*, foram construídos plasmídeos que permitem a expressão de fusões fluorescentes a proteínas de Staphylococcus, como descrito no capítulo três. Foi também construído um novo vector que permite a inserção de genes, sob o controlo do promotor indutivel P_{spac}, no locus ectópico *spa* do cromossoma de *S. aureus*. Este plasmídeo provou ser extremamente útil para estudos de complementação que requerem uma dose baixa do gene mutado, assim como para a expressão, em *S. aureus*, de derivados fluorescentes das proteínas de interesse, longe do seu *locus* nativo. Esta última aplicação é particularmente importante para proteínas codificadas em operões essenciais, onde é necessário evitar efeitos polares nos genes codificados a jusante do gene a alterar. Estas ferramentas foram depois usadas para estudar a divisão celular e a segregação de cromossomas em *S. aureus*.

A primeira questão abordada neste trabalho prende-se com o papel desempenhado pela segregação dos cromossomas na definição dos planos usados para a divisão. Antes de se iniciar a segregação dos cromossomas, a maior parte do volume da célula esférica de *S. aureus* está ocupado pelo nucleoide. Portanto, não é possível definir nenhum plano que divida a célula em duas partes iguais sem bissectar o DNA. Assim, só após a segregação dos cromossomas recém-formados é que fica disponível uma zona

essencialmente livre de DNA, onde pode começar a ser formada a maquinaria de divisão. Isto implica que o eixo de segregação dos cromossomas restringe o número de planos de divisão possíveis a apenas um. O capítulo quatro descreve a identificação do papel regulador do nucleoide na geometria da divisão celular, um processo que é mediado pela proteína Noc de *S. aureus,* homologa do factor mediador de "nucleoid occlusion" em *B. subtilis*. Utilizando um derivado fluorescente do FtsZ, a primeira proteína a posicionar-se no local de divisão, foi observado que na ausência de Noc, o FtsZ forma numerosas estruturas em forma de anel, que ocupam diferentes planos circulares da célula esférica. Estas estruturas de FtsZ, que não dão origem a uma maquinaria de divisão funcional e não originam septos completos, são formadas em cima do cromossoma e, eventualmente, resultam em quebras na cadeia dupla do DNA. Estes resultados mostram que, após a segregação dos cromossomas, a acção do Noc é necessária para confinar a localização do FtsZ, e consequentemente do septo, a apenas um único plano na célula.

Existem outros mecanismos de regulação, para além do processo de "nucleoid occlusion", que coordenam a segregação de cromossomas com a divisão celular. Um exemplo são as proteínas conservadas da família de translocases do DNA FtsK/SpoIIIE, que movem o DNA para longe do local de divisão antes da citocinese. No capítulo cinco desta tese, é mostrado que as duas proteínas FtsK/SpoIIIE de *S. aureus*, chamadas SpoIIIE e Slp (SpoIIIE-like protein), actuam através de vias independentes, e muito provavelmente de forma sinergística, para garantir que o local de divisão está livre de cromossomas. A proteína SpoIIIE é membranar e forma focos no centro do septo quando a sua actividade como translocase do DNA é necessária. A proteína Slp, por seu lado, é multifuncional e localiza-se cedo no septo. A ausência de SpoIIIE causa defeitos na segregação de cromossomas mesmo durante o crescimento normal. Em contraste, o domínio C-terminal da proteína Slp, responsável pela actividade de translocação do DNA, parece só ser necessário em situações de replicação/segregação anormal dos cromossomas. A segunda função da proteína Slp, desempenhada pelos seus domínios N-terminal e "linker", é contudo crítica para uma correcta divisão celular, pois a ausência de toda a proteína Slp resulta em sérios defeitos na divisão celular e na morfologia dos cromossomas.

Este estudo revelou o papel central do nucleoide no estabelecimento do plano de divisão onde se forma o septo de *S. aureus*. Este estudo também realçou o importante papel de reguladores, como a proteína Noc ou os motores para transporte de DNA SpoIIIE e Slp, na sincronização entre a segregação de cromossomas e a citocinese, que garante uma correcta progressão do ciclo celular bacteriano.

Chapter 1

General Introduction

Bacterial cell division

Bacterial cells are no longer viewed as simple and disorganized reaction chambers of homogenously distributed enzymes. It is now well established that these organisms possess a complex subcellular organization, maintained by specialized temporal and spatial regulatory systems. Moreover, the three classes of cytoskeletal elements (tubulin, actin and intermediate filaments), previously considered exclusive to eukaryotic cells, have also been shown to be present in bacteria. It is this dynamic and highly regulated interior organization that assures the orderly and coordinated progression of the cell cycle, essential for growth, chromosome segregation and cell division.

During the course of the cell cycle and in preparation for division, which usually occurs by binary fission, bacteria double their mass, replicate their DNA and segregate the two newly formed chromosomes. A division septum then assembles at a predetermined site between the chromosomes, the cell constricts and, ultimately, the mother cell splits into two identical daughters. This apparently straightforward mode of division has been studied since the beginning of the twentieth century. Early investigations, using light and electron microscopy, allowed the observation that, prior to bacterial division, an annular disc (now known as the septum) was formed in the middle of the cell. This structure closed, like an iris diaphragm, by invagination of the cytoplasmic membrane and ingrowth of the cell wall, until partitioning of the cell is complete (Knaysi, 1941, Chapman & Hillier, 1953). However, it was only at the end of the 1960s, with the application of genetic tools, that the essential proteins and molecular mechanisms behind bacterial division started to be characterized. In pioneering work, Hirota and co-workers isolated *Escherichia coli* thermosensitive mutants that failed to form the division septum when grown at nonpermissive temperatures (Hirota *et al.*, 1968). This group of mutant strains included filamentation temperature-sensitive (fts) mutants, which are unable to divide and therefore filament/elongate at the restrictive temperature, as well as partition (par) mutants that, in addition to the filamentation phenotype, had abnormally positioned nucleoids. The study of the *fts* mutants revealed many essential division proteins that

were later shown to localize at the septum. Critical among them is FtsZ (Bi & Lutkenhaus, 1991). This bacterial tubulin homologue is the earliest protein known to assemble at the division septum. It polymerizes into a cytoskeletal contractile structure, the Z-ring, which constitutes the initial position marker of the division site and is responsible for the recruitment of the other components of the division apparatus (Bi & Lutkenhaus, 1991, Goehring & Beckwith, 2005). Only a small group of bacteria divide in the absence of FtsZ (Reviewed in (Erickson & Osawa, 2010)).The Z-ring orchestrates cytokinesis in most prokaryotic cells, similarly to the actin-myosin ring in eukaryotes (Laporte *et al.*, 2010).

FtsZ polymerization has to be tightly regulated in space and time to ensure generation of equal progeny and faithful transmission of hereditary information. The spatial control of FtsZ polymerization usually involves the identification of the midpoint of the cell, although in some developmental situations, like sporulation, division occurs asymmetrically (Barak & Wilkinson, 2007). An efficient temporal coordination between septum placement and chromosome replication/segregation assures chromosome integrity.

In the following sections, the current knowledge on the different steps of the bacterial cell division cycle are summarized: (i) DNA replication and segregation; (ii) FtsZ ring assembly; (iii) Z-ring maturation after orderly recruitment of the divisome components; (iv) septal invagination with constriction of the envelope layers and (v) septum closure and splitting of the daughter cells.

Chromosome replication and segregation

The morphology of the bacterial chromosome

The majority of bacteria possess a single circular chromosome that is not enclosed in a specific membrane-surrounded compartment, but occupies a distinct region in the cytoplasm that, due to its functional resemblance to the eukaryotic nucleus, is termed the nucleoid. In *E. coli*, with a genome of approximately 4.6 Mb, the length of the

chromosome is approximately 1.6mm, which largely exceeds the size of the cell (2µm length and 1µm diameter). For that reason, the DNA is kept in a highly compacted architecture (Odijk, 1998).

The first level of compaction is ensured by topoisomerases that catalyze the coiling of the DNA duplex around itself (Wang, 2002). As a result of topoisomerases activity the circular bacterial chromosome is organized in numerous supercoiled domains that emanate from the central core and have an average length of 10Kb in *E. coli* (Postow *et al.*, 2004). The boundaries of these loops are variable and randomly distributed over the chromosome. Moreover, each domain is independent from the others, which means that, a break in a single loop does not affect the superhelicity of the others (Postow *et al.*, 2004). These supercoiled domains are disrupted during replication and transcription and rapidly reestablished afterwards, with the introduction of negative supercoils. The torcional tension accumulated in the loops is the source of valuable free energy used in the processes that requires melting of the DNA strands (Deng *et al.*, 2005).

Supercoiling by itself is very effective in the reduction of the cellular volume occupied by the chromosome. Nonetheless, other factors contribute to the balance of forces that establish the degree of compaction. Important roles are attributed to the forces exerted by a crowded cytoplasm (Zimmerman & Murphy, 1996) and to the transertion process (insertion of proteins into the membrane as their genes are transcribed and translated) that counteracts compaction by pulling DNA loops towards the cell membrane (Woldringh *et al.*, 1995). Additional very important players include a group of nucleoid-associated 'histone-like' proteins (NAPs) and the SMC/MukB complexes.

NAPs are a large and heterogeneous family of low molecular weight proteins that bend or twist the DNA, which includes: HU (heat unstable protein), H-NS (Histone-like nucleoid structuring protein), IHF (integration host factor) and Fis (factor for inversion stimulation). Regulation of DNA organization is dependent on the combined presence of

different NAPs, some acting as compacting agents, and others as antagonists of compaction (reviewed in (Rimsky & Travers, 2011, Browning *et al.*, 2010)).

The SMC (structural maintenance of chromosome) family, conserved in the different branches of life, includes the well-studied *B. subtilis* SMC protein (Britton *et al.*, 1998) and its *E. coli* homologue MukB (Niki *et al.*, 1991), both of which are involved in the condensation of the chromosome. SMCs function in complex with two non-SMC subunits. MukB interacts with MukE and MukF (Yamazoe *et al.*, 1999), while SMC complexes with ScpA and ScpB (Mascarenhas *et al.*, 2002, Soppa *et al.*, 2002). All prokaryotic SMC proteins have the same structure. They are composed of globular N- and C-terminal domains separated by two long coiled-coil regions and a central flexible hinge. The two coiled-coil motifs fold back onto each other so that the terminal domains form a functional ABC-type ATPase. Then the two SMC molecules dimerize through their hinges domains to form a V-shapped structure with a large opening angle (Melby *et al.*, 1998).

Inactivation of SMC or MukB causes temperature-sensitive growth, nucleoid decondensation and the formation of anucleated cells (Britton *et al.*, 1998, Moriya *et al.*, 1998, Niki *et al.*, 1991). In addition, *B. subtilis smc* mutant cells are hypersensitive to DNA gyrase inhibition (Lindow *et al.*, 2002). If excessive supercoiling is induced in these mutants, by elimination of topoisomerase I (Sawitzke & Austin, 2000) or overexpression of topoisomerase IV (Tadesse *et al.*, 2005), the phenotypes are suppressed. This shows that, as expected, the bacterial SMC/MukB complexes are involved in compaction of the chromosome, most likely by affecting DNA supercoilling. As discussed later, this SMC-mediated chromosome compaction also affects proper chromosome segregation (Danilova *et al.*, 2007, Sullivan *et al.*, 2009, Gruber & Errington, 2009).

The final V-shaped structure of SMC/MukB is the key for SMC-mediated DNA condensation. The two arms of the SMC dimer can embrace and join DNA segments upon ATP-induced closing of the V-shaped structure. The N/C-terminal domains of one dimer can also interact with other dimers originating large nucleoprotein complexes that interlock strands from different DNA loops (Hirano & Hirano, 2006, Hirano, 2006).

Spatial organization of the bacterial chromosome

In the 1990s, with the advent of site-specific DNA labeling methods, it became possible to investigate the three-dimensional arrangement of the bacterial chromosome inside the cell. These studies showed that, in *E. coli* and *B. subtilis,* the origin (*oriC*) and terminus (*ter*) of DNA replication present a bipolar and very reproducible pattern of localization during the cell cycle (Webb *et al.*, 1997, Niki *et al.*, 2000). Likewise, in several bacteria, individual loci of the circular chromosome were consistently found at defined cellular locations in between the regions occupied by the origin and terminus (Teleman *et al.*, 1998, Viollier *et al.*, 2004, Niki *et al.*, 2000). This shows that there is a correlation between the position of a locus on the chromosomal map and its spatial localization within the cell, i.e., that the bacterial chromosome is organized and orientated in the cell. This chromosomal organization is species dependent. Slow growing *E. coli* cells with a single non-replicative chromosome, have *oriC* and *ter* positioned close to the midcell with the left and right chromosome arms in opposite cell halves (Figure 1.1) (Wang *et al.*, 2005, Wang *et al.*, 2006, Nielsen *et al.*, 2006b). In contrast, *Caulobacter crescentus* chromosome is positioned along the long axis of the cell, with the origin and terminus near opposite cell poles (Figure 1.1) (Viollier *et al.*, 2004).

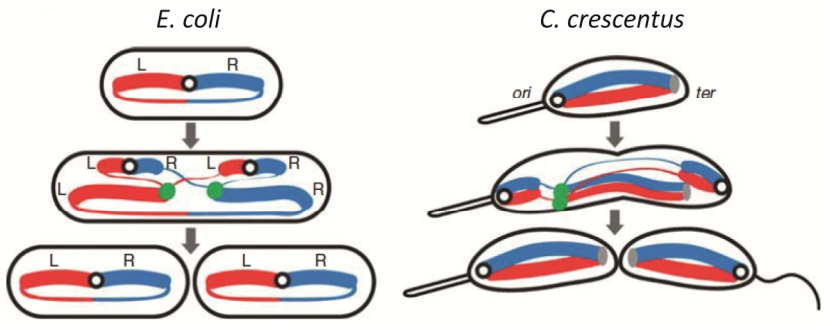

Figure 1.1. The bacterial chromosome is spatially organized inside the cell. Representation of the nucleoid organization inside slow growing *E. coli* and *C. crescentus* cells, showing *oriC* regions (open circles), the left (L, red) and right (R, blue) chromosomal arms and *ter* regions (closed gray circle in *C. crescentus* and thin lines in *E. coli*). The replisome is represented by closed green circles and the newly replicated DNA as thin lines. Three stages of *E. coli* and *C. crescentus* division cycles are represented, showing the localization of the different chromosomal regions as replication/segregation proceeds. Adapted from (Jackson *et al.*, 2012).

As discussed later, the linear chromosomal arrangement of loci is maintained in each cell cycle as a consequence of an orderly movement of the DNA regions during chromosome segregation.

Chromosome replication

Bacterial chromosome replication initiates at a single defined site, the origin of replication (*oriC*). Conserved motifs within this region, the DnaA boxes, are recognized by the AAA+ ATPase bacterial initiation protein, DnaA, which induces the local separation of the double-stranded DNA and is therefore required for initiation of DNA replication (Kaguni, 2006). This event is followed by DnaC-mediated assembling of two DnaB helicases that separate further the DNA duplex to open two symmetric replication forks. Replication starts after complete loading of the additional components of the bacterial replication complex, the replisome (reviewed in (Mott & Berger, 2007, Reyes-Lamothe *et al.*, 2012)).

It is essential that the chromosome is replicated to completion only once during each division cycle and that replication occurs before cell division. This is controlled at the level of DnaA-*oriC* interaction by the action of several restrictive mechanisms that modulate the DnaA ligation to *oriC* according to the physiological state of the cell (reviewed in (Boye *et al.*, 2000, Donachie & Blakely, 2003)).

The assembling of the complete replisome marks the beginning of the elongation phase of chromosome replication. The replication forks progress bi-directionally through each arm of the circular chromosome, until they meet at the terminus region (*ter*). With the movement of these forks the unreplicated DNA undergoes an increase in twist that can be accommodated as positive supercoiling ahead of the fork or, occasionally, due to the rotation of the replication fork, diffuse behind to form precatenanes (the two new sister chromosomes interwrapped) (Figure 1.2A). DNA gyrase acts to remove the positive supercoils accumulated ahead, whereas topoisomerase IV assumes two functions, removal of positive supercoils and decatenation (Postow *et al.*, 2001, Espeli & Marians, 2004, Schvartzman & Stasiak, 2004). As the replication forks approach each other near the terminus region, the short unreplicated DNA between them becomes more

inaccessible to DNA gyrase. Therefore, the positive supercoiling accumulated ahead of the forks, diffuses behind, forming precatenanes that, with the end of replication, are converted in catenanes (interlinked sister chromosomes). These final links have to be removed by topoisomerase IV (Sherratt, 2003).

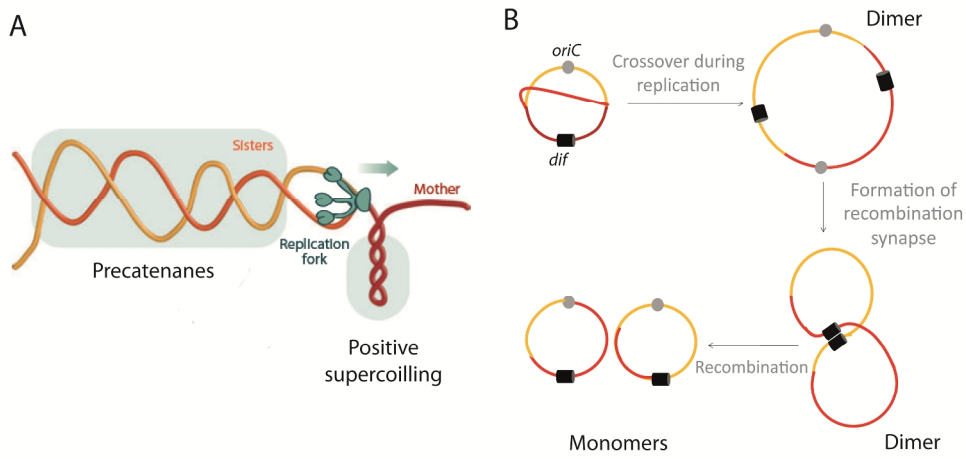

Figure 1.2. Precatenanes and dimers formed as a consequence of bacterial chromosome replication. (A) Progression of the replication fork (green arrow), with concomitant unwinding of the DNA double helix, results in the accumulation of positive supercoils ahead of the fork. When this positive tension diffuses behind the fork, the newly replicated strands of DNA rotate and interwrap, originating precatenanes. DNA gyrase and topoisomerase IV remove the positive supercoils accumulated ahead, while decatenation is only performed by topoisomerase IV. Adapted from (Reyes-Lamothe *et al.*, 2012). (B) Crossover events between two growing circular chromosomes produce chromosome dimers. Site-specific recombination at the *dif* site (black box), located in *ter* region, resolves dimers to monomers. This reaction is catalyzed by two tyrosine recombinases (XerC and XerD in *E. coli*) and requires FtsK in *E. coli*. Dark red: mother chromosome; light red and orange: newly replicated daughter chromosomes.

Replication of a circular chromosome can also produce chromosome dimers, as a consequence of crossing over during homologous recombination of the newly replicated sister chromosomes (Figure 1.2B) (Lesterlin *et al.*, 2004). This has been estimated to happen, in *E. coli*, once every six generations (10-15% of a population) (Steiner & Kuempel, 1998, Perals *et al.*, 2000). Before segregation, chromosome dimers have to be converted to monomers by the combined action of two tyrosine recombinases, XerC and XerD (RipX and CodV in *B. subtilis*), that act at the *dif* recombination site located in the *ter*

region (Lesterlin *et al.*, 2004). In *E. coli*, the action of the XerCD and Topoisomerase IV chromosome-resolving enzymes depends on the cell division protein FtsK that, as discussed in more detail later, establishes an important link between the end of chromosome replication/segregation and the beginning of cell division.

In rapid growth conditions, some bacteria, like *E. coli* and *B. subtilis*, can undergo multi-fork DNA replication to ensure that the time it takes to synthesize and segregate new chromosomes does not exceed the mass doubling time. During multi-fork replication, the mother cell initiates more than one round of replication before division. This gives a "head-start" to its progeny that, in this situation, are able to complete chromosome replication/segregation in fast dividing cells (Cooper & Helmstetter, 1968).

Chromosome segregation

In marked contrast with eukaryotic cells, bacteria segregate their chromosome progressively in parallel with replication. Immediately after *oriC* replication, the newly formed origin regions are separated and move to conserved positions in the incipient daughter cells. Subsequently, as each new locus is replicated, the two new copies are condensed and incorporated into the nucleoid in formation. In this way, the DNA loci are organized accordingly to the order of replication, which assures the maintenance of the original orderly arrangement of the chromosome (Figure 1.1) (Nielsen *et al.*, 2006a, Viollier *et al.*, 2004). Following decatenation and, eventually, chromosome dimer resolution, segregation is concluded, with the movement of the terminus regions away from the cell-division site, to allow cytokinesis.

Despite decades of study, the mechanisms that mediate bacterial chromosome segregation remain elusive. Bacteria do not possess an obvious mitotic-like apparatus. However, the observation of rapid and directed movement of the new *oriC* regions into opposite directions, led to the proposal of an active mechanism as the driving force for segregation, for which different models were proposed. It was hypothesized that chromosomes partition by bidirectional extrusion from the replisome, driven by the free

energy of DNA polymerization (Lemon & Grossman, 2001). However, this model required the two replisomes to be immobile in the cell and was therefore ruled out after the finding that replisomes in fact track along a stationary chromosome (Bates, 2008, Reyes-Lamothe *et al.*, 2008). Alternatively, the transcription process was implicated in DNA segregation. RNA polymerase is an abundant and powerful motor protein with a rather stationary behavior. Since the majority of genes are oriented away from the *oriC*, the combined effect of many transcription reactions could result into the movement of the newly synthesized DNA (Dworkin & Losick, 2002). However it is now known that inhibition of transcription with rifampicin, does not prevent proper chromosome segregation (Wang & Sherratt, 2010). For a short period, the actin-like cytoskeletal protein MreB, involved in the determination of the bacterial cell shape, was also implicated in the partioning process (Kruse *et al.*, 2006, Soufo & Graumann, 2003, Kruse *et al.*, 2003). However, it was shown that segregation defects observed upon MreB depletion result from the MreB-induced morphological cell alterations rather than from the direct involvement of this protein in DNA segregation (Karczmarek *et al.*, 2007, Wang & Sherratt, 2010).

Other studies provided evidence for the role of the chromosomal encoded ParABS system in chromosome segregation. The *parABS* locus, constituted by two *trans*-acting proteins, ParA and ParB, and a *cis*-acting site *parS*, was first identified in several low-copy number plasmids as the essential component of their partioning system. ParB protein binds to the *cis*-acting centromere analog *parS* and spreads into the flanking regions forming a nucleoprotein complex. This complex then recruits the ATPase ParA protein, which is thought to assemble into dynamic polymeric structures to mediate segregation (reviewed in (Schumacher, 2008, Gerdes *et al.*, 2010)). The presence of ParA and ParB homologues in the chromosome of several bacteria (but not *E. coli*) (Livny *et al.*, 2007, Gerdes *et al.*, 2000) raised the hypothesis that chromosomally encoded Par proteins might function in the process of chromosome segregation, in a similar way to the plasmid segregation system. This idea was reinforced by the observation that insertion of various chromosomal *parABS* homologues in an otherwise unstable plasmid increases its efficiency of transmission (Yamaichi & Niki, 2000, Godfrin-Estevenon *et al.*, 2002, Lin &

Grossman, 1998). However, there are only few cases in which this system was shown to be truly essential for chromosome segregation. The most compelling evidence was obtained in *Vibrio cholerae* and *Caulobacter crescentus,* which segregate their new chromosomes asymmetrically. In both of these bacteria, the *oriC* is located at one pole, (the "old" pole) and, after replication, one copy remains at that site while the other transverses the entire length of the cell, to be positioned at the opposite end (new pole). (Fogel & Waldor, 2005, Mohl & Gober, 1997). *V. cholerae,* one of the few bacteria with two circular chromosomes, each with their own ParABS system, was shown to require all Par components to properly segregate the *oriC* region of its larger chromosome (Chromosome I). $ParB_I$ binds to three *parS* sites in the vicinity of the *oriC,* and this region is moved across the cell by $ParA_I$. Time-lapse analysis shows that a $ParA_I$ structure stretches throughout the cell from the new pole to the old one and, then, progressively retracts, pulling the $ParB_I$-*parS* complex (Fogel & Waldor, 2006). In *C. crescentus*, ParA is also required for the segregation of a chromosomal region containing the *oriC* and the ParB-*parS* complex (Toro *et al.*, 2008). Notably, in both species, the ParABS systems are primarily involved in the segregation of the *oriC* regions. Most likely, segregation of the rest of the chromosome relies on other, still unknown, mechanisms.

Contrary to *V. cholerae* and *C. crescentus*, inactivation of Par system in *B. subtilis* has only mild effects in chromosome segregation. The *B. subtilis* genome encodes ten *parS* sites, eight of which have high affinity for Spo0J (ParB homologue) and are located in the origin-proximal region of the chromosome (Lin & Grossman, 1998, Breier & Grossman, 2007). Spo0J binds to these sites and spreads over a distance of approximately 800 Kb, to form a nucleoprotein complex that appears as a single fluorescent focus, when visualized by fluorescence microscopy using a fluorescent fusion to Spo0J (Lin *et al.*, 1997). A Spo0J mutant produces 1 to 2% of anucleated cells (Ireton *et al.*, 1994) while in 15% of new born cells *oriC* regions are positioned closer together than in the wild-type (Lee *et al.*, 2003, Lee & Grossman, 2006). Deletion of Spo0J disrupts the organization of a large (~1Mb) region surrounding the replication origin. On the contrary a Soj (ParA homologue) deletion mutant has no visible chromosome partioning defects

(Ireton *et al.*, 1994) and its absence only affects the position of a discrete region next to the *oriC* (Sullivan *et al.*, 2009). All these results argue against the idea that *B. subtilis* parABS system is responsible for exerting the motor force that drives chromosome segregation. In fact, it was recently shown that the role exerted by Spo0J in chromosome partitioning is rather indirect. The *parS*-Spo0J nucleoprotein complex targets the SMC complex to the origin of replication, which ensures proper condensation of the newly synthesized DNA, necessary for proper chromosome segregation (Sullivan *et al.*, 2009, Gruber & Errington, 2009). In an independent and complementary pathway, Spo0J also regulates Soj role in chromosome replication. Soj, due to its ability to alternate between a monomeric form and an ATP-bound dimeric form, functions as a dynamic molecular switch that regulates the activity of the replication initiator protein DnaA. Initiation of replication is stimulated by the Soj dimer and inhibited in the presence of the monomeric form. Generally, due to the presence of Spo0J, that stimulates Soj ATPase activity and therefore the conversion of the Soj dimer to monomer, the equilibrium favors the presence of the monomeric conformation (Murray & Errington, 2008, Scholefield *et al.*, 2011). However, at some point in the cell cycle, Soj "escapes" Spo0J control by an unknown mechanism, so replication can occur.

The effect of Spo0J/Soj in sporulation is also dependent on Soj-regulation of the initiation of replication. For a long time it has been known that in the absence of Spo0J, Soj represses the expression of genes required for the early stages of sporulation, which blocks the switch from vegetative growth to spore differentiation (Ireton *et al.*, 1994). This happens because, without Spo0J control, Soj dimer stimulates DnaA which, in turn, activates a sporulation checkpoint protein, Sda, that blocks sporulation (Murray & Errington, 2008). Spo0J and Soj not only regulate the entrance into sporulation but, as soon as this pathway is activated, also ensure that the future spore receives its copy of the chromosome. Soj drives the movement of *parS*-Spo0J nucleoprotein complex towards the pole (in the future spore) where it is tethered by the sporulation protein RacA to the polar protein DivIVA (Ben-Yehuda *et al.*, 2003b, Wu & Errington, 2003).

Until now, all efforts devoted to finding a conserved machine-like apparatus that depends on specific proteins to move bacterial DNA during segregation have not been successful. All of the identified systems seem to act more at the level of organization and proper positioning of the origin of replication than on the movement of the bulk of the chromosome. Therefore, alternative models, presenting chromosome segregation as a passive process that depends on physical aspects of DNA organization, have been receiving more attention. It has been suggested that chromosome segregation is a self-organizing process, driven by separation of transertion domains (Woldringh, 2002). As explained earlier, numerous chromosomal regions are linked to the cell envelope due to the coupling of co-transcriptional translation and translocation of membrane proteins (transertion). According to this model, the transertion complexes of each daughter chromosome create two independent proteolipid domains that are located in opposite cell halves. Continuing synthesis of DNA, and thus addition of new transertion complexes between the domains was thought to push the two nucleoids away from each other (Woldringh, 2002). However, this implies that the movement of each DNA region is gradual, which is not what is observed *in vivo* (Webb *et al.*, 1998, Viollier *et al.*, 2004).

The most plausible and attractive model presented to date, suggests that the driving force for chromosome segregation in bacteria is entropy (Jun & Mulder, 2006, Jun & Wright, 2010). Mathematical modeling analysis indicates that, when long polymeric chains (the chromosome molecules) are confined to a compartment with little free space (the bacterial cell), the polymers strongly self-avoid and therefore separate spontaneously, in a physiologically relevant timescale, without the need for any other force except entropy. That is, entropy alone can induce segregation of replicated chromosomes. The same is not applicable to plasmids. Due to their small size, plasmids do not suffer the same constriction forces as chromosomes inside the cell. Therefore, plasmids cannot solely rely on the entropic forces to segregate, which may explain why they possess such easily identifiable partitioning systems. This model of entropic exclusion of sister chromosomes also implies that the primary role SMC/MukB proteins is to maintain proper chromosome organization, necessary for the establishment of the

entropic forces. In agreement, aberrant chromosome organization, due to the absence of functional SMC/MukB, leads to altered chromosome segregation (Danilova *et al.*, 2007, Sullivan *et al.*, 2009, Gruber & Errington, 2009).

The FtsK/SpoIIIE family of DNA translocases

Bacteria possess regulatory mechanisms to ensure that septum formation only occurs after proper clearance of the chromosomes from the division site (discussed in more detail below). Nevertheless, in some situations, particularly when chromosome dimers are formed, the septum starts assembling over non-segregated DNA. To guarantee that all genetic material is properly cleared from the division site before septum closure, bacteria rely on the action of DNA translocases (Kaimer & Graumann, 2011). These double strand DNA transporters, which belong to the FtsK/SpoIIIE family, localize at the leading edge of the constricting septum, to remove all DNA standing in the way of the septum (Yu *et al.*, 1998a, Biller & Burkholder, 2009, Kaimer *et al.*, 2009) or form channels in already assembled septa, to rescue trapped DNA (Wu & Errington, 1994, Bath *et al.*, 2000). In addition, some FtsK/SpoIIIE proteins also help to resolve the physical chromosomal links formed during replication (Steiner *et al.*, 1999).

FtsK/SpoIIIE DNA translocases share a common organization in three domains (Bigot *et al.*, 2007). A membrane localized N-terminal domain, a soluble carboxy-terminal region and a central linker of variable length that connects the N- and C-terminal domains. The poorly conserved N-terminal domain often mediates functions unrelated with DNA translocation, while the C-terminal domain forms an ATP-dependent DNA pumping motor (Aussel *et al.*, 2002, Kaimer *et al.*, 2009, Massey *et al.*, 2006, Bath *et al.*, 2000).

The *E. coli* DNA translocase, FtsK, is a multifunctional protein that links three important cell division processes: resolution of chromosome links, segregation of sister chromosomes and formation of the division septum. Its amino-terminal membrane domain ($FtsK_N$), which contains 4 transmembrane helices (Dorazi & Dewar, 2000), serves

to localize the protein to the septum and is part of the divisome, the multiprotein complex that orchestrates bacterial cell division (see sections ahead). Cells expressing only $FtsK_N$ can survive, however, in the absence of this domain, the division machinery cannot properly assemble at the septum and thus, the cells present a filamentation phenotype, typical of *E. coli* cells that cannot divide (Wang & Lutkenhaus, 1998, Yu *et al.*, 1998a, Draper *et al.*, 1998). The FtsK linker domain ($FtsK_L$), with approximately 600 amino acids, extends from the septum into the cytoplasm, separating $FtsK_N$ from the soluble C-terminal motor domain ($FtsK_C$). This linker was initially considered to be just a spacer, however, it is now known to be required for the establishment of essential interactions between FtsK and several other components of the divisome (Bigot *et al.*, 2004, Dubarry & Barre, 2010). Contrary to the other two domains, the soluble C-terminal FtsK region ($FtsK_C$) is dispensable for septum synthesis. Instead, it bears the important roles of promoting chromosome dimer resolution and removing DNA from the site of constriction (Yu *et al.*, 1998b, Liu *et al.*, 1998, Steiner *et al.*, 1999). $FtsK_C$ can be subdivided into three regions (Yates *et al.*, 2003). The α and β domains, that constitute the DNA translocation motor, and the regulatory γ domain that interacts with DNA and with the XerD recombinase. The α/β subdomains of different FtsK molecules assemble into a hexameric ring with a large central channel capable of accommodating a double-stranded DNA molecule (Massey *et al.*, 2006). This complex uses the energy of ATP hydrolysis to move chromosomal DNA with a velocity of approximately 5 Kb/s (Massey *et al.*, 2006, Pease *et al.*, 2005, Saleh *et al.*, 2004). The correct direction of translocation, crucial for the accurate sorting of chromosomal DNA, is determined by FtsK ability to "read" sequences on the DNA. The chromosome carries preferential $FtsK_C$ loading sites, known as KOPS (FtsK-orienting polarized sequences), which are polarized towards the terminus region (Bigot *et al.*, 2005, Levy *et al.*, 2005). The γ subdomain, which is connected to the α/β motor by a small flexible linker (Sivanathan *et al.*, 2006), recognizes these KOPS motifs, promoting the loading of $FtsK_{\alpha/\beta}$ in a precise orientation. This interaction directs DNA translocation by FtsK towards the *dif* recombination site, a specific region within the replication terminus region where the final chromosome unlinking occurs (Pease *et al.*,

2005, Bigot *et al.*, 2006, Lowe *et al.*, 2008, Sivanathan *et al.*, 2009, Graham *et al.*, 2010). FtsK also promotes chromosome dimer resolution in combination with the site-specific tyrosine recombinases XerC and XerD. The $FtsK_C$-mediated DNA translocation, helps to align the sister *dif* sites, bound by XerC and XerD, to form a productive recombination synapse (Corre & Louarn, 2002, Steiner *et al.*, 1999, Capiaux *et al.*, 2002, Massey *et al.*, 2004). Additionally, FtsKγ interacts with and activates XerD to perform a first pair of strand exchanges that result in an intermediate Holliday junction. This Holliday junction is then resolved into a crossover product, independently of FtsK, by XerC-mediated strand exchanges (Yates *et al.*, 2003, Barre *et al.*, 2000, Aussel *et al.*, 2002, Yates *et al.*, 2006, Grainge *et al.*, 2011). FtsK, like other DNA translocases, strips off proteins from the DNA as translocation occurs (Crozat *et al.*, 2010). Interestingly however it stops DNA movement when it encounters the XerCD-*dif* complex, thus preventing the removal of the tyrosine recombinases (Graham *et al.*, 2010). FtsK is also thought to assist in the removal of the catenation links that persist between sister chromosomes after replication. $FtsK_C$ was shown *in vitro,* to interact with the ParC subunits of topoisomerase IV and stimulate the decatenation activity of this enzyme (Espeli *et al.*, 2003, Bigot & Marians, 2010). Moreover, the FtsK-XerCD-*dif* recombination machinery seems to be able to unlink catenated DNA monomers when topoisomerase IV function is compromised (Ip *et al.*, 2003, Grainge *et al.*, 2007).

The processes of chromosome unlinking, chromosome segregation and septum formation have to be tightly coordinated to ensure faithful transmission of the genetic material. Therefore, it seems very advantageous that regulation of these processes is centered on one protein. In the last few years, the role of FtsK as a cell division checkpoint has been the subject of discussion. Is FtsK able to delay septation until chromosomes are completely and correctly segregated? It seems plausible that it can do so, since otherwise it would have a short period to clear DNA from the division site. FtsK does not transport DNA before membrane fusion (Dubarry & Barre, 2010) and the XerCD-*dif* complex is only activated after septum invagination has already started (Kennedy *et al.*, 2008b). One possibility is that when chromosomes are at the division site and thus

$FtsK_C$ is enrolled in the process of DNA translocation, the linker becomes stretched and cannot establish the necessary contacts with the other cell division proteins, which delays septum constriction. Only once the activity of the C-terminal motor is no longer required, are the interactions reestablished, allowing division to proceed (Grainge, 2010, Dubarry *et al.*, 2010).

In *B. subtilis,* two DNA translocases, SpoIIIE and SftA, act at different stages to coordinate chromosome segregation and cell division. SftA, like FtsK, clears DNA before septum constriction and SpoIIIE moves trapped chromosomes after septum closure.

SpoIIIE is best known for its essential role in spore development (Wu & Errington, 1994). During sporulation, an asymmetric septum is formed at the cell pole, dividing the cell into a large mother cell and a small forespore (future spore). When this septum assembles, only the origin-proximal region of the chromosome is positioned in the forespore space. Approximately two-thirds of the chromosome are still located in the mother cell. SpoIIIE uses the energy of ATP hydrolysis to actively transport, through the closed septum, the remaining two-thirds of the DNA destined to the forespore (Wu & Errington, 1997, Wu *et al.*, 1995, Bath *et al.*, 2000, Burton *et al.*, 2007). This DNA crosses two membranes and a peptidoglycan layer (Becker & Pogliano, 2007) and, like with FtsK, the direction of translocation, is determined by SpoIIIEγ-mediated recognition of specific DNA sequences. These sequences, known as SRS (SpoIIIE recognition sequences), are polarized towards the terminus region (Ptacin *et al.*, 2008, Becker & Pogliano, 2007). SpoIIIE has a transmembrane N-terminal domain and is usually distributed throughout the cytoplasmic membrane. Only in the presence of septum-entrapped chromosomes, do the SpoIIIE molecules assemble around the DNA, forming multimeric translocation channels (Figure 1.3) (Wu & Errington, 1997, Ben-Yehuda *et al.*, 2003a). These are seen by fluorescence microscopy, as a bright foci at the middle of the septum (Wu & Errington, 1997, Ben-Yehuda *et al.*, 2003a). The two arms of the entrapped circular chromosome are transported, at the same time, by two independent SpoIIIE channels. Each channel is, most likely, composed of two communicating SpoIIIE hexamers (one within each

membrane) joined through their extracellular loops located in the peptidoglycan layer (Massey *et al.*, 2006, Burton *et al.*, 2007, Becker & Pogliano, 2007) (Figure 1.3). It is not yet clear, however, how the final loop of the circular DNA molecule is translocated across the septum. Since the DNA does not seem to break to allow translocation, most likely the channels open/merge for the chromosome to pass. Recent data suggests that the SpoIIIE molecules can only form stable channels when their C-terminal motor interacts with the DNA (Fleming *et al.*, 2010). Possibly, once the terminus region reaches the septum and the DNA motor dissociates from the DNA, the channels destabilize and a large pore is opened for final DNA translocation (Fleming *et al.*, 2010).

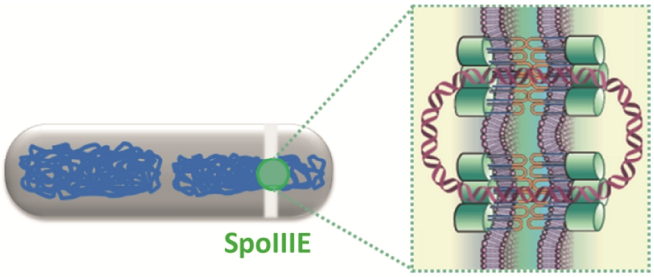

Figure 1.3. *B. subtilis* SpoIIIE forms DNA conducting channels. During sporulation, to translocate the last two thirds of the chromosome from the mother cell to the forespore, across two membranes and a peptidoglycan layer, SpoIIIE forms DNA conducting channels. Most likely, each SpoIIIE channel, is composed of two communicating SpoIIIE hexamers (one within each membrane) joined through their extracellular loops located in the peptidoglycan layer. Each channel can only transport one double-stranded chromosome arm. Green: SpoIIIE; Grey: Sporulating *B. subtilis* cell; Blue and Violet: DNA. Adapted from (Wu & Errington, 2008).

Importantly, SpoIIIE has a second function during sporulation. Its N-terminal domain mediates the two sporulation processes that require membrane fission: asymmetric division and spore engulfment (Sharp & Pogliano, 1999, Sharp & Pogliano, 2003, Liu *et al.*, 2006).

SpoIIIE is expressed not only during sporulation but also during *B. subtilis* vegetative cycle. However, it is only recruited to the vegetative division septum in the rare occasions in which a post-septational mechanism is required to rescue trapped DNA. In accordance with this, the absence of SpoIIIE impairs proper cell cycle progression only under conditions that induce a high frequency of septum assembling over unsegregated DNA, such as, in the absence of the chromosome organization protein SMC or in the presence of antibiotics that cause DNA damage or affect DNA replication/segregation (Sharpe & Errington, 1995, Britton & Grossman, 1999). The role of SpoIIIE as a post-septational DNA translocase is supplemented, during vegetative growth, by the presence of a second DNA motor protein, SftA, which localizes at the septum throughout the cell cycle (Kaimer *et al.*, 2009, Biller & Burkholder, 2009). Since SftA does not have a clear transmembrane domain, the localization at the septum most likely requires a direct interaction with a divisome protein. The exact nature of this interaction is not known, but it requires the N-terminal and linker domains of SftA and a properly assembled FtsZ-ring (Kaimer *et al.*, 2009). Lack of SftA, or of its C-terminal domain, causes only mild segregation defects. Nevertheless, as for SpoIIIE, the important SftA role becomes evident when DNA replication is blocked or chromosome segregation is compromised (Kaimer *et al.*, 2009, Biller & Burkholder, 2009). Accordingly, SftA mutant cells are more sensitive to DNA damaging agents than wild-type cells (Kaimer *et al.*, 2009). Most likely SftA, akin to *E. coli* FtsK, localizes at the septum to promote faithful chromosome partitioning before septum constriction. Its C-terminal domain forms hexamers and has ATPase activity (Kaimer *et al.*, 2009). To date, it is not known if SftA can recognize the same SRS sequences as SpoIIIE to direct DNA translocation.

Loss of both SpoIIIE and SftA exacerbates the single mutants' phenotypes (Kaimer *et al.*, 2009, Biller & Burkholder, 2009) indicating that SpoIIIE and SftA act synergistically to regulate chromosome segregation at different levels. SftA moves the DNA away from the closing septum, while SpoIIIE acts as a back-up system, ready to be activated whenever chromosomes get trapped by the septum. SpoIIIE and SftA seem also to be involved in chromosome dimer resolution. Although they do not activate RipX and CodV

(XerC and XerD homologues) (Sciochetti 2001), they presumably promote efficient chromosome dimers resolution by positioning the *dif* sites in close proximity (Biller & Burkholder, 2009, Kaimer *et al.*, 2011).

Assembling of the division septum

Formation of the division septum occurs in a multistep process that is initiated by the assembly of FtsZ into a ring-shaped structure, the Z-ring, at the division site. This cytoskeleton element then mediates the recruitment of all the other division proteins that compose the bacterial division apparatus, the divisome.

The divisome proteins can be separated into two distinct groups according to their temporal pattern of recruitment. The first group of proteins arrives early to stabilize the Z-ring and anchor it to the membrane. The latter components assemble after a considerable delay (20% of the cell cycle in *B. subtilis*), to form the mature constriction-competent septal ring (Gamba *et al.*, 2009, Aarsman *et al.*, 2005).

The Z-ring

FtsZ is the most conserved bacterial division protein and, despite the low sequence similarity, it is a homologue of the eukaryotic tubulin. Both proteins share a similar 3D structure and similar biochemical properties (Lowe & Amos, 1998, Erickson, 1995, Erickson *et al.*, 2010). FtsZ, like tubulin, undergoes GTP-dependent polymerization into protofilaments (Mukherjee & Lutkenhaus, 1994). The head-to-tail liner arrangement of FtsZ subunits, sandwiches GTP between its binding site, located in the lower subunit, and a cation-coordinating loop of the subunit above. Since this loop mediates GTP hydrolysis, FtsZ acts as its own GTPase-activating protein with GTP hydrolysis occurring only after the subunits come into contact in the protofilament (de Boer *et al.*, 1992, RayChaudhuri & Park, 1992, Mukherjee *et al.*, 1993, Scheffers *et al.*, 2001, Scheffers *et al.*, 2002, Oliva *et al.*, 2004).

Although tubulin protofilaments associate laterally to produce microtubules, FtsZ microtubule-like structures have never been observed. *In vitro*, FtsZ assembles predominantly into single-stranded protofilaments (Mukherjee & Lutkenhaus, 1998) and, depending on the experimental conditions, can also associate further into sheets, helices, minirings and tubules (Erickson *et al.*, 1996, Popp *et al.*, 2009, Bramhill & Thompson, 1994, Lu *et al.*, 2000). Despite all *in vitro* data, the *in vivo* Z-ring structure remains to be identified. It is currently thought that the Z-ring is formed by a large number of overlapping FtsZ protofilaments. Given that each protofilament is on average 120nm long (30 subunits), as a consequence of FtsZ high turnover rate (see below), a single protofilament should not be sufficient to encircle the entire division site (approximately 3000nm) (Chen *et al.*, 2005). Accordingly, FtsZ short arc-like filaments, lying underneath the inner membrane of *C. crescentus,* have been observed by cryo-electron microscopy tomography (Li *et al.*, 2007).

Different techniques that allow monitoring of cells expressing FtsZ-GFP have been used to explore the dynamics of the Z-ring (Anderson *et al.*, 2004, Stricker *et al.*, 2002, Thanedar & Margolin, 2004, Peters *et al.*, 2007). These studies demonstrated that the ring is not a static structure but rather suffers constant remodeling throughout its existence. FtsZ subunits continuously enter and exit the protofilament with an average half-life of 9 seconds (Anderson *et al.*, 2004, Stricker *et al.*, 2002). This flux is dictated by FtsZ intrinsic GTPase activity, with GTP hydrolysis promoting the disassembling of the protofilament. In addition, FtsZ filaments are also dynamic throughout the cell cycle. FtsZ spiral-like structures have been observed underneath the cell membrane, along the length of the cell, before a midcell ring is formed (Thanedar & Margolin, 2004, Peters *et al.*, 2007, Monahan *et al.*, 2009). This suggests that the Z-ring is not a closed circle but a dynamic helix able to extend from pole to pole. It is the condensation of this helix at mid cell that originates the ring observed at the time of division (Thanedar & Margolin, 2004, Peters *et al.*, 2007, Monahan *et al.*, 2009).

Assembling of the Z-ring – Recruitment of the first group of divisome proteins

Although proper assembling of the Z-ring requires its attachment to the membrane, FtsZ has no intrinsic affinity for phospholipids. Therefore, in the beginning of septum formation, adaptor proteins are recruited to anchor the FtsZ filaments to the membrane. In *E. coli* two essential proteins, FtsA and ZipA, collaborate in tethering FtsZ to the membrane (Pichoff & Lutkenhaus, 2002, Hale & de Boer, 1997, Hale & de Boer, 1999). These proteins seem to have overlapping roles, since Z-rings can assemble in the absence of FtsA or ZipA but not in the absence of both proteins (Pichoff & Lutkenhaus, 2002). However, the mutual presence of FtsA and ZipA is necessary for the recruitment of downstream divisome components (Pichoff & Lutkenhaus, 2002). Both FtsA and ZipA recognize a short conserved sequence at the extreme carboxy-end of FtsZ (Ma & Margolin, 1999, Mosyak *et al.*, 2000, Haney *et al.*, 2001) and are recruited independently to the septum membrane (Hale & de Boer, 1999). ZipA is embedded in the membrane via its N-terminal single trans-membrane anchor (Hale & de Boer, 1997) while FtsA possesses a C-terminal amphipathic helix that acts as a membrane targeting sequence (MTS) (Pichoff & Lutkenhaus, 2005). In contrast to FtsA MTS (Pichoff & Lutkenhaus, 2005, Shiomi & Margolin, 2008), the N-terminal membrane region of ZipA is more than a simple anchor and cannot be replaced by heterologous membrane domains (Hale *et al.*, 2000). Presumably, is necessary for the interaction with other division proteins. The cytoplasmic ZipA C-terminus domain also has an important function in promoting the lateral interaction of FtsZ protofilaments *in vitro* (Hale *et al.*, 2000). FtsA is a structural homologue of actin and is more widely conserved than ZipA (van den Ent & Lowe, 2000), which is only found in *E. coli* close relatives (Hale & de Boer, 1997). The *B. subtilis* FtsA also ensures correct formation of the Z-ring but its deletion does not cause a lethal phenotype (Beall & Lutkenhaus, 1992, Jensen *et al.*, 2005).

The formation of a stable Z-ring also requires the presence of proteins that modulate the polymerization, and so the dynamics, of this FtsZ structure. Many of these proteins are non-essential. However, the individual deletion of each one of them is synthetic lethal when combined with mutations in other early divisome components. Two

soluble proteins ZapA and ZapC interact with FtsZ to enhance polymerization. ZapA is a conserved protein (Gueiros-Filho & Losick, 2002) that was shown to promote the bundling of FtsZ monomers and protofilaments *in vitro* and the assembling and stability of Z-rings *in vivo* (Gueiros-Filho & Losick, 2002, Small *et al.*, 2007, Monahan *et al.*, 2009). ZapA also recruits the auxiliary protein ZapB to the division site (Ebersbach *et al.*, 2008, Galli & Gerdes, 2010). ZapC stabilizes FtsZ protofilaments by suppressing their GTPase activity and also promotes their lateral association (Hale *et al.*, 2011, Durand-Heredia *et al.*, 2011). The most widely conserved FtsZ regulator is ClpX, one of the components of the ClpXP protease. ClpX is a chaperone that recognizes and unfolds proteins to be degradated by ClpP serine protease. In addition, and independently of its role as a chaperone, ClpX inhibits Z-ring polymerization and seems to be required for the maintenance of the cytoplasmic pool of unassembled FtsZ, indispensable for the Z-ring dynamics (Haeusser *et al.*, 2009, Weart *et al.*, 2005).

SepF and EzrA are two additional important regulators of Z-ring assembly and dynamics in gram-positive bacteria. SepF is a positive regulator (Hamoen *et al.*, 2006, Ishikawa *et al.*, 2006) that promotes the bundling of FtsZ protofilaments and the suppression of their GTPase activity (Singh *et al.*, 2008). *In vitro*, SepF assembles into large rings, which induce the bundling of FtsZ protofilaments into tubular structures that have the same diameter as the SepF ring (Gundogdu *et al.*, 2011). EzrA is a negative regulator of FtsZ polymerization with multiple functions in division. Akin to ZipA, EzrA has a single N-terminal transmembrane domain and binds to the extreme C-terminus of FtsZ (Levin *et al.*, 1999, Haeusser *et al.*, 2004, Singh *et al.*, 2007). This protein prevents formation of aberrant Z-rings at the poles (Levin *et al.*, 1999) and, in its absence, the critical FtsZ concentration for Z-ring formation is reduced (Levin *et al.*, 1999, Levin *et al.*, 2001). EzrA not only reduces the affinity of FtsZ for GTP, but also increases the rate of GTP hydrolysis in the protofilaments, thus promoting the FtsZ polymer disassembly (Chung *et al.*, 2007). In the absence of EzrA, the FtsZ polymers become stabilized and can overcome the inhibitory activity of MinCD at the cell poles (see below) (Levin *et al.*, 1999, Levin *et al.*, 2001). EzrA is uniformly distributed throughout the plasma membrane in non-dividing

cells but, it concentrates at the Z-ring during division (Levin *et al.*, 1999). The localization at midcell seems to contradict its role as inhibitor of Z-ring formation, however, it reflects EzrA's second role. At the division site, EzrA promotes FtsZ subunit turnover and, in this way, maintains the proper FtsZ assembly dynamics within the ring (Haeusser *et al.*, 2007). EzrA was shown to have a third role in coordinating the switch between the two modes of growth/peptidoglycan synthesis in rod-shape bacteria: lateral synthesis for elongation and septal synthesis for division. Together with divisome protein GpsB, EzrA re-localizes the major cell transglycosylase/transpeptidase enzyme, PBP1, according to the cell cycle stage (Claessen *et al.*, 2008).

Maturation of the Z-ring

Maturation of the Z-ring involves the ordered recruitment of the late divisome components, many of which are membrane-anchored, some protruding into the cytoplasm, like the above mentioned FtsK, and others presenting extracellular domains (Errington *et al.*, 2003). The exact signal that triggers assembling of late proteins to the divisome remains to be discovered. However, it is known that their localization at the septum depends on interactions with other divisome components, on substrate availability and on the presence of transient forms of peptidoglycan (de Boer, 2010).

In *E. coli,* each late divisome protein is recruited in a hierarchically, interdependent manner, which implies that the depletion of one division protein abolishes the assembling of all downstream proteins. The known order is: FtsX/E, FtsK, FtsQ, [FtsL, YgbQ(FtsB)], FtsW, FtsI (PBP3), FtsN, AmiC, EnvC. FtsB and FtsL are both dependent on FtsQ for localization (Buddelmeijer & Beckwith, 2002, Aarsman *et al.*, 2005). In *B. subtilis* the recruitment seems to be cooperative, the DivIB, FtsL and DivIC proteins (homologues of *E. coli* FtsQ, FtsL and FtsB respectively) and the penicillin binding protein PBP2B (homologue of FtsI) are interdependent for assemble. The absence of any of these proteins prevents all the others from localizing (Errington *et al.*, 2003, Daniel *et al.*, 2006). A detailed description of the late divisome proteins and their interactions can

be found in the following reviews (Errington *et al.*, 2003, den Blaauwen *et al.*, 2008, Goehring & Beckwith, 2005).

The number of divisome components for which the exact function is known is still reduced. However, it is likely that several of them control the peptidoglycan synthetic machinery and regulate membrane dynamics during invagination (Errington *et al.*, 2003). In the group of proteins with clearly identified functions are *E. coli* FtsK, which clears DNA from the division site (see above), and the penicillin binding proteins (e.g. *E. coli* PBP3 and *B. subtilis* PBP2B) involved in the localized synthesis of septal peptidoglycan (Scheffers & Pinho, 2005, den Blaauwen *et al.*, 2008). This polymer is the major component of cell wall, it surrounds the bacterial cell, confers resistance and protection against the high internal osmotic pressure and maintains cell shape. Peptidoglycan is composed of repeating units of *N*-acetylglucosamine (GlcNAc) and N-Acetylmuramic acid (MurNAc) linked by a β-1,4-glycosidic bond. A short peptide is attached to the MurNAc sugar used to form cross-links between adjacent glycan strands (Vollmer *et al.*, 2008a).

Constriction and closure of the division septum

Once the septal ring is completely assembled, the divisome proteins promote the coordinated annular constriction of the cytoplasmic membrane and any other envelope layer present (such as the outer membrane of gram-negative bacteria). The constriction process seems to be energy-dependent but, until now, no constriction motor has been identified in bacteria. In the past decade, the idea that the Z-ring is the origin of the constriction force has gained more strength. Osawa and Erickson showed that when FtsZ molecules, engineered to tether themselves to the membrane, are incorporated inside lipid tubular vesicles, they can form Z-rings that are able to produce constriction of liposomes (Osawa *et al.*, 2008). Therefore, the Z-ring can exert a constriction force onto the membrane without additional proteins. Interestingly, membrane-tethered FtsZ protofilaments have a fixed direction of curvature. Therefore, if the FtsZ molecules bind

to the outside of the liposome through their C-terminal domain, they distort the surface into multiple concave depressions. Conversely, if the amphipathic tethering helix is switched to FtsZ N-terminus, convex protrusions are formed (Osawa *et al.*, 2009). This suggests that bending of the protofilaments is the source of the mechanical constriction force applied, by the Z-ring, to the membrane. Remodeling of the protofilaments, from a straight to a curved conformation, depends on GTP hydrolysis (Lu *et al.*, 2000). Accordingly, without conversion of GTP to GDP, the protofilaments become rigidly stabilized and, even though Z-rings can assemble and initiate constriction, the constriction process is quickly stopped (Osawa & Erickson, 2011).

At the end of constriction, the membranes close to compartmentalize the daughter cells. However, almost nothing is known about the mechanisms that promote these fusion events, except that, during sporulation, they require SpoIIIE (Liu *et al.*, 2006, Sharp & Pogliano, 1999, Sharp & Pogliano, 2003).

After septum synthesis, the septal cell wall material has to be hydrolyzed so that the two daughter cells can split. In *E. coli*, the hydrolysis of the peptidoglycan layer occurs concomitantly with membrane invagination. On the contrary, in *B. subtilis* and many other gram-positive bacteria, the daughter cells stay connected by a non-constricted cell wall layer, which is cleaved only after membrane fission. The localized hydrolysis of the septal peptidoglycan is catalyzed by a specific group of proteins know as peptidoglycan hydrolases or autolysins (reviewed in (Vollmer *et al.*, 2008b)). These potentially lethal enzymes must be tightly controlled to prevent opening of breaches in the cell wall protective layer. However, it is still unclear how their action is restricted to a specific time and cellular location (Uehara & Bernhardt, 2011). In gram-positive bacteria, expression of the autolysins genes is controlled by the WalK/WalR two component system that recognize environmental signals (Dubrac *et al.*, 2007) and downregulated in the absence of PBP1 responsible for peptidoglycan synthesis (Pereira *et al.*, 2009). Additionally, their activity and localization seem to depend upon the absence of fully formed wall teichoic acids (Yamamoto *et al.*, 2008, Schlag *et al.*, 2010) and on the acetylation status of the

peptidoglycan sugar backbone (Laaberki *et al.*, 2011, Moynihan & Clarke, 2011). In *E. coli*, a fail-safe mechanism exists to prevent premature hydrolysis. The LytM factors, EnvC and NlpD, which are activators of the principal peptidoglycan hydrolases AmiA, AmiB and AmiC (Uehara *et al.*, 2010), are recruited to the septum well before the three amidases and before initiation of peptidoglycan synthesis (Yang *et al.*, 2011). In contrast, their cognate autolysins do not go to the septum if peptidoglycan synthesis is blocked (Peters *et al.*, 2011). In this way, activators and autolysins are only concentrated at the septum at a specific time, ensuring that septal peptidoglycan synthesis precedes peptidoglycan hydrolysis (Peters *et al.*, 2011).

Regulation of Z-ring assembling

Z-ring assembling is the earliest and most crucial event in cytokinesis and therefore has to be strictly regulated in space and time, in coordination with DNA segregation, the cell cycle status and environmental conditions.

Spatial and temporal regulation of the Z-ring

One central aspect of cell division is the establishment of the time and place for division to occur. Two complementary systems are required for precise selection of the division site in rod-shaped bacteria: the Min system and nucleoid occlusion. In combination, these systems inhibit Z-ring formation in nearly all cell places, with the exception of the midcell region, after chromosome segregation.

- **Min system**

The Min system prevents Z-ring assembly away from midcell, especially near the poles, and relies on the FtsZ inhibitor MinC, the MinC carrier MinD and topological factors, different in *E. coli* and *B. subtilis,* that spatially organize the MinCD complex. Mutations in the Min system result in the production of several anucleated minicells,

reflecting frequent aberrant septation near the cell poles (de Boer *et al.*, 1989, Lee & Price, 1993).

MinC inhibits the assembly of the Z-ring. It functions as a homodimer and has two functionally distinct domains connected by a flexible linker (Hu & Lutkenhaus, 2000, Cordell *et al.*, 2001). The N-terminal domain ($MinC^N$) is essential for the inhibitory action of MinC. It antagonizes FtsZ polymer assembly without affecting the GTPase activity (Hu *et al.*, 1999, Hu & Lutkenhaus, 2000, Dajkovic *et al.*, 2008, Shen & Lutkenhaus, 2010). The C-terminal domain ($MinC^C$) mediates dimerization and interaction with MinD (Hu & Lutkenhaus, 2000) which is required to position $MinC^N$ near FtsZ (Shen & Lutkenhaus, 2009). $MinC^C$ in complex with MinD, has inhibitory activity (Shiomi & Margolin, 2007). *In vitro* $MinC^C$/MinD prevent lateral interactions between FtsZ filaments (Dajkovic *et al.*, 2008). $MinC^C$/MinD complex also inhibits Z-ring formation by competing with the FtsZ stabilizers FtsA and ZipA, as it recognizes a region of the FtsZ C-terminus (Shen & Lutkenhaus, 2009) that is also the target site for these proteins. MinD is an ATPase with an amphiphatic membrane targeting sequence. In its ATP-bound state, MinD forms membrane-bound dimers (de Boer *et al.*, 1991, Szeto *et al.*, 2002, Zhou & Lutkenhaus, 2003, Szeto *et al.*, 2003) that recruit MinC from the cytoplasm, forming the MinCD complex (Hu & Lutkenhaus, 2003, Lackner *et al.*, 2003). The inhibitory activity of MinCD is spatially restricted by the action of MinE in *E. coli* (de Boer *et al.*, 1989) and MinJ/DivIVA in *B. subtilis* (Edwards & Errington, 1997, Marston *et al.*, 1998, Bramkamp *et al.*, 2008, Patrick & Kearns, 2008).

The MinC/MinD proteins, in *E. coli*, are stimulated by MinE to oscillate from pole-to-pole (Figure 1.4) (Raskin & de Boer, 1999). MinD-ATP interacts with the membrane and with itself (Hu *et al.*, 2002, Suefuji *et al.*, 2002) forming an inhibitory zone that "grows" from the pole towards the cell center. Once this area approaches midcell, a ring of MinE molecules (E ring) is formed at its leading edge (Raskin & de Boer, 1997, Fu *et al.*, 2001, Hale *et al.*, 2001). This E-ring stimulates MinD ATPase activity causing the release of MinD-ADP (together with MinC) into the cytoplasm and the gradual shrinking of the inhibitory zone (Hale *et al.*, 2001, Hu *et al.*, 2002, Wu *et al.*, 2011). In the meantime, the

released MinD-ADP is converted into MinD-ATP and re-binds the membrane at the opposite pole, where the concentration of MinE is lowest. Only when all MinD molecules have been disassembled from the “first" pole, MinE is released from its location, towards the opposite pole, to form an E-ring at the border of the new inhibitory zone (Fu *et al.*, 2001). This remarkable self-organized oscillation continues indefinitely and results in a higher time-averaged concentration of MinCD division inhibitor at the cell poles (Raskin & de Boer, 1999). In contrast, *B. subtilis* MinCD topological specificity is provided by DivIVA protein, which preferentially assembles on concave (negatively curved) membranes (Lenarcic *et al.*, 2009, Ramamurthi & Losick, 2009) and mediates the subcellular localization of this complex, via MinJ (Bramkamp *et al.*, 2008, Patrick & Kearns, 2008). DivIVA forms ring-like structures on either side of the invaginating septum and recruits MinJ which, in turns, recruits MinCD (Eswaramoorthy *et al.*, 2011). It was recently proposed that these rings prevent the immediate reassembly of FtsZ released from the septum, at the end of constriction. Therefore, they avoid the formation of irregular Z-rings in the vicinity of the region that will become the new daughter cell poles (Eswaramoorthy *et al.*, 2011, Gregory *et al.*, 2008). These DivIVA rings are maintained as septation proceeds. However, when the flat septum is converted into hemispherical poles, they collapse into patches, eventually releasing DivIVA to assemble new rings at the next division site (Eswaramoorthy *et al.*, 2011).

- **Nucleoid occlusion**

The concept of nucleoid occlusion, originally proposed by Woldringh and coworkers (Mulder & Woldringh, 1989, Woldringh *et al.*, 1991), postulated a negative effect of the nucleoid on septum assembly. We now know that this effect is based on the activity of DNA-associated proteins that act as inhibitors of Z-ring formation in the vicinity of the nucleoid (Figure 1.4) (Wu & Errington, 2004, Bernhardt & de Boer, 2005). The inhibition of division over the chromosomes not only provides a protective mechanism to the DNA, which otherwise would be bisected by the septa, but it also contributes to the precise temporal and spatial positioning of the division septum.

The nucleoid occlusion effect is mediated in *B. subtilis* by Noc (Wu & Errington, 2004) and in *E. coli* by SlmA protein (Bernhardt & de Boer, 2005). The deletion of either *noc* or *slmA* genes, does not alter cell division, presumably because the Min system is sufficient to compensate for their absence. However, depletion of these nucleoid occlusion effectors in combination with a Min deficiency results in impaired septation. Without the restrictions imposed by the two negative regulators, multiple FtsZ structures are formed throughout the cell, including on top of the DNA, which leads to insufficient FtsZ molecules in each structure to support proper Z-ring formation. Accordingly, this phenotype can be suppressed by FtsZ overexpression (Wu & Errington, 2004, Bernhardt & de Boer, 2005). The role of Noc and SlmA, in rod-shaped bacteria, also becomes evident in cases of abnormal chromosome replication and segregation. In these situations, both nucleoid occlusion effectors are required to prevent FtsZ assembling over the unpartitioned nucleoids (Wu & Errington, 2004, Bernhardt & de Boer, 2005).

Noc and SlmA have similar localization patterns (Wu & Errington, 2004, Bernhardt & de Boer, 2005). Both proteins recognize specific DNA sequences that are scattered throughout the chromosome but absent from the terminus region (Wu *et al.*, 2009, Cho *et al.*, 2011, Tonthat *et al.*, 2011). Noc binds the *B. subtilis* Noc-binding DNA sequences (NBSs) (Wu *et al.*, 2009) while SlmA associates with the consensus SlmA-binding sequences (SBSs) in the *E. coli* chromosome, in an event essential for SlmA activity (Cho *et al.*, 2011, Tonthat *et al.*, 2011). This asymmetric distribution of the nucleoid occlusion effectors in the chromosome is essential for their role and for correct timing of cell division in coordination with chromosomes segregation. The reason for this is that the formation of the division septum is prevented during the major part of chromosome replication/segregation process, due to the presence of the nucleoid occlusion effector at midcell. Only when the Noc/SlmA-protected area is completely segregated and the terminus proximal region, which is unprotected, starts to segregate, can FtsZ start to polymerize at the division site.

The mechanism by which Noc prevents Z-ring assembly over the nucleoid is still unclear. Although its role requires binding to the NBS sites (Wu *et al.*, 2009), Noc may not

interact with FtsZ (Wu & Errington, 2011). On the contrary, SBS-bound SlmA is known to directly interact with FtsZ (Bernhardt & de Boer, 2005, Cho *et al.*, 2011, Tonthat *et al.*, 2011) but the mechanistic basis for SlmA inhibition of Z-ring formation is also still theme of discussion. Two recent studies addressing this question present different conclusions (Cho *et al.*, 2011, Tonthat *et al.*, 2011). According to one model, SlmA acts as a dimer (or higher order oligomer) to disrupt FtsZ-FtsZ interactions and antagonize FtsZ polymerization. It possibly does so by stimulating the conversion of GTP into GDP within the FtsZ polymers (Cho *et al.*, 2011). A second contradictory model, proposes that SlmA does not prevent the liner polymerization of FtsZ but rather the interactions between FtsZ polymers required to form higher-order structures. It also suggests that SlmA interacts with FtsZ without affecting its GTPase activity. According to this study, the FtsZ protofilaments bound to the two sides of the SlmA dimer are forced to grow in anti-parallel directions which would prevent bundling of FtsZ polymers and inhibition of assembly (Tonthat *et al.*, 2011).

In the absence of the Min system and nucleoid occlusion, FtsZ still has a modest bias for polymerizing at mid-cell (Wu & Errington, 2004, Bernhardt & de Boer, 2005). Therefore, it is likely that additional mechanisms exist to regulate septum formation. This idea is supported by the observation that a Noc-independent nucleoid occlusion effect is responsible for preventing bisectation of the chromosome during replication fork arrest (Bernard *et al.*, 2010). Also, using an outgrown *B. subtilis* spore system, it was shown that in the absence of Min and Noc, Z-rings are positioned precisely at midcell, although at a later stage and less efficiently (Rodrigues & Harry, 2012). In fact, Morya *et al* proposed an additional mechanism for Z-ring positioning at midcell, the so called “ready-set-go” model, which states that the midcell region becomes increasingly “potenciated” for division as the initial phase of chromosome replication reaches completion (Moriya *et al.*, 2010).

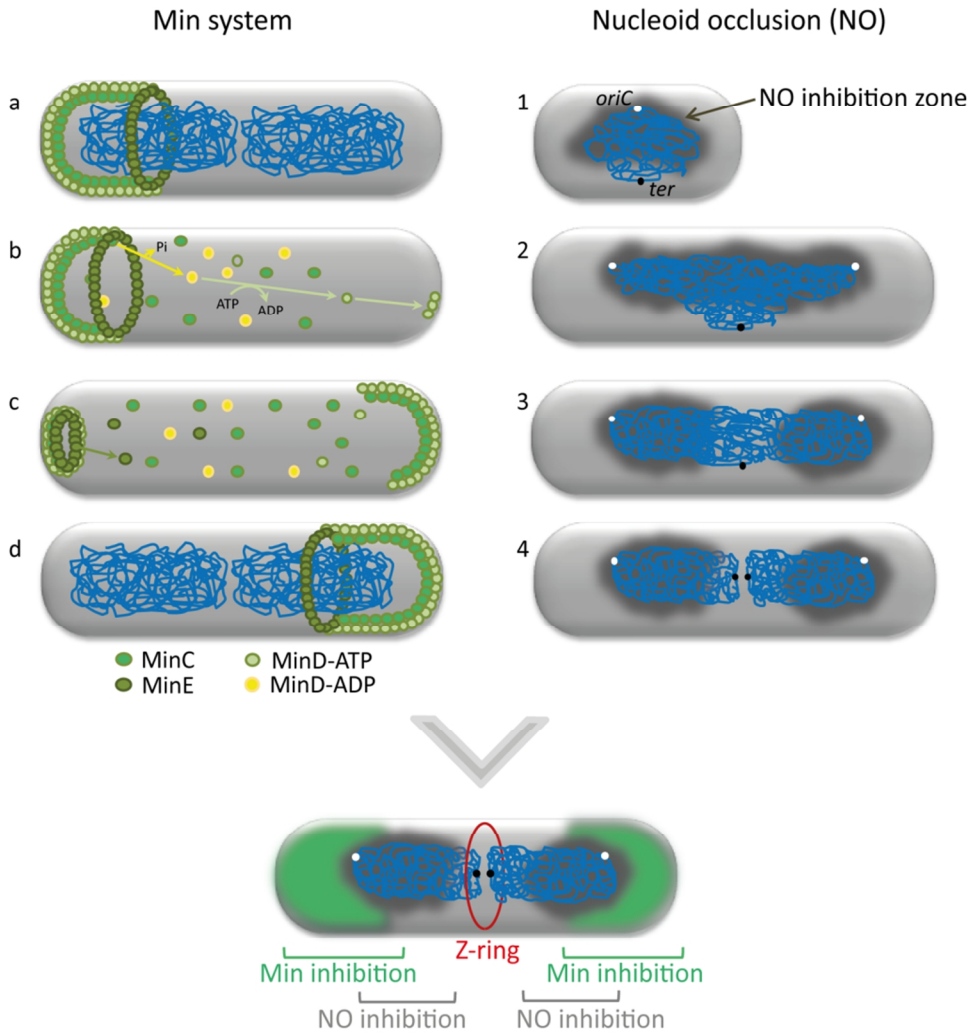

Figure 1.4. Temporal and spatial regulation of Z-ring assembly by Min system and Nucleoid occlusion. The Min system prevents Z-ring formation at the cell poles. In *E. coli*, the inhibitory activity of MinC is concentrated at the poles as a consequence of a MinE-mediated oscillation. (a) MinD-ATP binds to the membrane and recruits MinC, forming an inhibitory zone that "grows" from the pole towards the cell center. As the MinCD complex approaches midcell, the MinE molecules form a ring-like structure (E ring) at its leading edge. (b) This E ring gradually releases MinD, and thus MinC, from the membrane by stimulating MinD ATPase activity. The released MinD-ADP undergoes nucleotide exchange in the cytoplasm, generating MinD-ATP that then binds to the membrane at the opposite pole. (c) When the "initial" inhibitory zone has been disassembled, the E-ring is released and (d) a new ring is formed at the border of the other inhibitory zone, restarting the cycle. The oscillation results in a higher time-averaged concentration of MinC at cell poles. For simplicity, the chromosomes were omitted in figures b and c. **(Continued on next page)**

Figure 1.4 (continuation) Nucleoid occlusion (NO) prevents septum formation on top of the DNA. (1) The nucleoid occlusion effectors (SlmA in *E. coli* and Noc in *B. subtilis*) are inhibitors of FtsZ assembly that bind specific sequences in the chromosome that are essentially absent from the *ter* region. At the onset of *E. coli* division cycle, before DNA replication, Z-ring cannot form at midcell since this area is occupied by the NO-protected *oriC*-proximal region. (2) As replication/segregation proceeds the nucleoid occlusion effectors are moved away from the mid cell region. (3) When the NO-protected regions are completely segregated and the last third of the chromosome starts to replicate/segregate, a NO-free zone is opened at midcell, allowing for the initiation of Z-ring polymerization. (4) With the complete segregation of the chromosomes, the septum can be fully assembled. The bottom panel shows the combined inhibition exerted by the Min system and nucleoid occlusion, which restricts Z-ring formation to the midcell, after chromosomes segregation.

Regulation of Z-ring assembly according to cell cycle status

- **Nutrient dependent inhibition of FtsZ assembly**

Bacterial cells are considerably larger when growing at a fast growth rate, in a nutrient-rich media, than when growing in conditions of poor nutrient availability (discussed in more detail in (Young, 2010)). This occurs, most likely, because in rich media cells have to reach a longer length to permit chromosome segregation during multi-fork DNA replication (see DNA replication section) (Weart *et al.*, 2007). The ability to modulate cell size accordingly to the growth rate requires cells to detect nutrient availability and communicate this information to the division apparatus so that FtsZ will assemble and therefore initiate division after the cells reached a certain size.

A growth rate-dependent inhibitor of FtsZ, UgtP, was identified in *B. subtilis* and may constitute the link between metabolism and cell division (Weart *et al.*, 2007). UgtP is an enzyme of the glucolipid biosynthesis pathway that uses UDP-Glucose in the synthesis of the diglucosyl diacylglycerol anchor of lipoteichoic acids. Under nutrient rich conditions, in response to high levels of its substrate, UgtP is present in the cytoplasm but concentrated at the Z-ring. There it inhibits FtsZ assembling, by disruption of the lateral protofilaments interactions, delaying division until cells reach the appropriate size. In contrast, during growth on a poor carbon source, the levels of UgtP are reduced and the

remaining protein is sequestered in randomly distributed foci where, presumably, it has no effect on FtsZ polymerization. This allows earlier formation of the division septum and thus generation of smaller cells (Weart *et al.*, 2007). A mutation in one of the enzymes of the *E. coli* UDP-Glucose synthetic pathway results in the same phenotype observed in *B. subtilis* mutant, i.e. short cells in rich medium. Although no UgtP homologue can be found in *E. coli*, most likely the biosynthesis of UDP-Glucose is a conserved mechanism that coordinates growth rate and cell division in bacteria (Weart *et al.*, 2007).

- **Regulation of Z-ring formation in response to DNA damage**

When DNA is damaged, an SOS response is activated to repair the DNA and cease cell division until normality is restored. This response is mediated by the RecA protein that induces the autoproteolysis of the repressor LexA leading to the expression of several SOS genes under its control (reviewed in (Erill *et al.*, 2007)). In *E. coli,* one of these SOS genes is *sulA,* which is sufficient to halt cell division (Huisman *et al.*, 1984, Huisman & D'Ari, 1981). SulA is an inhibitor of FtsZ polymerization (Trusca *et al.*, 1998, Mukherjee *et al.*, 1998) that binds to the surface of the FtsZ cation-coordinating loop, blocking the interaction between the FtsZ monomers (Cordell *et al.*, 2003).

SulA is not well conserved. However, different SOS-induced proteins, with the same division arrest role, are known in other organisms. *B. subtilis* has a functional counterpart of SulA, YneA, which inhibits Z-ring formation (Kawai *et al.*, 2003) without establishing a direct contact with FtsZ. Whereas SulA is a cytosolic protein, YneA has a single-pass transmembrane domain and also a protease cleavage site. Its transmembrane domain is necessary to delay division, most likely because it is involved in interactions with yet unidentified transmembrane targets. Since YneA is rapidly cleaved, cells quickly resume division when transcription of *yneA* is repressed (Mo & Burkholder, 2010).

Most of the knowledge obtained in the bacterial cell division field was based on studies done with a few model organisms such as *E. coli, B. subtilis* or, more recently, *Caulobacter crescentus.* Lessons learned from these studies are obviously of very high value, but they cannot always be directly extrapolated to other bacteria with different morphologies. In this thesis, we studied the mode of division of the pathogenic bacteria *Staphylococcus aureus* which, like other cocci, divides by placing the division septum in three perpendicular planes in successive division cycles.

Staphylococcus aureus

Staphylococcus aureus cell division

The name *Staphylococci* originates from the spherical shape and characteristic grape-like clusters organization (Greek word "staphyle" means bunch of grapes) of the members of this genus, including *Staphylococcus aureus.* These clusters result from a mode of division in three sequential orthogonal planes over successive division cycles (Figure 1.5) (Koyama *et al.*, 1977, Tzagoloff & Novick, 1977), which, in theory, should result in cuboidal packets of eight cells. However, under the light microscope, *S. aureus* cells appear as irregular bunched clusters, probably due to irregular post-fissional movements of the sister cells, associated with the activity of lytic enzymes (Koyama *et al.*, 1977). Although known for more than three decades, this bacterial mode of division in three perpendicular planes is still poorly understood. The continuous selection of three orthogonal planes, apparently in the same order, implies that bacteria somehow store information from the two last divisions. It also implies that, from the infinite number of planes that divide a spherical cell in equal halves, only three are selected for division. Moreover, in order to ensure a faithful transmission of the genetic material, the segregation of the chromosomes has to occur also along three perpendicular axes.

Recently, the existence of bands of peptidoglycan material, with a piecrust texture, demarking the position of *S. aureus* previous and current planes of division was

shown by atomic force microscopy. These structures were proposed to hold the necessary information to define *S. aureus* next division plane (Turner *et al.*, 2010). However, it is not yet clear how this epigenetic information is translated to the cell cycle mechanisms. Interestingly, similar division-marker "scars", located at the cell surface, were also observed in an immunoelectron microscopy study of the localization of the major *S. aureus* autolysin, Atl (Yamada *et al.*, 1996).

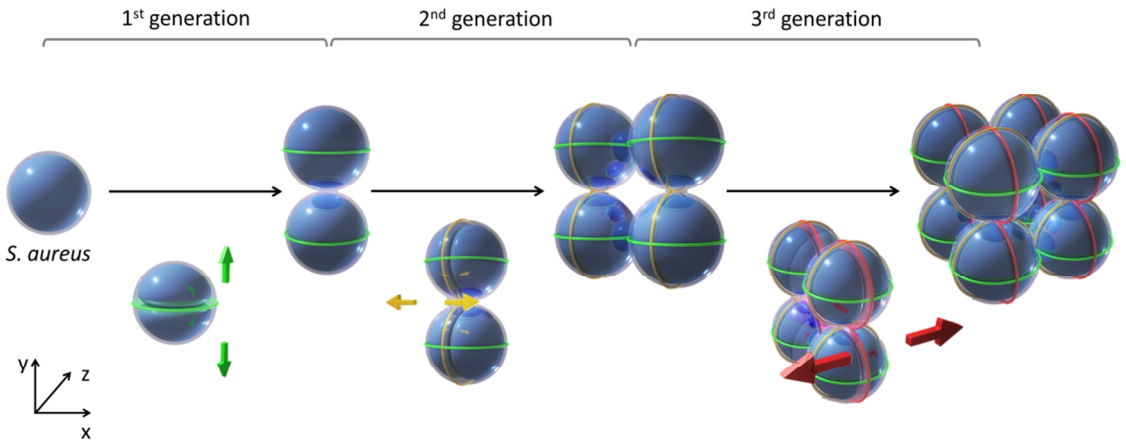

Figure 1.5. *Staphylococcus aureus* mode of division. Representation of three *S. aureus* division cycles. *S. aureus* cells divide along three ordered orthogonal planes (represented by the green yellow and red discs) over three successive division cycles. In preparation for division, the replicated chromosomes segregate in a specific axis (indicated by the green, yellow and red arrows). DNA is represented in blue. All the previous division planes are represented by green, yellow and red lines around the cell.

In contrast to *S. aureus*, the better studied rod-shaped organisms *Escherichia coli* and *Bacillus subtilis*, place the septum always at the same medial plane and segregate the chromosome in the same direction (Barak & Wilkinson, 2007, Bramkamp & van Baarle, 2009), parallel to the long axis of the cell. Therefore, it is not likely that all the knowledge obtained from the study of these organisms, could be directly applied to *S. aureus.* In fact,

for example, while the Min system is necessary to regulate FtsZ localization in *B. subtilis* and *E. coli,* it is absent from *S. aureus* and other cocci. Also, DivIVA, an important regulator of *oriC* positioning during *B. subtilis* sporulation (Thomaides *et al.*, 2001), is not required for normal chromosome segregation and cell division in *S. aureus* (Pinho & Errington, 2004) .

Staphylococcus aureus pathogenicity and resistance to antibiotics

The gram-positive cocci *Staphylococcus aureus* is both a commensal organism and a pathogen responsible for nosocomial and community-acquired infections. It colonizes persistently about 30% of human population, preferentially in the skin and nasopharynx, and it is intermittently carried by a further 60% of individuals (Edwards *et al.*, 2012). Colonization is often asymptomatic but if the skin barrier or the mucous membranes are breached, *S. aureus* may enter the soft tissues and establish an invasive infection. Therefore *S. aureus* can cause from relatively minor skin lesions to more serious and life-threatening diseases, such bacteremia, endocarditis or haemolytic pneumonia (Tenover & Gorwitz, 2006).

S. aureus is a very versatile and virulent pathogen. It expresses several virulence factors, such as surface-attached and secreted proteins, which are involved in human tissue adherence and invasion, resistance to innate defences and toxin secretion (Gordon & Lowy, 2008). It is also a serious health-care problem due to its remarkable potential to develop antimicrobial resistance (Boucher & Corey, 2008). Within a few years after the introduction of the β-lactam penicillin, the first drug effective against *S. aureus,* penicillin resistance was encountered in hospital settings (Barber & Rozwadowska-Dowzenko, 1948). The resistant strains carried plasmid-encoded β-lactamases that catalysed the hydrolysis of the penicillin β-lactam ring, thus rendering the antibiotic inactive.

The first β-lactamase-insensitive penicillin derivative, methicillin, for the treatment of infections caused by penicillin-resistant *S. aureus* strains, was introduced in the sixties. However, soon after, the first methicillin resistant *S. aureus* (MRSA) strains were identified in the UK (Jevons *et al.*, 1963). By the 1980s, infections caused by *S.*

aureus MRSA strains, an acronym that now identifies strains resistant to virtually all classes of β-lactam antibiotics, reached global proportions and, nowadays, MRSAs are one of the leading causes of nosocomial infections worldwide (Chambers & Deleo, 2009, Klevens *et al.*, 2007). In fact, a recent report puts the number of deaths attributable to MRSA, in the United States, in front of those related to HIV/AIDS and tuberculosis combined (Boucher & Corey, 2008). In the past decade, MRSA strains have also been emerging in community settings, affecting even healthy individuals lacking predisposing risk factors (Conly & Johnston, 2003, Kennedy *et al.*, 2008a).

The central determinant of MRSA resistance is the *mecA* gene, contained in an exogenous mobile genetic element called staphylococcal cassette chromosome *mec* (SCC*mec*), which codes for an alternative penicillin binding protein, PBP2A (Hartman & Tomasz, 1984, Reynolds & Brown, 1985). β-lactam antibiotics bind the active site of penicillin-binding proteins (PBPs), blocking access to their substrates and thus causing inhibition of cell wall biosynthesis (Zapun *et al.*, 2008). PBP2A, however, has lower affinity for these antibiotics and so maintains, in their presence, its transpeptidase activity which, in cooperation with the glycosyltransferase activity of PBP2 (whose transpeptidase domain is inactivated by the presence of β-lactams), ensures proper cell wall synthesis (Pinho *et al.*, 2001).

MRSA strains are frequently susceptible to the glycopeptide antibiotic vancomycin. However, its intensive clinical use created a selective pressure for the emergence of vancomycin-intermediate *S. aureus* strains (VISA, not susceptible to vancomycin concentrations below 4-8 μg/ml) (Hiramatsu *et al.*, 1997) and vancomycin-resistant *S. aureus* strains (VRSA, resistant to concentrations equal or higher than 16 μg/ml) (Chang *et al.*, 2003), further confirming the ability of *S. aureus* to adapt and develop resistance. Therefore, there is an increasing need for the development of new antimicrobial agents against *S. aureus* infections, which largely depends on an intensive knowledge of this organism.

The main goal of the work described in this thesis, was to identify the molecular factors and morphodynamic principles underlying *S. aureus* spherical cells division, namely regarding placement of the septum and chromosome segregation along three perpendicular planes.

References

Aarsman, M. E., A. Piette, C. Fraipont, T. M. Vinkenvleugel, M. Nguyen-Disteche & T. den Blaauwen, (2005) Maturation of the *Escherichia coli* divisome occurs in two steps. *Mol Microbiol* **55**: 1631-1645.

Anderson, D. E., F. J. Gueiros-Filho & H. P. Erickson, (2004) Assembly dynamics of FtsZ rings in *Bacillus subtilis* and *Escherichia coli* and effects of FtsZ-regulating proteins. *J Bacteriol* **186**: 5775-5781.

Aussel, L., F. X. Barre, M. Aroyo, A. Stasiak, A. Z. Stasiak & D. Sherratt, (2002) FtsK is a DNA motor protein that activates chromosome dimer resolution by switching the catalytic state of the XerC and XerD recombinases. *Cell* **108**: 195-205.

Barak, I. & A. J. Wilkinson, (2007) Division site recognition in *Escherichia coli* and *Bacillus subtilis*. *FEMS Microbiol Rev* **31**: 311-326.

Barber, M. & M. Rozwadowska-Dowzenko, (1948) Infection by penicillin-resistant *Staphylococci*. *Lancet* **2**: 641-644.

Barre, F. X., M. Aroyo, S. D. Colloms, A. Helfrich, F. Cornet & D. J. Sherratt, (2000) FtsK functions in the processing of a Holliday junction intermediate during bacterial chromosome segregation. *Genes Dev* **14**: 2976-2988.

Bates, D., (2008) The bacterial replisome: back on track? *Mol Microbiol* **69**: 1341-1348.

Bath, J., L. J. Wu, J. Errington & J. C. Wang, (2000) Role of *Bacillus subtilis* SpoIIIE in DNA transport across the mother cell-prespore division septum. *Science* **290**: 995-997.

Beall, B. & J. Lutkenhaus, (1992) Impaired cell division and sporulation of a *Bacillus subtilis* strain with the ftsA gene deleted. *J Bacteriol* **174**: 2398-2403.

Becker, E. C. & K. Pogliano, (2007) Cell-specific SpoIIIE assembly and DNA translocation polarity are dictated by chromosome orientation. *Mol Microbiol* **66**: 1066-1079.

Ben-Yehuda, S., D. Z. Rudner & R. Losick, (2003a) Assembly of the SpoIIIE DNA translocase depends on chromosome trapping in *Bacillus subtilis*. *Curr Biol* **13**: 2196-2200.

Ben-Yehuda, S., D. Z. Rudner & R. Losick, (2003b) RacA, a bacterial protein that anchors chromosomes to the cell poles. *Science* **299**: 532-536.

Bernard, R., K. A. Marquis & D. Z. Rudner, (2010) Nucleoid occlusion prevents cell division during replication fork arrest in *Bacillus subtilis*. *Mol Microbiol* **78**: 866-882.

Bernhardt, T. G. & P. A. de Boer, (2005) SlmA, a nucleoid-associated, FtsZ binding protein required for blocking septal ring assembly over Chromosomes in *E. coli*. *Mol Cell* **18**: 555-564.

Bi, E. F. & J. Lutkenhaus, (1991) FtsZ ring structure associated with division in *Escherichia coli*. *Nature* **354**: 161-164.

Bigot, S., J. Corre, J. M. Louarn, F. Cornet & F. X. Barre, (2004) FtsK activities in Xer recombination, DNA mobilization and cell division involve overlapping and separate domains of the protein. *Mol Microbiol* **54**: 876-886.

Bigot, S. & K. J. Marians, (2010) DNA chirality-dependent stimulation of topoisomerase IV activity by the C-terminal AAA+ domain of FtsK. *Nucl Acids Res* **38**: 3031-3040.

Bigot, S., O. A. Saleh, F. Cornet, J. F. Allemand & F. X. Barre, (2006) Oriented loading of FtsK on KOPS. *Nat Struct Mol Biol* **13**: 1026-1028.

Bigot, S., O. A. Saleh, C. Lesterlin, C. Pages, M. El Karoui, C. Dennis, M. Grigoriev, J. F. Allemand, F. X. Barre & F. Cornet, (2005) KOPS: DNA motifs that control *E. coli* chromosome segregation by orienting the FtsK translocase. *EMBO J* **24**: 3770-3780.

Bigot, S., V. Sivanathan, C. Possoz, F. X. Barre & F. Cornet, (2007) FtsK, a literate chromosome segregation machine. *Mol Microbiol* **64**: 1434-1441.

Biller, S. J. & W. F. Burkholder, (2009) The *Bacillus subtilis* SftA (YtpS) and SpoIIIE DNA translocases play distinct roles in growing cells to ensure faithful chromosome partitioning. *Mol Microbiol* **74**: 790-809.

Boucher, H. W. & G. R. Corey, (2008) Epidemiology of methicillin-resistant *Staphylococcus aureus*. *Clin Infect Dis* **46 Suppl 5**: S344-349.

Boye, E., A. Lobner-Olesen & K. Skarstad, (2000) Limiting DNA replication to once and only once. *EMBO Rep* **1**: 479-483.

Bramhill, D. & C. M. Thompson, (1994) GTP-dependent polymerization of *Escherichia coli* FtsZ protein to form tubules. *Proc Natl Acad Sci U S A* **91**: 5813-5817.

Bramkamp, M., R. Emmins, L. Weston, C. Donovan, R. A. Daniel & J. Errington, (2008) A novel component of the division-site selection system of *Bacillus subtilis* and a new mode of action for the division inhibitor MinCD. *Mol Microbiol* **70**: 1556-1569.

Bramkamp, M. & S. van Baarle, (2009) Division site selection in rod-shaped bacteria. *Curr Opin Microbiol* **12**: 683-688.

Breier, A. M. & A. D. Grossman, (2007) Whole-genome analysis of the chromosome partitioning and sporulation protein Spo0J (ParB) reveals spreading and origin-distal sites on the *Bacillus subtilis* chromosome. *Mol Microbiol* **64**: 703-718.

Britton, R. A. & A. D. Grossman, (1999) Synthetic lethal phenotypes caused by mutations affecting chromosome partitioning in *Bacillus subtilis*. *J Bacteriol* **181**: 5860-5864.

Britton, R. A., D. C. Lin & A. D. Grossman, (1998) Characterization of a prokaryotic SMC protein involved in chromosome partitioning. *Genes Dev* **12**: 1254-1259.

Browning, D. F., D. C. Grainger & S. J. Busby, (2010) Effects of nucleoid-associated proteins on bacterial chromosome structure and gene expression. *Curr Opin Microbiol* **13**: 773-780.

Buddelmeijer, N. & J. Beckwith, (2002) Assembly of cell division proteins at the *E. coli* cell center. *Curr Opin Microbiol* **5**: 553-557.

Burton, B. M., K. A. Marquis, N. L. Sullivan, T. A. Rapoport & D. Z. Rudner, (2007) The ATPase SpoIIIE transports DNA across fused septal membranes during sporulation in *Bacillus subtilis*. *Cell* **131**: 1301-1312.

Capiaux, H., C. Lesterlin, K. Perals, J. M. Louarn & F. Cornet, (2002) A dual role for the FtsK protein in *Escherichia coli* chromosome segregation. *EMBO Rep* **3**: 532-536.

Chambers, H. F. & F. R. Deleo, (2009) Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nat Rev Microbiol* **7**: 629-641.

Chang, S., D. M. Sievert, J. C. Hageman, M. L. Boulton, F. C. Tenover, F. P. Downes, S. Shah, J. T. Rudrik, G. R. Pupp, W. J. Brown, D. Cardo & S. K. Fridkin, (2003) Infection with vancomycin-resistant *Staphylococcus aureus* containing the *vanA* resistance gene. *N Engl J Med* **348**: 1342-1347.

Chapman, G. B. & J. Hillier, (1953) Electron microscopy of ultra-thin sections of bacteria I. Cellular division in *Bacillus cereus*. *J Bacteriol* **66**: 362-373.

Chen, Y., K. Bjornson, S. D. Redick & H. P. Erickson, (2005) A rapid fluorescence assay for FtsZ assembly indicates cooperative assembly with a dimer nucleus. *Biophys J* **88**: 505-514.

Cho, H., H. R. McManus, S. L. Dove & T. G. Bernhardt, (2011) Nucleoid occlusion factor SlmA is a DNA-activated FtsZ polymerization antagonist. *Proc Natl Acad Sci U S A* **108**: 3773-3778.

Chung, K. M., H. H. Hsu, H. Y. Yeh & B. Y. Chang, (2007) Mechanism of regulation of prokaryotic tubulin-like GTPase FtsZ by membrane protein EzrA. *J Biol Chem* **282**: 14891-14897.

Claessen, D., R. Emmins, L. W. Hamoen, R. A. Daniel, J. Errington & D. H. Edwards, (2008) Control of the cell elongation-division cycle by shuttling of PBP1 protein in *Bacillus subtilis*. *Mol Microbiol* **68**: 1029-1046.

Conly, J. M. & B. L. Johnston, (2003) The emergence of methicillin-resistant *Staphylococcus aureus* as a community-acquired pathogen in Canada. *Can J Infect Dis* **14**: 249-251.

Cooper, S. & C. E. Helmstetter, (1968) Chromosome replication and the division cycle of *Escherichia coli* B/r. *J Mol Biol* **31**: 519-540.

Cordell, S. C., R. E. Anderson & J. Lowe, (2001) Crystal structure of the bacterial cell division inhibitor MinC. *EMBO J* **20**: 2454-2461.

Cordell, S. C., E. J. Robinson & J. Lowe, (2003) Crystal structure of the SOS cell division inhibitor SulA and in complex with FtsZ. *Proc Natl Acad Sci U S A* **100**: 7889-7894.

Corre, J. & J. M. Louarn, (2002) Evidence from terminal recombination gradients that FtsK uses replichore polarity to control chromosome terminus positioning at division in *Escherichia coli*. *J Bacteriol* **184**: 3801-3807.

Crozat, E., A. Meglio, J. F. Allemand, C. E. Chivers, M. Howarth, C. Venien-Bryan, I. Grainge & D. J. Sherratt, (2010) Separating speed and ability to displace roadblocks during DNA translocation by FtsK. *EMBO J* **29**: 1423-1433.

Dajkovic, A., G. Lan, S. X. Sun, D. Wirtz & J. Lutkenhaus, (2008) MinC spatially controls bacterial cytokinesis by antagonizing the scaffolding function of FtsZ. *Curr Biol* **18**: 235-244.

Daniel, R. A., M. F. Noirot-Gros, P. Noirot & J. Errington, (2006) Multiple interactions between the transmembrane division proteins of *Bacillus subtilis* and the role of FtsL instability in divisome assembly. *J Bacteriol* **188**: 7396-7404.

Danilova, O., R. Reyes-Lamothe, M. Pinskaya, D. Sherratt & C. Possoz, (2007) MukB colocalizes with the *oriC* region and is required for organization of the two *Escherichia coli* chromosome arms into separate cell halves. *Mol Microbiol* **65**: 1485-1492.

de Boer, P., R. Crossley & L. Rothfield, (1992) The essential bacterial cell-division protein FtsZ is a GTPase. *Nature* **359**: 254-256.

de Boer, P. A., (2010) Advances in understanding *E. coli* cell fission. *Curr Opin Microbiol* **13**: 730-737.

de Boer, P. A., R. E. Crossley, A. R. Hand & L. I. Rothfield, (1991) The MinD protein is a membrane ATPase required for the correct placement of the *Escherichia coli* division site. *EMBO J* **10**: 4371-4380.

de Boer, P. A., R. E. Crossley & L. I. Rothfield, (1989) A division inhibitor and a topological specificity factor coded for by the minicell locus determine proper placement of the division septum in *E. coli*. *Cell* **56**: 641-649.

den Blaauwen, T., M. A. de Pedro, M. Nguyen-Disteche & J. A. Ayala, (2008) Morphogenesis of rod-shaped sacculi. *FEMS Microbiol Rev* **32**: 321-344.

Deng, S., R. A. Stein & N. P. Higgins, (2005) Organization of supercoil domains and their reorganization by transcription. *Mol Microbiol* **57**: 1511-1521.

Donachie, W. D. & G. W. Blakely, (2003) Coupling the initiation of chromosome replication to cell size in *Escherichia coli*. *Curr Opin Microbiol* **6**: 146-150.

Dorazi, R. & S. J. Dewar, (2000) Membrane topology of the N-terminus of the *Escherichia coli* FtsK division protein. *FEBS lett* **478**: 13-18.

Draper, G. C., N. McLennan, K. Begg, M. Masters & W. D. Donachie, (1998) Only the N-terminal domain of FtsK functions in cell division. *J Bacteriol* **180**: 4621-4627.

Dubarry, N. & F. X. Barre, (2010) Fully efficient chromosome dimer resolution in *Escherichia coli* cells lacking the integral membrane domain of FtsK. *EMBO J* **29**: 597-605.

Dubarry, N., C. Possoz & F. X. Barre, (2010) Multiple regions along the *Escherichia coli* FtsK protein are implicated in cell division. *Mol Microbiol* **78**: 1088-1100.

Dubrac, S., I. G. Boneca, O. Poupel & T. Msadek, (2007) New insights into the WalK/WalR (YycG/YycF) essential signal transduction pathway reveal a major role in controlling cell wall metabolism and biofilm formation in *Staphylococcus aureus*. *J Bacteriol* **189**: 8257-8269.

Durand-Heredia, J. M., H. H. Yu, S. De Carlo, C. F. Lesser & A. Janakiraman, (2011) Identification and characterization of ZapC, a stabilizer of the FtsZ ring in *Escherichia coli*. *J Bacteriol* **193**: 1405-1413.

Dworkin, J. & R. Losick, (2002) Does RNA polymerase help drive chromosome segregation in bacteria? *Proc Natl Acad Sci U S A* **99**: 14089-14094.

Ebersbach, G., E. Galli, J. Moller-Jensen, J. Lowe & K. Gerdes, (2008) Novel coiled-coil cell division factor ZapB stimulates Z ring assembly and cell division. *Mol Microbiol* **68**: 720-735.

Edwards, A. M., R. C. Massey & S. R. Clarke, (2012) Molecular mechanisms of *Staphylococcus aureus* nasopharyngeal colonization. *Mol Oral Microbiol* **27**: 1-10.

Edwards, D. H. & J. Errington, (1997) The *Bacillus subtilis* DivIVA protein targets to the division septum and controls the site specificity of cell division. *Mol Microbiol* **24**: 905-915.

Erickson, H. P., (1995) FtsZ, a prokaryotic homolog of tubulin? *Cell* **80**: 367-370.

Erickson, H. P., D. E. Anderson & M. Osawa, (2010) FtsZ in bacterial cytokinesis: cytoskeleton and force generator all in one. *Microbiol Mol Biol Rev* **74**: 504-528.

Erickson, H. P. & M. Osawa, (2010) Cell division without FtsZ--a variety of redundant mechanisms. *Mol Microbiol* **78**: 267-270.

Erickson, H. P., D. W. Taylor, K. A. Taylor & D. Bramhill, (1996) Bacterial cell division protein FtsZ assembles into protofilament sheets and minirings, structural homologs of tubulin polymers. *Proc Natl Acad Sci U S A* **93**: 519-523.

Erill, I., S. Campoy & J. Barbe, (2007) Aeons of distress: an evolutionary perspective on the bacterial SOS response. *FEMS Microbiol Rev* **31**: 637-656.

Errington, J., R. A. Daniel & D. J. Scheffers, (2003) Cytokinesis in bacteria. *Microbiol Mol Biol Rev* **67**: 52-65.

Espeli, O., C. Lee & K. J. Marians, (2003) A physical and functional interaction between *Escherichia coli* FtsK and topoisomerase IV. *J Biol Chem* **278**: 44639-44644.

Espeli, O. & K. J. Marians, (2004) Untangling intracellular DNA topology. *Mol Microbiol* **52**: 925-931.

Eswaramoorthy, P., M. L. Erb, J. A. Gregory, J. Silverman, K. Pogliano, J. Pogliano & K. S. Ramamurthi, (2011) Cellular architecture mediates DivIVA ultrastructure and regulates min activity in *Bacillus subtilis*. *mBio* **2**: e00257-11.

Fleming, T. C., J. Y. Shin, S. H. Lee, E. Becker, K. C. Huang, C. Bustamante & K. Pogliano, (2010) Dynamic SpoIIIE assembly mediates septal membrane fission during *Bacillus subtilis* sporulation. *Genes Dev* **24**: 1160-1172.

Fogel, M. A. & M. K. Waldor, (2005) Distinct segregation dynamics of the two Vibrio cholerae chromosomes. *Mol Microbiol* **55**: 125-136.

Fogel, M. A. & M. K. Waldor, (2006) A dynamic, mitotic-like mechanism for bacterial chromosome segregation. *Genes Dev* **20**: 3269-3282.

Fu, X., Y. L. Shih, Y. Zhang & L. I. Rothfield, (2001) The MinE ring required for proper placement of the division site is a mobile structure that changes its cellular location during the *Escherichia coli* division cycle. *Proc Natl Acad Sci U S A* **98**: 980-985.

Galli, E. & K. Gerdes, (2010) Spatial resolution of two bacterial cell division proteins: ZapA recruits ZapB to the inner face of the Z-ring. *Mol Microbiol* **76**: 1514-1526.

Gamba, P., J. W. Veening, N. J. Saunders, L. W. Hamoen & R. A. Daniel, (2009) Two-step assembly dynamics of the *Bacillus subtilis* divisome. *J Bacteriol* **191**: 4186-4194.

Gerdes, K., M. Howard & F. Szardenings, (2010) Pushing and pulling in prokaryotic DNA segregation. *Cell* **141**: 927-942.

Gerdes, K., J. Moller-Jensen & R. Bugge Jensen, (2000) Plasmid and chromosome partitioning: surprises from phylogeny. *Mol Microbiol* **37**: 455-466.

Godfrin-Estevenon, A. M., F. Pasta & D. Lane, (2002) The parAB gene products of *Pseudomonas putida* exhibit partition activity in both *P. putida* and *Escherichia coli*. *Mol Microbiol* **43**: 39-49.

Goehring, N. W. & J. Beckwith, (2005) Diverse paths to midcell: assembly of the bacterial cell division machinery. *Curr Biol* **15**: R514-526.

Gordon, R. J. & F. D. Lowy, (2008) Pathogenesis of methicillin-resistant *Staphylococcus aureus* infection. *Clin Infect Dis* **46 Suppl 5**: S350-359.

Graham, J. E., D. J. Sherratt & M. D. Szczelkun, (2010) Sequence-specific assembly of FtsK hexamers establishes directional translocation on DNA. *Proc Natl Acad Sci U S A* **107**: 20263-20268.

Grainge, I., (2010) FtsK--a bacterial cell division checkpoint? *Mol Microbiol* **78**: 1055-1057.

Grainge, I., M. Bregu, M. Vazquez, V. Sivanathan, S. C. Ip & D. J. Sherratt, (2007) Unlinking chromosome catenanes *in vivo* by site-specific recombination. *EMBO J* **26**: 4228-4238.

Grainge, I., C. Lesterlin & D. J. Sherratt, (2011) Activation of XerCD-*dif* recombination by the FtsK DNA translocase. *Nucl Acids Res* **39**: 5140-5148.

Gregory, J. A., E. C. Becker & K. Pogliano, (2008) *Bacillus subtilis* MinC destabilizes FtsZ-rings at new cell poles and contributes to the timing of cell division. *Genes Dev* **22**: 3475-3488.

Gruber, S. & J. Errington, (2009) Recruitment of condensin to replication origin regions by ParB/SpoOJ promotes chromosome segregation in *B. subtilis*. *Cell* **137**: 685-696.

Gueiros-Filho, F. J. & R. Losick, (2002) A widely conserved bacterial cell division protein that promotes assembly of the tubulin-like protein FtsZ. *Genes Dev* **16**: 2544-2556.

Gundogdu, M. E., Y. Kawai, N. Pavlendova, N. Ogasawara, J. Errington, D. J. Scheffers & L. W. Hamoen, (2011) Large ring polymers align FtsZ polymers for normal septum formation. *EMBO J* **30**: 617-626.

Haeusser, D. P., A. C. Garza, A. Z. Buscher & P. A. Levin, (2007) The division inhibitor EzrA contains a seven-residue patch required for maintaining the dynamic nature of the medial FtsZ ring. *J Bacteriol* **189**: 9001-9010.

Haeusser, D. P., A. H. Lee, R. B. Weart & P. A. Levin, (2009) ClpX inhibits FtsZ assembly in a manner that does not require its ATP hydrolysis-dependent chaperone activity. *J Bacteriol* **191**: 1986-1991.

Haeusser, D. P., R. L. Schwartz, A. M. Smith, M. E. Oates & P. A. Levin, (2004) EzrA prevents aberrant cell division by modulating assembly of the cytoskeletal protein FtsZ. *Mol Microbiol* **52**: 801-814.

Hale, C. A. & P. A. de Boer, (1997) Direct binding of FtsZ to ZipA, an essential component of the septal ring structure that mediates cell division in *E. coli. Cell* **88**: 175-185.

Hale, C. A. & P. A. de Boer, (1999) Recruitment of ZipA to the septal ring of *Escherichia coli* is dependent on FtsZ and independent of FtsA. *J Bacteriol* **181**: 167-176.

Hale, C. A., H. Meinhardt & P. A. de Boer, (2001) Dynamic localization cycle of the cell division regulator MinE in *Escherichia coli*. *EMBO J* **20**: 1563-1572.

Hale, C. A., A. C. Rhee & P. A. de Boer, (2000) ZipA-induced bundling of FtsZ polymers mediated by an interaction between C-terminal domains. *J Bacteriol* **182**: 5153-5166.

Hale, C. A., D. Shiomi, B. Liu, T. G. Bernhardt, W. Margolin, H. Niki & P. A. de Boer, (2011) Identification of *Escherichia coli* ZapC (YcbW) as a component of the division apparatus that binds and bundles FtsZ polymers. *J Bacteriol* **193**: 1393-1404.

Hamoen, L. W., J. C. Meile, W. de Jong, P. Noirot & J. Errington, (2006) SepF, a novel FtsZ-interacting protein required for a late step in cell division. *Mol Microbiol* **59**: 989-999.

Haney, S. A., E. Glasfeld, C. Hale, D. Keeney, Z. He & P. de Boer, (2001) Genetic analysis of the *Escherichia coli* FtsZ.ZipA interaction in the yeast two-hybrid system. Characterization of FtsZ residues essential for the interactions with ZipA and with FtsA. *J Biol Chem* **276**: 11980-11987.

Hartman, B. J. & A. Tomasz, (1984) Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus*. *J Bacteriol* **158**: 513-516.

Hiramatsu, K., H. Hanaki, T. Ino, K. Yabuta, T. Oguri & F. C. Tenover, (1997) Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. *J Antimicrob Chemother* **40**: 135-136.

Hirano, M. & T. Hirano, (2006) Opening closed arms: long-distance activation of SMC ATPase by hinge-DNA interactions. *Mol Cell* **21**: 175-186.

Hirano, T., (2006) At the heart of the chromosome: SMC proteins in action. *Nat Rev Mol Cell Biol* **7**: 311-322.

Hirota, Y., A. Ryter & F. Jacob, (1968) Thermosensitive mutants of *E. coli* affected in the processes of DNA synthesis and cellular division. *Cold Spring Harb Symp Quant Biol* **33**: 677-693.

Hu, Z., E. P. Gogol & J. Lutkenhaus, (2002) Dynamic assembly of MinD on phospholipid vesicles regulated by ATP and MinE. *Proc Natl Acad Sci U S A* **99**: 6761-6766.

Hu, Z. & J. Lutkenhaus, (2000) Analysis of MinC reveals two independent domains involved in interaction with MinD and FtsZ. *J Bacteriol* **182**: 3965-3971.

Hu, Z. & J. Lutkenhaus, (2003) A conserved sequence at the C-terminus of MinD is required for binding to the membrane and targeting MinC to the septum. *Mol Microbiol* **47**: 345-355.

Hu, Z., A. Mukherjee, S. Pichoff & J. Lutkenhaus, (1999) The MinC component of the division site selection system in *Escherichia coli* interacts with FtsZ to prevent polymerization. *Proc Natl Acad Sci U S A* **96**: 14819-14824.

Huisman, O. & R. D'Ari, (1981) An inducible DNA replication-cell division coupling mechanism in *E. coli*. *Nature* **290**: 797-799.

Huisman, O., R. D'Ari & S. Gottesman, (1984) Cell-division control in *Escherichia coli*: specific induction of the SOS function SfiA protein is sufficient to block septation. *Proc Natl Acad Sci U S A* **81**: 4490-4494.

Ip, S. C., M. Bregu, F. X. Barre & D. J. Sherratt, (2003) Decatenation of DNA circles by FtsK-dependent Xer site-specific recombination. *EMBO J* **22**: 6399-6407.

Ireton, K., N. W. t. Gunther & A. D. Grossman, (1994) spo0J is required for normal chromosome segregation as well as the initiation of sporulation in *Bacillus subtilis*. *J Bacteriol* **176**: 5320-5329.

Ishikawa, S., Y. Kawai, K. Hiramatsu, M. Kuwano & N. Ogasawara, (2006) A new FtsZ-interacting protein, YlmF, complements the activity of FtsA during progression of cell division in *Bacillus subtilis*. *Mol Microbiol* **60**: 1364-1380.

Jackson, D., X. Wang & D. Z. Rudner, (2012) Spatio-temporal organization of replication in bacteria and eukaryotes (nucleoids and nuclei). *Cold Spring Harb Perspect Biol* **4**: a010389.

Jensen, S. O., L. S. Thompson & E. J. Harry, (2005) Cell division in *Bacillus subtilis*: FtsZ and FtsA association is Z-ring independent, and FtsA is required for efficient midcell Z-Ring assembly. *J Bacteriol* **187**: 6536-6544.

Jevons, M. P., A. W. Coe & M. T. Parker, (1963) Methicillin resistance in *Staphylococci*. *Lancet* **1**: 904-907.

Jun, S. & B. Mulder, (2006) Entropy-driven spatial organization of highly confined polymers: lessons for the bacterial chromosome. *Proc Natl Acad Sci U S A* **103**: 12388-12393.

Jun, S. & A. Wright, (2010) Entropy as the driver of chromosome segregation. *Nat Rev Microbiol* **8**: 600-607.

Kaguni, J. M., (2006) DnaA: controlling the initiation of bacterial DNA replication and more. *Annu Rev Microbiol* **60**: 351-375.

Kaimer, C., J. E. Gonzalez-Pastor & P. L. Graumann, (2009) SpoIIIE and a novel type of DNA translocase, SftA, couple chromosome segregation with cell division in *Bacillus subtilis*. *Mol Microbiol* **74**: 810-825.

Kaimer, C. & P. L. Graumann, (2011) Players between the worlds: multifunctional DNA translocases. *Curr Opin Microbiol* **14**: 719-725.

Kaimer, C., K. Schenk & P. L. Graumann, (2011) Two DNA translocases synergistically affect chromosome dimer resolution in *Bacillus subtilis*. *J Bacteriol* **193**: 1334-1340.

Karczmarek, A., R. Martinez-Arteaga, S. Alexeeva, F. G. Hansen, M. Vicente, N. Nanninga & T. den Blaauwen, (2007) DNA and origin region segregation are not affected by the transition from rod to sphere after inhibition of *Escherichia coli* MreB by A22. *Mol Microbiol* **65**: 51-63.

Kawai, Y., S. Moriya & N. Ogasawara, (2003) Identification of a protein, YneA, responsible for cell division suppression during the SOS response in *Bacillus subtilis*. *Mol Microbiol* **47**: 1113-1122.

Kennedy, A. D., M. Otto, K. R. Braughton, A. R. Whitney, L. Chen, B. Mathema, J. R. Mediavilla, K. A. Byrne, L. D. Parkins, F. C. Tenover, B. N. Kreiswirth, J. M. Musser

& F. R. DeLeo, (2008a) Epidemic community-associated methicillin-resistant *Staphylococcus aureus*: recent clonal expansion and diversification. *Proc Natl Acad Sci U S A* **105**: 1327-1332.

Kennedy, S. P., F. Chevalier & F. X. Barre, (2008b) Delayed activation of Xer recombination at *dif* by FtsK during septum assembly in *Escherichia coli*. *Mol Microbiol* **68**: 1018-1028.

Klevens, R. M., M. A. Morrison, J. Nadle, S. Petit, K. Gershman, S. Ray, L. H. Harrison, R. Lynfield, G. Dumyati, J. M. Townes, A. S. Craig, E. R. Zell, G. E. Fosheim, L. K. McDougal, R. B. Carey & S. K. Fridkin, (2007) Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *J Amer Med Assoc* **298**: 1763-1771.

Knaysi, G., (1941) Observations on the cell division of some yeasts and bacteria. *J Bacteriol* **41**: 141-153.

Koyama, T., M. Yamada & M. Matsuhashi, (1977) Formation of regular packets of *Staphylococcus aureus* cells. *J Bacteriol* **129**: 1518-1523.

Kruse, T., B. Blagoev, A. Lobner-Olesen, M. Wachi, K. Sasaki, N. Iwai, M. Mann & K. Gerdes, (2006) Actin homolog MreB and RNA polymerase interact and are both required for chromosome segregation in *Escherichia coli*. *Genes Dev* **20**: 113-124.

Kruse, T., J. Moller-Jensen, A. Lobner-Olesen & K. Gerdes, (2003) Dysfunctional MreB inhibits chromosome segregation in *Escherichia coli. EMBO J* **22**: 5283-5292.

Laaberki, M. H., J. Pfeffer, A. J. Clarke & J. Dworkin, (2011) O-Acetylation of peptidoglycan is required for proper cell separation and S-layer anchoring in *Bacillus anthracis*. *J Biol Chem* **286**: 5278-5288.

Lackner, L. L., D. M. Raskin & P. A. J. de Boer, (2003) ATP-Dependent interactions between *Escherichia coli* Min proteins and the phospholipid membrane *in vitro*. *J Bacteriol* **185**: 735-749.

Laporte, D., R. Zhao & J. Q. Wu, (2010) Mechanisms of contractile-ring assembly in fission yeast and beyond. *Semin Cell Dev Biol* **21**: 892-898.

Lee, P. S. & A. D. Grossman, (2006) The chromosome partitioning proteins Soj (ParA) and Spo0J (ParB) contribute to accurate chromosome partitioning, separation of replicated sister origins, and regulation of replication initiation in *Bacillus subtilis*. *Mol Microbiol* **60**: 853-869.

Lee, P. S., D. C. H. Lin, S. Moriya & A. D. Grossman, (2003) Effects of the chromosome partitioning protein Spo0J (ParB) on *oriC* positioning and replication initiation in *Bacillus subtilis*. *J Bacteriol* **185**: 1326-1337.

Lee, S. & C. W. Price, (1993) The *minCD* locus of *Bacillus subtilis* lacks the *minE* determinant that provides topological specificity to cell division. *Mol Microbiol* **7**: 601-610.

Lemon, K. P. & A. D. Grossman, (2001) The extrusion-capture model for chromosome partitioning in bacteria. *Genes Dev* **15**: 2031-2041.

Lenarcic, R., S. Halbedel, L. Visser, M. Shaw, L. J. Wu, J. Errington, D. Marenduzzo & L. W. Hamoen, (2009) Localisation of DivIVA by targeting to negatively curved membranes. *EMBO J* **28**: 2272-2282.

Lesterlin, C., F. X. Barre & F. Cornet, (2004) Genetic recombination and the cell cycle: what we have learned from chromosome dimers. *Mol Microbiol* **54**: 1151-1160.

Levin, P. A., I. G. Kurtser & A. D. Grossman, (1999) Identification and characterization of a negative regulator of FtsZ ring formation in *Bacillus subtilis*. *Proc Natl Acad Sci U S A* **96**: 9642-9647.

Levin, P. A., R. L. Schwartz & A. D. Grossman, (2001) Polymer stability plays an important role in the positional regulation of FtsZ. *J Bacteriol* **183**: 5449-5452.

Levy, O., J. L. Ptacin, P. J. Pease, J. Gore, M. B. Eisen, C. Bustamante & N. R. Cozzarelli, (2005) Identification of oligonucleotide sequences that direct the movement of the *Escherichia coli* FtsK translocase. *Proc Natl Acad Sci U S A* **102**: 17618-17623.

Li, Z., M. J. Trimble, Y. V. Brun & G. J. Jensen, (2007) The structure of FtsZ filaments *in vivo* suggests a force-generating role in cell division. *EMBO J* **26**: 4694-4708.

Lin, D. C. & A. D. Grossman, (1998) Identification and characterization of a bacterial chromosome partitioning site. *Cell* **92**: 675-685.

Lin, D. C., P. A. Levin & A. D. Grossman, (1997) Bipolar localization of a chromosome partition protein in *Bacillus subtilis*. *Proc Natl Acad Sci U S A* **94**: 4721-4726.

Lindow, J. C., R. A. Britton & A. D. Grossman, (2002) Structural maintenance of chromosomes protein of *Bacillus subtilis* affects supercoiling *in vivo*. *J Bacteriol* **184**: 5317-5322.

Liu, G., G. C. Draper & W. D. Donachie, (1998) FtsK is a bifunctional protein involved in cell division and chromosome localization in *Escherichia coli*. *Mol Microbiol* **29**: 893-903.

Liu, N. J., R. J. Dutton & K. Pogliano, (2006) Evidence that the SpoIIIE DNA translocase participates in membrane fusion during cytokinesis and engulfment. *Mol Microbiol* **59**: 1097-1113.

Livny, J., Y. Yamaichi & M. K. Waldor, (2007) Distribution of centromere-like *parS* sites in bacteria: insights from comparative genomics. *J Bacteriol* **189**: 8693-8703.

Lowe, J. & L. A. Amos, (1998) Crystal structure of the bacterial cell-division protein FtsZ. *Nature* **391**: 203-206.

Lowe, J., A. Ellonen, M. D. Allen, C. Atkinson, D. J. Sherratt & I. Grainge, (2008) Molecular mechanism of sequence-directed DNA loading and translocation by FtsK. *Mol Cell* **31**: 498-509.

Lu, C., M. Reedy & H. P. Erickson, (2000) Straight and curved conformations of FtsZ are regulated by GTP hydrolysis. *J Bacteriol* **182**: 164-170.

Ma, X. & W. Margolin, (1999) Genetic and functional analyses of the conserved C-terminal core domain of *Escherichia coli* FtsZ. *J Bacteriol* **181**: 7531-7544.

Marston, A. L., H. B. Thomaides, D. H. Edwards, M. E. Sharpe & J. Errington, (1998) Polar localization of the MinD protein of *Bacillus subtilis* and its role in selection of the mid-cell division site. *Genes Dev* **12**: 3419-3430.

Mascarenhas, J., J. Soppa, A. V. Strunnikov & P. L. Graumann, (2002) Cell cycle-dependent localization of two novel prokaryotic chromosome segregation and condensation proteins in *Bacillus subtilis* that interact with SMC protein. *Embo J* **21**: 3108-3118.

Massey, T. H., L. Aussel, F. X. Barre & D. J. Sherratt, (2004) Asymmetric activation of Xer site-specific recombination by FtsK. *EMBO Rep* **5**: 399-404.

Massey, T. H., C. P. Mercogliano, J. Yates, D. J. Sherratt & J. Lowe, (2006) Double-stranded DNA translocation: structure and mechanism of hexameric FtsK. *Mol Cell* **23**: 457-469.

Melby, T. E., C. N. Ciampaglio, G. Briscoe & H. P. Erickson, (1998) The symmetrical structure of structural maintenance of chromosomes (SMC) and MukB proteins: long, antiparallel coiled coils, folded at a flexible hinge. *J Cell Biol* **142**: 1595-1604.

Mo, A. H. & W. F. Burkholder, (2010) YneA, an SOS-induced inhibitor of cell division in *Bacillus subtilis*, is regulated posttranslationally and requires the transmembrane region for activity. *J Bacteriol* **192**: 3159-3173.

Mohl, D. A. & J. W. Gober, (1997) Cell cycle-dependent polar localization of chromosome partitioning proteins in *Caulobacter crescentus*. *Cell* **88**: 675-684.

Monahan, L. G., A. Robinson & E. J. Harry, (2009) Lateral FtsZ association and the assembly of the cytokinetic Z ring in bacteria. *Mol Microbiol* **74**: 1004-1017.

Moriya, S., R. A. Rashid, C. D. Rodrigues & E. J. Harry, (2010) Influence of the nucleoid and the early stages of DNA replication on positioning the division site in *Bacillus subtilis*. *Mol Microbiol* **76**: 634-647.

Moriya, S., E. Tsujikawa, A. K. Hassan, K. Asai, T. Kodama & N. Ogasawara, (1998) A *Bacillus subtilis* gene-encoding protein homologous to eukaryotic SMC motor protein is necessary for chromosome partition. *Mol Microbiol* **29**: 179-187.

Mosyak, L., Y. Zhang, E. Glasfeld, S. Haney, M. Stahl, J. Seehra & W. S. Somers, (2000) The bacterial cell-division protein ZipA and its interaction with an FtsZ fragment revealed by X-ray crystallography. *EMBO J* **19**: 3179-3191.

Mott, M. L. & J. M. Berger, (2007) DNA replication initiation: mechanisms and regulation in bacteria. *Nat Rev Microbiol* **5**: 343-354.

Moynihan, P. J. & A. J. Clarke, (2011) O-Acetylated peptidoglycan: controlling the activity of bacterial autolysins and lytic enzymes of innate immune systems. *Int J Biochem Cell Biol* **43**: 1655-1659.

Mukherjee, A., C. Cao & J. Lutkenhaus, (1998) Inhibition of FtsZ polymerization by SulA, an inhibitor of septation in *Escherichia coli*. *Proc Natl Acad Sci U S A* **95**: 2885-2890.

Mukherjee, A., K. Dai & J. Lutkenhaus, (1993) *Escherichia coli* cell division protein FtsZ is a guanine nucleotide binding protein. *Proc Natl Acad Sci U S A* **90**: 1053-1057.

Mukherjee, A. & J. Lutkenhaus, (1994) Guanine nucleotide-dependent assembly of FtsZ into filaments. *J Bacteriol* **176**: 2754-2758.

Mukherjee, A. & J. Lutkenhaus, (1998) Dynamic assembly of FtsZ regulated by GTP hydrolysis. *EMBO J* **17**: 462-469.

Mulder, E. & C. L. Woldringh, (1989) Actively replicating nucleoids influence positioning of division sites in *Escherichia coli* filaments forming cells lacking DNA. *J Bacteriol* **171**: 4303-4314.

Murray, H. & J. Errington, (2008) Dynamic control of the DNA replication initiation protein DnaA by Soj/ParA. *Cell* **135**: 74-84.

Nielsen, H. J., Y. Li, B. Youngren, F. G. Hansen & S. Austin, (2006a) Progressive segregation of the *Escherichia coli* chromosome. *Mol Microbiol* **61**: 383-393.

Nielsen, H. J., J. R. Ottesen, B. Youngren, S. J. Austin & F. G. Hansen, (2006b) The *Escherichia coli* chromosome is organized with the left and right chromosome arms in separate cell halves. *Mol Microbiol* **62**: 331-338.

Niki, H., A. Jaffe, R. Imamura, T. Ogura & S. Hiraga, (1991) The new gene *mukB* codes for a 177 kDa protein with coiled-coil domains involved in chromosome partitioning of *E. coli*. *EMBO J* **10**: 183-193.

Niki, H., Y. Yamaichi & S. Hiraga, (2000) Dynamic organization of chromosomal DNA in *Escherichia coli*. *Genes Dev* **14**: 212-223.

Odijk, T., (1998) Osmotic compaction of supercoiled DNA into a bacterial nucleoid. *Biophys Chem* **73**: 23-29.

Oliva, M. A., S. C. Cordell & J. Lowe, (2004) Structural insights into FtsZ protofilament formation. *Nat Struct Mol Biol* **11**: 1243-1250.

Osawa, M., D. E. Anderson & H. P. Erickson, (2008) Reconstitution of contractile FtsZ rings in liposomes. *Science* **320**: 792-794.

Osawa, M., D. E. Anderson & H. P. Erickson, (2009) Curved FtsZ protofilaments generate bending forces on liposome membranes. *EMBO J* **28**: 3476-3484.

Osawa, M. & H. P. Erickson, (2011) Inside-out Z rings--constriction with and without GTP hydrolysis. *Mol Microbiol* **81**: 571-579.

Patrick, J. E. & D. B. Kearns, (2008) MinJ (YvjD) is a topological determinant of cell division in *Bacillus subtilis*. *Mol Microbiol* **70**: 1166-1179.

Pease, P. J., O. Levy, G. J. Cost, J. Gore, J. L. Ptacin, D. Sherratt, C. Bustamante & N. R. Cozzarelli, (2005) Sequence-directed DNA translocation by purified FtsK. *Science* **307**: 586-590.

Perals, K., F. Cornet, Y. Merlet, I. Delon & J. M. Louarn, (2000) Functional polarization of the *Escherichia coli* chromosome terminus: the *dif* site acts in chromosome dimer resolution only when located between long stretches of opposite polarity. *Mol Microbiol* **36**: 33-43.

Pereira, S. F., A. O. Henriques, M. G. Pinho, H. de Lencastre & A. Tomasz, (2009) Evidence for a dual role of PBP1 in the cell division and cell separation of *Staphylococcus aureus*. *Mol Microbiol* **72**: 895-904.

Peters, N. T., T. Dinh & T. G. Bernhardt, (2011) A fail-safe mechanism in the septal ring assembly pathway generated by the sequential recruitment of cell separation amidases and their activators. *J Bacteriol* **193**: 4973-4983.

Peters, P. C., M. D. Migocki, C. Thoni & E. J. Harry, (2007) A new assembly pathway for the cytokinetic Z ring from a dynamic helical structure in vegetatively growing cells of *Bacillus subtilis*. *Mol Microbiol* **64**: 487-499.

Pichoff, S. & J. Lutkenhaus, (2002) Unique and overlapping roles for ZipA and FtsA in septal ring assembly in *Escherichia coli*. *EMBO J* **21**: 685-693.

Pichoff, S. & J. Lutkenhaus, (2005) Tethering the Z ring to the membrane through a conserved membrane targeting sequence in FtsA. *Mol Microbiol* **55**: 1722-1734.

Pinho, M. G., H. de Lencastre & A. Tomasz, (2001) An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant *Staphylococci*. *Proc Natl Acad Sci U S A* **98**: 10886-10891.

Pinho, M. G. & J. Errington, (2004) A *divIVA* null mutant of *Staphylococcus aureus* undergoes normal cell division. *FEMS Microbiol Lett* **240**: 145-149.

Popp, D., M. Iwasa, A. Narita, H. P. Erickson & Y. Maeda, (2009) FtsZ condensates: an *in vitro* electron microscopy study. *Biopolymers* **91**: 340-350.

Postow, L., N. J. Crisona, B. J. Peter, C. D. Hardy & N. R. Cozzarelli, (2001) Topological challenges to DNA replication: conformations at the fork. *Proc Natl Acad Sci U S A* **98**: 8219-8226.

Postow, L., C. D. Hardy, J. Arsuaga & N. R. Cozzarelli, (2004) Topological domain structure of the *Escherichia coli* chromosome. *Genes Dev* **18**: 1766-1779.

Ptacin, J. L., M. Nollmann, E. C. Becker, N. R. Cozzarelli, K. Pogliano & C. Bustamante, (2008) Sequence-directed DNA export guides chromosome translocation during sporulation in *Bacillus subtilis*. *Nat Struct Mol Biol* **15**: 485-493.

Ramamurthi, K. S. & R. Losick, (2009) Negative membrane curvature as a cue for subcellular localization of a bacterial protein. *Proc Natl Acad Sci U S A* **106**: 13541-13545.

Raskin, D. M. & P. A. de Boer, (1997) The MinE ring: an FtsZ-independent cell structure required for selection of the correct division site in *E. coli*. *Cell* **91**: 685-694.

Raskin, D. M. & P. A. de Boer, (1999) Rapid pole-to-pole oscillation of a protein required for directing division to the middle of *Escherichia coli*. *Proc Natl Acad Sci U S A* **96**: 4971-4976.

RayChaudhuri, D. & J. T. Park, (1992) *Escherichia coli* cell-division gene *ftsZ* encodes a novel GTP-binding protein. *Nature* **359**: 251-254.

Reyes-Lamothe, R., E. Nicolas & D. J. Sherratt, (2012) Chromosome replication and segregation in bacteria. *Annu Rev Genet*. Epub ahead of print, doi:10.1146/annurev-genet-110711-155421.

Reyes-Lamothe, R., C. Possoz, O. Danilova & D. J. Sherratt, (2008) Independent positioning and action of *Escherichia coli* replisomes in live cells. *Cell* **133**: 90-102.

Reynolds, P. E. & D. F. Brown, (1985) Penicillin-binding proteins of beta-lactam-resistant strains of *Staphylococcus aureus*. Effect of growth conditions. *FEBS Lett* **192**: 28-32.

Rimsky, S. & A. Travers, (2011) Pervasive regulation of nucleoid structure and function by nucleoid-associated proteins. *Curr Opin Microbiol* **14**: 136-141.

Rodrigues, C. D. & E. J. Harry, (2012) The Min system and nucleoid occlusion are not required for identifying the division site in *Bacillus subtilis* but ensure its efficient utilization. *PLoS Genet* **8**: e1002561.

Saleh, O. A., C. Perals, F. X. Barre & J. F. Allemand, (2004) Fast, DNA-sequence independent translocation by FtsK in a single-molecule experiment. *EMBO J* **23**: 2430-2439.

Sawitzke, J. A. & S. Austin, (2000) Suppression of chromosome segregation defects of *Escherichia coli* muk mutants by mutations in topoisomerase I. *Proc Natl Acad Sci U S A* **97**: 1671-1676.

Scheffers, D. J., J. G. de Wit, T. den Blaauwen & A. J. Driessen, (2001) Substitution of a conserved aspartate allows cation-induced polymerization of FtsZ. *FEBS lett* **494**: 34-37.

Scheffers, D. J., J. G. de Wit, T. den Blaauwen & A. J. Driessen, (2002) GTP hydrolysis of cell division protein FtsZ: evidence that the active site is formed by the association of monomers. *Biochemistry* **41**: 521-529.

Scheffers, D. J. & M. G. Pinho, (2005) Bacterial cell wall synthesis: new insights from localization studies. *Microbiol Mol Biol Rev* **69**: 585-607.

Schlag, M., R. Biswas, B. Krismer, T. Kohler, S. Zoll, W. Yu, H. Schwarz, A. Peschel & F. Gotz, (2010) Role of staphylococcal wall teichoic acid in targeting the major autolysin Atl. *Mol Microbiol* **75**: 864-873.

Scholefield, G., R. Whiting, J. Errington & H. Murray, (2011) Spo0J regulates the oligomeric state of Soj to trigger its switch from an activator to an inhibitor of DNA replication initiation. *Mol Microbiol* **79**: 1089-1100.

Schumacher, M. A., (2008) Structural biology of plasmid partition: uncovering the molecular mechanisms of DNA segregation. *Biochem J* **412**: 1-18.

Schvartzman, J. B. & A. Stasiak, (2004) A topological view of the replicon. *EMBO Rep* **5**: 256-261.

Sharp, M. D. & K. Pogliano, (1999) An *in vivo* membrane fusion assay implicates SpoIIIE in the final stages of engulfment during *Bacillus subtilis* sporulation. *Proc Natl Acad Sci U S A* **96**: 14553-14558.

Sharp, M. D. & K. Pogliano, (2003) The membrane domain of SpoIIIE is required for membrane fusion during *Bacillus subtilis* sporulation. *J Bacteriol* **185**: 2005-2008.

Sharpe, M. E. & J. Errington, (1995) Postseptational chromosome partitioning in bacteria. *Proc Natl Acad Sci U S A* **92**: 8630-8634.

Shen, B. & J. Lutkenhaus, (2009) The conserved C-terminal tail of FtsZ is required for the septal localization and division inhibitory activity of MinC(C)/MinD. *Mol Microbiol* **72**: 410-424.

Shen, B. & J. Lutkenhaus, (2010) Examination of the interaction between FtsZ and $MinC^{N}$ in *E. coli* suggests how MinC disrupts Z rings. *Mol Microbiol* **75**: 1285-1298.

Sherratt, D. J., (2003) Bacterial chromosome dynamics. *Science* **301**: 780-785.

Shiomi, D. & W. Margolin, (2007) The C-terminal domain of MinC inhibits assembly of the Z ring in *Escherichia coli*. *J Bacteriol* **189**: 236-243.

Shiomi, D. & W. Margolin, (2008) Compensation for the loss of the conserved membrane targeting sequence of FtsA provides new insights into its function. *Mol Microbiol* **67**: 558-569.

Singh, J. K., R. D. Makde, V. Kumar & D. Panda, (2007) A membrane protein, EzrA, regulates assembly dynamics of FtsZ by interacting with the C-terminal tail of FtsZ. *Biochemistry* **46**: 11013-11022.

Singh, J. K., R. D. Makde, V. Kumar & D. Panda, (2008) SepF increases the assembly and bundling of FtsZ polymers and stabilizes FtsZ protofilaments by binding along its length. *J Biol Chem* **283**: 31116-31124.

Sivanathan, V., M. D. Allen, C. de Bekker, R. Baker, L. K. Arciszewska, S. M. Freund, M. Bycroft, J. Lowe & D. J. Sherratt, (2006) The FtsK gamma domain directs oriented DNA translocation by interacting with KOPS. *Nat Struct Mol Biol* **13**: 965-972.

Sivanathan, V., J. E. Emerson, C. Pages, F. Cornet, D. J. Sherratt & L. K. Arciszewska, (2009) KOPS-guided DNA translocation by FtsK safeguards *Escherichia coli* chromosome segregation. *Mol Microbiol* **71**: 1031-1042.

Small, E., R. Marrington, A. Rodger, D. J. Scott, K. Sloan, D. Roper, T. R. Dafforn & S. G. Addinall, (2007) FtsZ polymer-bundling by the *Escherichia coli* ZapA orthologue, YgfE, involves a conformational change in bound GTP. *J Mol Biol* **369**: 210-221.

Soppa, J., K. Kobayashi, M. F. Noirot-Gros, D. Oesterhelt, S. D. Ehrlich, E. Dervyn, N. Ogasawara & S. Moriya, (2002) Discovery of two novel families of proteins that are proposed to interact with prokaryotic SMC proteins, and characterization of the *Bacillus subtilis* family members ScpA and ScpB. *Mol Microbiol* **45**: 59-71.

Soufo, H. J. D. & P. L. Graumann, (2003) Actin-like proteins MreB and Mbl from *Bacillus subtilis* are required for bipolar positioning of replication origins. *Curr Biol* **13**: 1916-1920.

Steiner, W., G. Liu, W. D. Donachie & P. Kuempel, (1999) The cytoplasmic domain of FtsK protein is required for resolution of chromosome dimers. *Mol Microbiol* **31**: 579-583.

Steiner, W. W. & P. L. Kuempel, (1998) Sister chromatid exchange frequencies in *Escherichia coli* analyzed by recombination at the *dif* resolvase site. *J Bacteriol* **180**: 6269-6275.

Stricker, J., P. Maddox, E. D. Salmon & H. P. Erickson, (2002) Rapid assembly dynamics of the *Escherichia coli* FtsZ-ring demonstrated by fluorescence recovery after photobleaching. *Proc Natl Acad Sci U S A* **99**: 3171-3175.

Suefuji, K., R. Valluzzi & D. RayChaudhuri, (2002) Dynamic assembly of MinD into filament bundles modulated by ATP, phospholipids, and MinE. *Proc Natl Acad Sci U S A* **99**: 16776-16781.

Sullivan, N. L., K. A. Marquis & D. Z. Rudner, (2009) Recruitment of SMC by ParB-*parS* organizes the origin region and promotes efficient chromosome segregation. *Cell* **137**: 697-707.

Szeto, T. H., S. L. Rowland, C. L. Habrukowich & G. F. King, (2003) The MinD membrane targeting sequence is a transplantable lipid-binding helix. *J Biol Chem* **278**: 40050-40056.

Szeto, T. H., S. L. Rowland, L. I. Rothfield & G. F. King, (2002) Membrane localization of MinD is mediated by a C-terminal motif that is conserved across eubacteria, archaea, and chloroplasts. *Proc Natl Acad Sci U S A* **99**: 15693-15698.

Tadesse, S., J. Mascarenhas, B. Kosters, A. Hasilik & P. L. Graumann, (2005) Genetic interaction of the SMC complex with topoisomerase IV in *Bacillus subtilis*. *Microbiology* **151**: 3729-3737.

Teleman, A. A., P. L. Graumann, D. C. Lin, A. D. Grossman & R. Losick, (1998) Chromosome arrangement within a bacterium. *Curr Biol* **8**: 1102-1109.

Tenover, F. C. & R. J. Gorwitz, (2006) The epidemiology of *Staphylococcus* infections. In: Gram-positive pathogens. V. A. Fischetti, R. P. Novick, J. J. Ferretti, D. A. Portnoy & J. I. Rood (eds). Washington DC: ASM Press, pp. 526-534.

Thanedar, S. & W. Margolin, (2004) FtsZ exhibits rapid movement and oscillation waves in helix-like patterns in *Escherichia coli*. *Curr Biol* **14**: 1167-1173.

Thomaides, H. B., M. Freeman, M. El Karoui & J. Errington, (2001) Division site selection protein DivIVA of *Bacillus subtilis* has a second distinct function in chromosome segregation during sporulation. *Genes Dev* **15**: 1662-1673.

Tonthat, N. K., S. T. Arold, B. F. Pickering, M. W. Van Dyke, S. Liang, Y. Lu, T. K. Beuria, W. Margolin & M. A. Schumacher, (2011) Molecular mechanism by which the nucleoid occlusion factor, SlmA, keeps cytokinesis in check. *EMBO J* **30**: 154-164.

Toro, E., S. H. Hong, H. H. McAdams & L. Shapiro, (2008) *Caulobacter* requires a dedicated mechanism to initiate chromosome segregation. *Proc Natl Acad Sci U S A* **105**: 15435-15440.

Trusca, D., S. Scott, C. Thompson & D. Bramhill, (1998) Bacterial SOS checkpoint protein SulA inhibits polymerization of purified FtsZ cell division protein. *J Bacteriol* **180**: 3946-3953.

Turner, R. D., E. C. Ratcliffe, R. Wheeler, R. Golestanian, J. K. Hobbs & S. J. Foster, (2010) Peptidoglycan architecture can specify division planes in *Staphylococcus aureus*. *Nat Commun* **1**: 26.

Tzagoloff, H. & R. Novick, (1977) Geometry of cell division in *Staphylococcus aureus*. *J Bacteriol* **129**: 343-350.

Uehara, T. & T. G. Bernhardt, (2011) More than just lysins: peptidoglycan hydrolases tailor the cell wall. *Curr Opin Microbiol* **14**: 698-703.

Uehara, T., K. R. Parzych, T. Dinh & T. G. Bernhardt, (2010) Daughter cell separation is controlled by cytokinetic ring-activated cell wall hydrolysis. *EMBO J* **29**: 1412-1422.

van den Ent, F. & J. Lowe, (2000) Crystal structure of the cell division protein FtsA from *Thermotoga maritima*. *EMBO J* **19**: 5300-5307.

Viollier, P. H., M. Thanbichler, P. T. McGrath, L. West, M. Meewan, H. H. McAdams & L. Shapiro, (2004) Rapid and sequential movement of individual chromosomal loci to specific subcellular locations during bacterial DNA replication. *Proc Natl Acad Sci U S A* **101**: 9257-9262.

Vollmer, W., D. Blanot & M. A. de Pedro, (2008a) Peptidoglycan structure and architecture. *FEMS Microbiol Rev* **32**: 149-167.

Vollmer, W., B. Joris, P. Charlier & S. Foster, (2008b) Bacterial peptidoglycan (murein) hydrolases. *FEMS Microbiol Rev* **32**: 259-286.

Wang, J. C., (2002) Cellular roles of DNA topoisomerases: a molecular perspective. *Nat Rev Mol Cell Biol* **3**: 430-440.

Wang, L. & J. Lutkenhaus, (1998) FtsK is an essential cell division protein that is localized to the septum and induced as part of the SOS response. *Mol Microbiol* **29**: 731-740.

Wang, X., X. Liu, C. Possoz & D. J. Sherratt, (2006) The two *Escherichia coli* chromosome arms locate to separate cell halves. *Genes Dev* **20**: 1727-1731.

Wang, X., C. Possoz & D. J. Sherratt, (2005) Dancing around the divisome: asymmetric chromosome segregation in *Escherichia coli*. *Genes Dev* **19**: 2367-2377.

Wang, X. & D. J. Sherratt, (2010) Independent segregation of the two arms of the *Escherichia coli ori* region requires neither RNA synthesis nor MreB dynamics. *J Bacteriol* **192**: 6143-6153.

Weart, R. B., A. H. Lee, A. C. Chien, D. P. Haeusser, N. S. Hill & P. A. Levin, (2007) A metabolic sensor governing cell size in bacteria. *Cell* **130**: 335-347.

Weart, R. B., S. Nakano, B. E. Lane, P. Zuber & P. A. Levin, (2005) The ClpX chaperone modulates assembly of the tubulin-like protein FtsZ. *Mol Microbiol* **57**: 238-249.

Webb, C. D., P. L. Graumann, J. A. Kahana, A. A. Teleman, P. A. Silver & R. Losick, (1998) Use of time-lapse microscopy to visualize rapid movement of the replication origin region of the chromosome during the cell cycle in *Bacillus subtilis*. *Mol Microbiol* **28**: 883-892.

Webb, C. D., A. Teleman, S. Gordon, A. Straight, A. Belmont, D. C. Lin, A. D. Grossman, A. Wright & R. Losick, (1997) Bipolar localization of the replication origin regions of chromosomes in vegetative and sporulating cells of *B. subtilis*. *Cell* **88**: 667-674.

Woldringh, C. L., (2002) The role of co-transcriptional translation and protein translocation (transertion) in bacterial chromosome segregation. *Mol Microbiol* **45**: 17-29.

Woldringh, C. L., P. R. Jensen & H. V. Westerhoff, (1995) Structure and partitioning of bacterial DNA: determined by a balance of compaction and expansion forces? *FEMS Microbiol Lett* **131**: 235-242.

Woldringh, C. L., E. Mulder, P. G. Huls & N. Vischer, (1991) Toporegulation of bacterial division according to the nucleoid occlusion model. *Res Microbiol* **142**: 309-320.

Wu, L. J. & J. Errington, (1994) *Bacillus subtilis* SpoIIIE protein required for DNA segregation during asymmetric cell division. *Science* **264**: 572-575.

Wu, L. J. & J. Errington, (1997) Septal localization of the SpoIIIE chromosome partitioning protein in *Bacillus subtilis*. *EMBO J* **16**: 2161-2169.

Wu, L. J. & J. Errington, (2003) RacA and the Soj-Spo0J system combine to effect polar chromosome segregation in sporulating *Bacillus subtilis*. *Mol Microbiol* **49**: 1463-1475.

Wu, L. J. & J. Errington, (2004) Coordination of cell division and chromosome segregation by a nucleoid occlusion protein in *Bacillus subtilis*. *Cell* **117**: 915-925.

Wu, L. J. & J. Errington, (2008) Cell biology: DNA versus membrane. *Nature* **451**: 900-901.

Wu, L. J. & J. Errington, (2011) Nucleoid occlusion and bacterial cell division. *Nat Rev Microbiol* **10**: 8-12.

Wu, L. J., S. Ishikawa, Y. Kawai, T. Oshima, N. Ogasawara & J. Errington, (2009) Noc protein binds to specific DNA sequences to coordinate cell division with chromosome segregation. *EMBO J* **28**: 1940-1952.

Wu, L. J., P. J. Lewis, R. Allmansberger, P. M. Hauser & J. Errington, (1995) A conjugation-like mechanism for prespore chromosome partitioning during sporulation in *Bacillus subtilis*. *Genes Dev* **9**: 1316-1326.

Wu, W., K. T. Park, T. Holyoak & J. Lutkenhaus, (2011) Determination of the structure of the MinD-ATP complex reveals the orientation of MinD on the membrane and the relative location of the binding sites for MinE and MinC. *Mol Microbiol* **79**: 1515-1528.

Yamada, S., M. Sugai, H. Komatsuzawa, S. Nakashima, T. Oshida, A. Matsumoto & H. Suginaka, (1996) An autolysin ring associated with cell separation of *Staphylococcus aureus*. *J Bacteriol* **178**: 1565-1571.

Yamaichi, Y. & H. Niki, (2000) Active segregation by the *Bacillus subtilis* partitioning system in *Escherichia coli*. *Proc Natl Acad U S A* **97**: 14656-14661.

Yamamoto, H., Y. Miyake, M. Hisaoka, S. Kurosawa & J. Sekiguchi, (2008) The major and minor wall teichoic acids prevent the sidewall localization of vegetative DL-endopeptidase LytF in *Bacillus subtilis*. *Mol Microbiol* **70**: 297-310.

Yamazoe, M., T. Onogi, Y. Sunako, H. Niki, K. Yamanaka, T. Ichimura & S. Hiraga, (1999) Complex formation of MukB, MukE and MukF proteins involved in chromosome partitioning in *Escherichia coli*. *EMBO J* **18**: 5873-5884.

Yang, D. C., N. T. Peters, K. R. Parzych, T. Uehara, M. Markovski & T. G. Bernhardt, (2011) An ATP-binding cassette transporter-like complex governs cell-wall hydrolysis at the bacterial cytokinetic ring. *Proc Natl Acad Sci U S A* **108**: 1052-1060.

Yates, J., M. Aroyo, D. J. Sherratt & F. X. Barre, (2003) Species specificity in the activation of Xer recombination at *dif* by FtsK. *Mol Microbiol* **49**: 241-249.

Yates, J., I. Zhekov, R. Baker, B. Eklund, D. J. Sherratt & L. K. Arciszewska, (2006) Dissection of a functional interaction between the DNA translocase, FtsK, and the XerD recombinase. *Mol Microbiol* **59**: 1754-1766.

Young, K. D., (2010) Bacterial shape: two-dimensional questions and possibilities. *Annu Rev Microbiol* **64**: 223-240.

Yu, X. C., A. H. Tran, Q. Sun & W. Margolin, (1998a) Localization of cell division protein FtsK to the *Escherichia coli* septum and identification of a potential N-terminal targeting domain. *J Bacteriol* **180**: 1296-1304.

Yu, X. C., E. K. Weihe & W. Margolin, (1998b) Role of the C terminus of FtsK in *Escherichia coli* chromosome segregation. *J Bacteriol* **180**: 6424-6428.

Zapun, A., C. Contreras-Martel & T. Vernet, (2008) Penicillin-binding proteins and beta-lactam resistance. *FEMS Microbiol Rev* **32**: 361-385.

Zhou, H. & J. Lutkenhaus, (2003) Membrane binding by MinD involves insertion of hydrophobic residues within the C-terminal amphipathic helix into the bilayer. *J Bacteriol* **185**: 4326-4335.

Zimmerman, S. B. & L. D. Murphy, (1996) Macromolecular crowding and the mandatory condensation of DNA in bacteria. *FEBS lett* **390**: 245-248.

Chapter 2

Inactivation of the SauI Type I restriction-modification system is not sufficient to generate *Staphylococcus aureus* strains capable of efficiently accepting foreign DNA

AUTHOR CONTRIBUTIONS

All experiments described in this chapter were performed by H. Veiga.

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Abstract

Genetic manipulation of *Staphylococcus aureus* is limited by the availability of only a single strain, RN4220, that is capable of easily accepting foreign DNA. Inactivation of the *hsdR* gene of the SauI type I restriction modification system was shown previously to be responsible for the high transformation efficiency of RN4220 (D. E. Waldron and J. A. Lindsay, J. Bacteriol. 188:5578–5585, 2006). However, deletion of this gene in three different *S. aureus* strains was not sufficient to make them readily transformable, which would be remarkably useful for genetic studies of this pathogenic organism. These results indicate that another unknown factor(s) is required for the transformable phenotype in *S. aureus*.

Introduction

Staphylococcus aureus is a major pathogen that causes both nosocomial and community-acquired infections, which range from superficial skin infections to severe systemic diseases. It is an extremely versatile pathogen which has developed resistance to virtually all known classes of antibiotics and which expresses various virulence factors that allow it to cause infection in different environments.

Molecular genetic studies of *S. aureus* have resulted in a better understanding of both the virulence and antibiotic resistance mechanisms of this organism, which is crucial for discovery of new approaches to treat staphylococcal infections. One of the limitations in genetically manipulating *S. aureus* is the fact that there is only a single strain available which can easily accept plasmid DNA isolated from *Escherichia coli.* This strain, RN4220, is a chemical mutant obtained in the early 1980s by highly mutagenizing NCTC8325-4 (=RN450) with nitrosoguanidine and selecting for a mutant that was able to accept and maintain *S. aureus* plasmids ((Kreiswirth *et al.*, 1983); B. Kreiswirth, personal communication).

One of the most important bacterial defenses against uptake of foreign DNA is restriction-modification (R-M) systems. These systems, comprising restriction endonucleases and methyltransferases, recognize and modify specific DNA sequences, protecting “self” DNA from restriction while eliminating potentially harmful foreign DNA which lacks appropriate modification (Murray, 2000). There are three distinct well-characterized types of classical R-M systems, including type II restriction enzymes, which cut DNA within specific recognition sequences and are therefore widely used as molecular biology tools (Bickle & Kruger, 1993). *S. aureus* strains may contain different R-M systems, including the Sau3AI and Sau96I type II systems (present in isolates of particular lytic groups) (Sussenbach *et al.*, 1976, Sussenbach *et al.*, 1978) or the Sau42I BcgI-like R-M system expressed by *S. aureus* ϕ42 lysogens (Dempsey *et al.*, 2005). However, the only chromosomal R-M system widely distributed in the sequenced *S. aureus* isolates is the SauI type I system (Waldron & Lindsay, 2006). TypeI R-M systems require the products of

three genes, *hsdR* (restriction), *hsdM* (modification), and *hsdS* (sequence specificity), and cut DNA at sites remote from the recognition sequence (Murray, 2000). The staphylococcal SauI system includes a single *hsdR* gene and two copies of the *hsdM* and *hsdS* genes, and there is substantial variation between the *hsdS* genes from different isolates (Waldron & Lindsay, 2006). It was recently shown that transformable RN4220 carries a stop mutation in the *sauI hsdR* gene and that complementation with a functional copy of this gene restores a nontransformable phenotype (Waldron & Lindsay, 2006). An *hsdR* mutant does not cleave foreign unmodified DNA, but it does modify incoming DNA, which therefore is not cleaved when it is transferred to other *S. aureus* strains that contain an identical R-M system. For this reason, RN4220 has become an essential intermediate for laboratory manipulation of *S. aureus*, despite its limited clinical relevance. DNA first introduced into RN4220 by electroporation can then be transferred into other laboratory strains (for example, by phage transduction).

The relevance of the SauI type I R-M system for horizontal transfer of foreign DNA in different *S. aureus* isolates in nature has been confirmed with bovine isolates that are hypersusceptible to gene transfer from enterococci and have stop mutations in each of the two SauI *hsdS* gene copies (Sung & Lindsay, 2007). These “hyperrecipient” strains may be ideal backgrounds for the acquisition of new antibiotic resistance markers, such as the *vanA* gene complex present in vancomycin-resistant *S. aureus* strains (Sung & Lindsay, 2007). However, the existence of a second pathway in *S. aureus* that blocks horizontal transfer of foreign DNA or of an unknown but necessary factor essential for SauI activity has also been proposed based on the fact that some strains that are hypersusceptible to gene transfer (such as B111 used by Noble *et al.* (Noble *et al.*, 1992)) do not have a mutation in any of the five *hsd* genes (Sung & Lindsay, 2007).

The availability of laboratory and clinical strains other than RN4220 that are capable of accepting foreign DNA would be remarkably useful for genetic studies of *S. aureus*, including studies of virulence and antibiotic resistance mechanisms present only in relevant clinical isolates. Therefore, we have tried to reproduce the *hsdR* mutation in

RN4220 in different backgrounds of widely used laboratory strains in order to generate useful, easily transformable strains.

Experimental procedures

Bacterial strains and growth conditions

The following bacterial strains were used in this study: methicillin-susceptible *S. aureus* strains NCTC8325-4 (Oshida & Tomasz, 1992) and SH1000 (Horsburgh *et al.*, 2002), methicillin-resistant *S. aureus* strain COL (Dyke *et al.*, 1966, Gill *et al.*, 2005), and restriction-deficient *S. aureus* strain 879R4RF (Stobberingh & Winkler, 1977). Unless otherwise stated, all *S. aureus* strains were grown in tryptic soy broth (TSB) (Difco) at 37°C with aeration. When required, the medium was supplemented with erythromycin (10 µg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 100 µg/ml), or chloramphenicol (10 µg/ml). Sequences of primers used are listed in Table 2.1.

Construction of *hsdR* null mutants

hsdR null mutants were constructed using the backgrounds of strains NCTC8325-4, SH1000, and COL and the thermosensitive plasmid pMAD (Arnaud *et al.*, 2004), which contains an erythromycin resistance marker as well as the *lacZ* reporter gene under control of a constitutive promoter. Two PCR fragments, corresponding to the upstream and downstream regions of the *hsdR* gene, were amplified from chromosomal DNA of strain NCTC8325-4 using Phusion highfidelity DNA polymerase (Finnzymes) and primer pairs HsdRP1/HsdRP2 and HsdRP3/HsdRP4, respectively (Figure 2.2A). In the second step, the two fragments were joined by overlapping PCR using primers HsdRP1 and HsdRP4. The 2kb PCR final product was restricted with BamHI and EcoRI (NEB), cloned into pMAD, resulting in pΔHsdR-1, and sequenced (STABVida). The latter plasmid was electroporated, as previously described (McNamara, 2008), into the transformable strain RN4220 at 30°C with erythromycin selection and was subsequently transduced into NCTC8325-4, SH1000,

and COL using phage 80α (Oshida & Tomasz, 1992). Strains containing pΔHsdR-1 were incubated at a nonpermissive temperature (43°C) in the presence of erythromycin to select for recombinants in which the plasmid had integrated into the chromosome, and then they were incubated at a permissive temperature (30°C) in the absence of antibiotic selection to select for white colonies in which the pΔHsdR-1 plasmid (and consequently *lacZ* and *erm* genes) had been excised (Figure 2.1). To identify mutants in which the *hsdR* gene had been deleted, chromosomal DNA was extracted from erythromycin-sensitive, white colonies and tested by performing PCR using GoTaq polymerase (Promega) and primers HsdRP5 and HsdRP6, which hybridize 211bp upstream and 84bp downstream of the *hsdR* gene, respectively (Figure 2.2A).

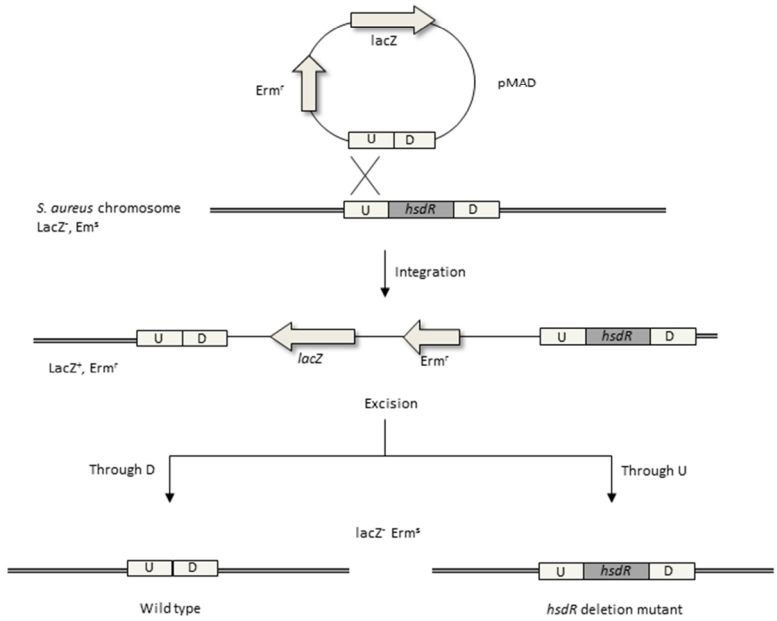

Figure 2.1. Strategy to delete *hsdR* using pMAD vector. A PCR product containing the upstream (U) and downstream (D) region of the *hsdR* gene was cloned into plasmid pMAD. This thermosensitive plasmid contains an erythromycin resistance marker as well as the *lacZ* reporter gene under the control of a constitutive promoter. The plasmid was introduced into NCTC8325-4, SH1000 and COL backgrounds. After growth at 43°C blue (LacZ+), erythromycin resistant colonies, which had the plasmid integrated into the chromosomal locus of *hsdR* were chosen. These colonies were then grown at 30°C in the absence of antibiotic selection to promote the loss of the plasmid and, consequently, of the *lacZ* and *erm* genes. White, LacZ- colonies were analysed by PCR to determine whether they have a wild type genotype or if the *hsdR* was deleted.

The transformable RN4220 strain expresses a truncated HsdR product containing only the first 192 amino acids of the wild-type protein (Waldron & Lindsay, 2006). Therefore, we constructed an NCTC8325-4 strain in which the sequence coding for the C terminus of HsdR was deleted. For this purpose, a 2598bp DNA fragment encompassing the upstream region, the first 576bp (192 codons) followed by a stop codon, and the downstream region of the *hsdR* gene was amplified in the following way. The initial PCRs were performed using primer pairs HsdRP1/HsdRP9 and HsdRP10/HsdRP11 (Figure 2.2A). The two resulting fragments were joined by overlap PCR using primers HsdRP1 and HsdRP11 and cloned into pMAD, resulting in plasmid pΔHsdR-2, which was sequenced. This plasmid was electroporated into RN4220 at 30°C and transduced into NCTC8325-4, where exchange of the wild-type copy of *hsdR* with the truncated copy was promoted, using the procedure described above for generation of the null mutants. The resulting strain, designated NCTC8325-4ΔC-termHsdR, was verified by performing PCR using primers HsdRP5 and HsdRP6 (Figure 2.2A).

Table 2.1. Primers used in this study

Primer Name	Primer Sequence (5'- 3')*
HsdRP1	tgaggatcctttgtgacgaatcaaggtagtcatagtg
HsdRP2	gtatttttcagtattcactttggtatgccattcatatccc
HsdRP3	aagtgaatactgaaaaatacggtgtgtaatgattcagccc
HsdRP4	gctgaattctataacaagaacttaatttcagccggcg
HsdRP5	caagtccctccattaatcgtag
HsdRP6	tgatggttgccaaacacatg
HsdRP7	gatcgtgatggtgaagtgcc
HsdRP8	ggttacgacgttgttccgc
HsdRP9	gtatttttcaactcttcaatagttcgctatcattattaga
HsdRP10	attgaagagttgaaaaatacggtgtgtaatgattcagccc
HsdRP11	gctgaattcgatgcctttgtttggaatattacgc

* Underlined sequences correspond to restriction sites

All genomic constructs were also verified by Southern blot hybridization using a specific probe for the 3' region of the *hsdR* gene. For this purpose, genomic DNA of *S. aureus* wild-type and *hsdR* mutant strains was digested with SmaI and separated by

pulsed-field gel electrophoresis (PFGE) as previously described (Chung *et al.*, 2000). The DNA was then transferred to a positively charged nylon membrane (Amersham), hybridized with the *hsdR* internal probe, a 817-bp DNA fragment amplified by PCR using primers HsdRP7 and HsdRP8 (Figure 2.2A), and labeled using the Gene Images AlkPhos direct labeling system (Amersham) according to the manufacturer's instructions.

Electroporation

Electroporation was performed as previously described (McNamara, 2008). Briefly, 45µl portions of competent cells of RN4220, of wild-type strains NCTC8325-4, SH1000, and COL, and of the corresponding *hsdR* mutants were mixed with 0.2µg of the replicative plasmid pGC2 (Wu *et al.*, 1996) extracted from *E. coli* DH5α in a 2.0mm electroporation cuvette (Bio-Rad) and subjected to an electroporation pulse in a Gene Pulser Xcell apparatus (Bio-Rad) set to 2.5kV, 25µF, and 100Ω. Following electroporation, cells were immediately resuspended in 955 µl of SMMP medium (5.5 parts SMM buffer [1 M sucrose, 0.04 M maleic acid, 0.04 M $MgCl_2$; pH 6.5], 4 parts 7% antibiotic medium number 3 [Difco], 0.5 parts 10% bovine serum albumin) and incubated for 1 h at 37°C before they were plated on tryptic soy agar (TSA) supplemented with chloramphenicol. The number of transformants was determined after 16 h of incubation at 37°C. Three independent batches of competent cells were prepared for each strain, and each batch was used in two different electroporation experiments.

The competent cells were also electroporated (in duplicate) with 50 ng of pGC2 extracted from RN4220 using a Wizard Plus SV minipreps kit (Promega) after 15 min of incubation with 100 µg/ml lysostaphin at 37°C to lyse the cells.

To determine the transformation efficiency of the wild-type and mutant strains after heat treatment, the competent cells were incubated for 2 min at 56°C immediately before they were subjected to the electroporation pulse. Two independent experiments were performed, in which heat-treated cells were transformed with 0.2µg of plasmid pGC2 DNA extracted from *E. coli*.

Transduction

Phages 80α and ϕ75 were used to transduce pGC2 from strain 879R4RF into NCTC8325-4 and SH1000 wild-type and *hsdR* mutant strains. The transduction experiments were performed essentially as previously described (Oshida & Tomasz, 1992). To prepare the phage lysates, recipient 879R4RF cells containing plasmid pGC2 were grown on TSA (Difco) plates for 16 h at 37°C and resuspended in 1ml of TSB supplemented with 5 mM $CaCl_2$. Dilutions (10^{-1} to 10^{-6}) of the 80α and ϕ75 stock lysates were prepared in phage buffer (1 mM MgSO4, 4 mM CaCl2, 50 mM Tris-HCl, 0.1 M NaCl, 0.1% gelatin; pH 7.8), and 10 µl of each dilution was mixed with 10 µl of the recipient cells and 3 ml of top phage agar (3 g/liter Casamino Acids, 3 g/liter yeast extract, 5.9 g/liter NaCl, 5 mM $CaCl_2$, 5 g/liter Bacto agar; pH 7.8). The mixture was plated on bottom phage agar (having the same composition as top phage agar except for 15 g/liter Bacto agar) and incubated for 16 h at 30°C. Phage buffer (3 ml) was added to the plates showing confluent lysis, which were subsequently incubated at 4°C for 1 h. The phage buffer and top phage agar were collected and incubated for 1 h at 4°C. After a centrifugation step the phage lysate was recovered and filtered through a sterile 0.45 µm filter.

For transduction, recipient cells were plated on TSA, grown overnight at 37°C, and resuspended in 1 ml of TSB supplemented with 5 mM $CaCl_2$. Cells (100 µl) were mixed with phage lysate (1 µl and 10 µl), and phage buffer was added to obtain a final volume of 300 µl. The mixture was incubated at 37°C for 20 min, added to 3 ml of 0.3GL top agar (3 g/l Casamino Acids, 3 g/l yeast extract, 5.9 g/l NaCl, 3.3 ml/l sodium lactate, 2 ml/l 50% glycerol, 0.5 g/l trisodium citrate, 7.5 g/l Bacto agar; pH 7.8) preheated to 50°C, and plated in plates containing 0.3GL bottom agar (having the same composition as 0.3GL top agar except for 15 g/l Bacto agar) with a layer of 20 ml of 0.3GL bottom agar without antibiotics over a layer of 10 ml of 0.3GL bottom agar with chloramphenicol (30 µg/ml). The plates were used within 1 h after preparation. The number of transductants carrying pGC2 was determined after overnight incubation at 37°C.

Bacteriophage susceptibility assays

NCTC8325-4 and SH1000 wild-type and *hsdR* mutant strains were tested for susceptibility to bacteriophages 80α and ϕ75 propagated in the nonlysogenic *S. aureus* 879R4RF strain.

Recipient cells were grown on TSA plates for 16 h at 37°C and resuspended in 1 ml of TSB supplemented with 5 mM $CaCl_2$. Serial dilutions of the 80α and ϕ75 stock lysates (10 µl of 10^{-1} to 10^{-6} dilutions) were mixed with 10 µl of the recipient cells, added to 3 ml of top phage agar, plated on bottom phage agar, and incubated overnight at 30°C. The efficiency of plaquing was calculated by dividing the number of phage PFU per milliliter of phage lysate for each strain tested by the number of phage PFU per milliliter of phage lysate for RN4220.

Results and discussion

Effect of *hsdR* gene deletion on the transformation efficiency of *S. aureus* strains

Developing a method to generate various clinical and laboratory *S. aureus* strains capable of easily accepting foreign DNA would be an important step forward in our ability to genetically manipulate *S. aureus*. With this aim, we decided to reproduce the *hsdR* mutation present in transformable *S. aureus* strain RN4220 in three different backgrounds of widely used laboratory strains, methicillin-susceptible *S. aureus* strains NCTC8325-4 (Novick, 1967) and SH1000 (Horsburgh *et al.*, 2002) and methicillin-resistant *S. aureus* strain COL (Dyke *et al.*, 1966, Gill *et al.*, 2005). The approach used consisted of constructing a complete null mutant in the three backgrounds by removing the *hsdR* gene, leaving no resistance marker in the chromosome (see experimental procedures section), so that the resulting strains would be more versatile for future genetic studies. The absence of the *hsdR* gene in strains NCTC8325-4ΔHsdR, SH1000ΔHsdR, and COLΔHsdR was confirmed by PCR (Figure 2.2B) using primers HsdRP5 and HsdRP6, which flank the *hsdR* gene (Figure 2.2A). Furthermore, SmaI-digested genomic DNA of wild-type

and *hsdR* mutant strains was separated by PFGE and hybridized with an *hsdR* gene internal probe. The PFGE restriction patterns of the wild-type strains and corresponding *hsdR* null mutants were identical, as expected (data not shown), and Southern blotting showed that the *hsdR* gene is not present in the genomes of the three null mutant strains (Figure 2.2C). Overexposure of the Southern blot autoradiography film revealed two weak bands corresponding to other SmaI DNA fragments in all wild-type and knockout strains (data not shown). However, as no *hsdR* homologue is present in either the NCTC8325-4 or COL genome, these weak bands probably resulted from nonspecific hybridization.

In order to assess the ability of the *hsdR* null mutants to accept foreign DNA, electroporation was performed using plasmid pGC2 DNA extracted from *E. coli* DH5α. Contrary to our expectations, complete deletion of the *hsdR* gene was not sufficient to generate readily transformable NCTC8325-4, SH1000, and COL strains (Table 2.2). Similar results were obtained when cells were transformed with the temperature-sensitive pMAD plasmid at 30°C (data not shown).

As the transformable RN4220 strain is not an *hsdR* null mutant but expresses a truncated HsdR product containing the first 192 amino acids (approximately 20%) of the wild-type protein (Waldron & Lindsay, 2006), we hypothesized that this peptide could somehow have a role in the transformable phenotype of RN4220. Therefore, we replaced the wild-type copy of the *hsdR* gene in the chromosome of NCTC8325-4, a strain closely related to RN4220, with an open reading frame containing only the first 192 codons of *hsdR*. The 3' *hsdR* gene deletion genotype was confirmed by PCR and Southern blotting (Figure 2.2). Failure to electroporate NCTC8325-4ΔC-termHsdR with replicative plasmid pCG2 extracted from *E. coli* (Table 2.2) showed that the ability of NCTC8325-4 to accept foreign DNA was not improved by the presence of the truncated form of the HsdR protein.

Importantly, *hsdR* mutant strains had growth rates identical to those of the parental strains (data not shown) and were competent if they were transformed with appropriately modified *S. aureus* DNA. This was tested by electroporating *hsdR* mutants with the pGC2 plasmid mentioned above but extracted from *S. aureus* strain RN4220

instead of *E. coli.* Since RN4220 belongs to the same lineage (CC8) as NCTC8325-4, SH1000, and COL, it was expected that its DNA, modified by its R-M system(s), would be accepted by the other strains. In fact, in contrast to the results for pGC2 extracted from *E. coli*, all strains tested were able to accept pGC2 DNA from RN4220 (Table2.2), suggesting that *hsdR* mutants are competent but do not accept foreign DNA due to the presence of an additional factor(s) that degrades unmodified DNA.

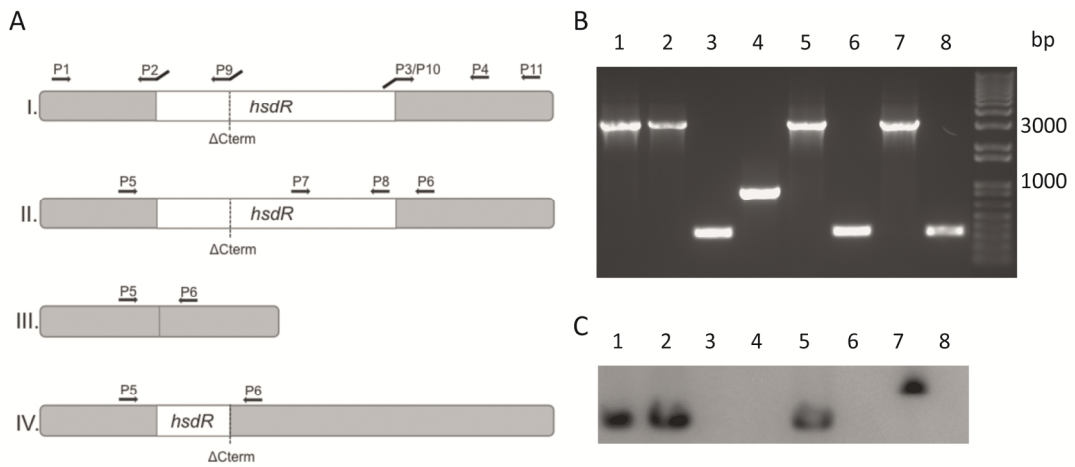

Figure 2.2. Genetic characterization of *hsdR* mutants. The strains used were RN4220 (lane 1), NCTC8325-4 (lane 2), NCTC8325-4ΔHsdR (lane3), NCTC8325-4ΔC-term HsdR (lane 4), SH1000 (lane 5), SH1000ΔHsdR (lane 6), COL (lane 7), and COLΔHsdR (lane 8). (A) Localization of primers used to construct *hsdR* mutants (panel I) (see experimental procedures section for details); of primers used to characterize the *hsdR* mutants by PCR for wild-type strains RN4220, NCTC8325-4, SH1000, and COL (panel II), for *hsdR* null mutants NCTC8325-4ΔHsdR, SH1000ΔHsdR, and COLΔHsdR (panel III), and for strain NCTC8325-4ΔC-term HsdR, which contains a truncated version of the *hsdR* gene (panel IV); and of primers HsdRP7 and HsdRP8 used to amplify the *hsdR* probe used for Southern blotting (panel II). (B) PCR fragments obtained by amplifying the DNA of the eight strains indicated above using primers HsdRP5 and HsdRP6, which flank the *hsdR* gene. (C) Results of Southern blot hybridization of SmaI-restricted genomic DNA of the eight strains indicated above with an internal probe for the *hsdR* gene amplified using primers HsdRP7 and HsdRP8, showing that this gene is absent from the *hsdR* mutant strains. Note that the probe hybridized with the DNA fragment which was deleted in strain NCTC8325-4ΔC-term HsdR.

Table 2.2. Electroporation efficiency of *S. aureus* wild type and *hsdR* mutant strains transformed with plasmid (pGC2) DNA extracted from *E. coli* DH5α or from *S. aureus* RN4220.

Strain	pGC2 from *E. coli* DH5α		pGC2 from *S. aureus* RN4220		pGC2 from *E. coli* DH5α (heat treated competent cells[c])	
	Transformation efficiency (cfu/µg DNA)[a]	Standard deviation (cfu/µg DNA)	Transformation efficiency (cfu/µg DNA)[b]	Standard deviation (cfu/µg DNA)	Transformation efficiency (cfu/µg DNA)[b]	Standard deviation (cfu/µg DNA)
RN4220	3.7×10^5	2.3×10^5	6.1×10^5	3.8×10^5	2.0×10^3	4.7×10^2
NCTC8325-4	1.7	2.6	3.0×10^4	0.2×10^4	0.0	0.0
NCTC8325-4 ΔHsdR	3.4	6.2	3.9×10^4	1.9×10^4	8.1×10^1	3.6×10^1
NCTC8325-4 ΔC-term HsdR	2.5	2.8	1.9×10^4	0.3×10^4	1.0×10^3	9.7×10^2
SH1000	0.0	0.0	7.0×10^4	2.6×10^4	0.0	0.0
SH1000 ΔHsdR	0.0	0.0	2.7×10^4	0.3×10^4	3.0×10^1	0.0
COL	0.0	0.0	3.0×10^1	4.2×10^1	0.0	0.0
COL ΔHsdR	0.0	0.0	5.0×10^1	4.2×10^1	0.0	0.0

[a] average value of 6 electroporation experiments

[b] average value of 2 electroporation experiments

[c] *S. aureus* competent cells were incubated at 56°C for 2 min, prior to electroporation

Effect of deletion of the *hsdR* gene on *S. aureus* susceptibility to infection by bacteriophages

One of the proposed roles of R-M systems is protection of bacterial strains against phage infections (Bickle & Kruger, 1993). Additionally, phage transduction is a method commonly used in the laboratory to move DNA between staphylococcal strains. Therefore, in an experiment complementary to the electroporation experiment, we tested if we could introduce foreign DNA into the *hsdR* mutants by transduction. For this, we used phages 80α and ϕ75 to transduce plasmid pGC2 from *S. aureus* strain 879R4RF into NCTC8325-4 and SH1000 wild-type and *hsdR* mutant strains. Strain 879R4RF belongs to the CC51 lineage, and DNA propagated in this strain is recognized as "foreign" DNA by members of the CC8 lineage, such as NCTC8325-4 and SH1000 (Waldron & Lindsay, 2006). In agreement with the electroporation data, NCTC8325-4ΔHsdR and SH1000ΔHsdR were not capable of accepting pGC2 DNA coming from 879R4RF by transduction (Table 2.3).

We also tested the susceptibility of the *hsdR* mutants to infection by phages propagated in 879R4RF. When NCTC8325-4 and SH1000 and the corresponding *hsdR* mutants were infected with phage 80α, again there was no significant increase (threefold or less) in the efficiency of infection associated with deletion of *hsdR* (Table 2.3). However, when the same strains were infected with phage ϕ75, there was a 100 to 1000 fold increase in the efficiency of infection of the *hsdR* mutants compared with their parental strains (Table 2.3). It is possible that phage ϕ75 (but not phage 80α) is able to resist the action of the additional factor(s) that degrades foreign DNA (possibly a second R-M system) which we postulate exists in NCTC8325-4 and SH1000. In fact, many phages have some means of reducing their susceptibility to R-M systems, such as unusual modifications of DNA, a low frequency of target sequences, or the production of a protein that inhibits the activity of an R-M system (Bickle & Kruger, 1993, Murray, 2000).

Table 2.3. Efficiency of transduction and infection of *S. aureus* wild type and *hsdR* mutant strains using phages 80α and ϕ75

Strain	Transduction of pGC2 plasmid from 879R4RF[1]		Efficiency of plaquing (EOP)[2]	
	Phage 80α	Phage ϕ75	Phage 80α from 879R4RF	Phage ϕ75 from 879R4RF
RN4220	158	620	1.0	1.0
NCTC8325-4	0	0	3.1×10^{-5}	3.7×10^{-6}
NCTC8325-4 ΔHsdR	0	0	5.8×10^{-5}	3.9×10^{-3}
NCTC8325-4 ΔC-term HsdR	0	0	1.0×10^{-4}	6.1×10^{-3}
SH1000	0	0	4.5×10^{-5}	4.7×10^{-5}
SH1000 ΔHsdR	0	0	1.6×10^{-4}	3.6×10^{-3}

[1]number of transductants/10^6 phage particles

[2] EOP was calculated by dividing the number of phage plaque forming units (PFU) per millilitre of phage lysate in each tested strain by the corresponding number in RN4220

Heat treatment of competent cells increases the transformation efficiency of *hsdR* mutants.

Some restriction enzymes (including type II enzymes) can be inactivated by heat, and brief heat treatment of staphylococcal cells has been used to increase phage sensitivity (Asheshov & Jevons, 1963, Stobberingh & Winkler, 1977). In order to determine if the restriction activity which remains in the *hsdR* mutants can be inactivated by heat, we examined the transformation efficiency of wild-type and *hsdR* mutant strains after exposure of competent cells to 56°C for 2 min. Indeed, after the heat treatment we were finally able to introduce foreign DNA (pGC2 extracted from *E. coli*) into the NCTC8325-4ΔHsdR and SH1000ΔHsdR strains by electroporation, while the parental strains remained nontransformable (Table 2.2). For unknown reasons, we were not able to transform COLΔHsdR. Importantly, the heat treatment resulted in a 100 fold decrease in the transformation efficiency of control strain RN4220 (Table 2.2). The low efficiencies obtained in the NCTC8325-4 and SH1000 backgrounds do not allow us to propose the use of heat-treated *hsdR* mutants as standard tools for transformation experiments with *S.*

aureus, particularly when transformation of nonreplicative plasmids is required as a step in procedures for genetically manipulating *S. aureus.*

Conclusion

It seems to be unambiguous that a mutation in SauI *hsdR* is required for the transformable phenotype of RN4220; this strain does not have mutations in any of the other *hsd* genes or their promoters, and complementation with a plasmid containing the intact SauI *hsdR* gene completely blocks uptake of *E. coli* DNA by RN4220 (Waldron & Lindsay, 2006). However our data indicate that deleting or truncating SauI *hsdR* is not enough to allow the *S. aureus* strains tested to easily accept foreign DNA either via electroporation or via phage infection or transduction, and therefore the ability of RN4220 to accept foreign DNA must be dependent on an additional unknown factor(s), possibly a second R-M system. Interestingly, NCTC8325 (the parental strain of the prophage-cured strain NCTC8325-4) has been suggested to have two distinct R-M systems based on analysis of restriction- and modification deficient mutants (Iordanescu & Surdeanu, 1976, Sjostrom *et al.*, 1978), but the second R-M system could be associated with phages present in this strain and is not annotated in the genome.

Constructing *S. aureus* mutants of relevant laboratory and clinical strains that are capable of efficiently accepting foreign DNA by inactivating the SauI type I R-M system does not seem to be a viable option. Full sequencing of the RN4220 genome should indicate which additional, unidentified factors contribute to its transformable phenotype. The information obtained should contribute to development of better genetic tools for manipulating *S. aureus*, as well as to a better understanding of gene transfer in this pathogenic organism.

References

Arnaud, M., A. Chastanet & M. Debarbouille, (2004) New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, Gram-positive bacteria. *Appl Environ Microbiol* **70**: 6887-6891.

Asheshov, E. A. & M. P. Jevons, (1963) The effect of heat on the ability of a host strain to support the growth of a *Staphylococcus* phage. *J Gen Microbiol* **31**: 97-107.

Bickle, T. A. & D. H. Kruger, (1993) Biology of DNA restriction. *Microbiol Rev* **57**: 434-450.

Chung, M., H. de Lencastre, P. Matthews, A. Tomasz, I. Adamsson, M. Aires de Sousa, T. Camou, C. Cocuzza, A. Corso, I. Couto, A. Dominguez, M. Gniadkowski, R. Goering, A. Gomes, K. Kikuchi, A. Marchese, R. Mato, O. Melter, D. Oliveira, R. Palacio, R. Sa-Leao, I. Santos Sanches, J. H. Song, P. T. Tassios & P. Villari, (2000) Molecular typing of methicillin-resistant *Staphylococcus aureus* by pulsed-field gel electrophoresis: comparison of results obtained in a multilaboratory effort using identical protocols and MRSA strains. *Microb Drug Resist* **6**: 189-198.

Dempsey, R. M., D. Carroll, H. Kong, L. Higgins, C. T. Keane & D. C. Coleman, (2005) Sau42I, a BcgI-like restriction-modification system encoded by the *Staphylococcus aureus* quadruple-converting phage Phi42. *Microbiology* **151**: 1301-1311.

Dyke, K. G., M. P. Jevons & M. T. Parker, (1966) Penicillinase production and intrinsic resistance to penicillins in *Staphylococcus aureus*. *Lancet* **1**: 835-838.

Gill, S. R., D. E. Fouts, G. L. Archer, E. F. Mongodin, R. T. Deboy, J. Ravel, I. T. Paulsen, J. F. Kolonay, L. Brinkac, M. Beanan, R. J. Dodson, S. C. Daugherty, R. Madupu, S. V. Angiuoli, A. S. Durkin, D. H. Haft, J. Vamathevan, H. Khouri, T. Utterback, C. Lee, G. Dimitrov, L. Jiang, H. Qin, J. Weidman, K. Tran, K. Kang, I. R. Hance, K. E. Nelson & C. M. Fraser, (2005) Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *J Bacteriol* **187**: 2426-2438.

Horsburgh, M. J., J. L. Aish, I. J. White, L. Shaw, J. K. Lithgow & S. J. Foster, (2002) sigmaB modulates virulence determinant expression and stress resistance: characterization of a functional *rsbU* strain derived from *Staphylococcus aureus* 8325-4. *J Bacteriol* **184**: 5457-5467.

Iordanescu, S. & M. Surdeanu, (1976) Two restriction and modification systems in *Staphylococcus aureus* NCTC8325. *J Gen Microbiol* **96**: 277-281.

Kreiswirth, B. N., S. Lofdahl, M. J. Betley, M. O'Reilly, P. M. Schlievert, M. S. Bergdoll & R. P. Novick, (1983) The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature* **305**: 709-712.

McNamara, P. J., (2008) Genetic Manipulation of *Staphylococcus aureus*. In: *Staphylococcus* molecular genetics. J. A. Lindsay (ed). Norfolk, UK: Caister Academic Press, pp. 89-129.

Murray, N. E., (2000) Type I restriction systems: sophisticated molecular machines (a legacy of Bertani and Weigle). *Microbiol Mol Biol Rev* **64**: 412-434.

Noble, W. C., Z. Virani & R. G. Cree, (1992) Co-transfer of vancomycin and other resistance genes from *Enterococcus faecalis* NCTC 12201 to *Staphylococcus aureus*. *FEMS Microbiol Lett* **72**: 195-198.

Novick, R., (1967) Properties of a cryptic high-frequency transducing phage in *Staphylococcus aureus*. *Virology* **33**: 155-166.

Oshida, T. & A. Tomasz, (1992) Isolation and characterization of a Tn*551*-autolysis mutant of *Staphylococcus aureus*. *J Bacteriol* **174**: 4952-4959.

Sjostrom, J. E., S. Lofdahl & L. Philipson, (1978) Biological characteristics of a type I restriction-modification system in *Staphylococcus aureus*. *J Bacteriol* **133**: 1144-1149.

Stobberingh, E. E. & K. C. Winkler, (1977) Restriction-deficient mutants of *Staphylococcus aureus*. *J Gen Microbiol* **99**: 359-367.

Sung, J. M. & J. A. Lindsay, (2007) *Staphylococcus aureus* strains that are hypersusceptible to resistance gene transfer from *Enterococci*. *Antimicrob Agents Chemother* **51**: 2189-2191.

Sussenbach, J. S., C. H. Monfoort, R. Schiphof & E. E. Stobberingh, (1976) A restriction endonuclease from *Staphylococcus aureus*. *Nucleic Acids Res* **3**: 3193-3202.

Sussenbach, J. S., P. H. Steenbergh, J. A. Rost, W. J. van Leeuwen & J. D. van Embden, (1978) A second site-specific restriction endonuclease from *Staphylococcus aureus*. *Nucleic Acids Res* **5**: 1153-1163.

Waldron, D. E. & J. A. Lindsay, (2006) Sau1: a novel lineage-specific type I restriction-modification system that blocks horizontal gene transfer into *Staphylococcus aureus* and between *S. aureus* isolates of different lineages. *J Bacteriol* **188**: 5578-5585.

Wu, S., H. de Lencastre, A. Sali & A. Tomasz, (1996) A phosphoglucomutase-like gene essential for the optimal expression of methicillin resistance in *Staphylococcus aureus*: molecular cloning and DNA sequencing. *Microb Drug Resist* **2**: 277-286.

Chapter 3

Fluorescent reporters for studies of cellular localization of proteins in *Staphylococcus aureus*

AUTHOR CONTRIBUTIONS

H. Veiga performed all experiments related with pBCB13 plasmid. P. M. Pereira performed all experiments related with pBCB1-8 plasmids. A. M. Jorge constructed plasmid pEzrA-CFP and strain RNpEzrA-CFP.

Dr. S. Foster, Dr. D. Sherratt and Dr. J. Errington kindly provided plasmids pDG792, pROD17 and pHM232, respectively. The authors thank Dr. W. Schumann for maiking the pMUTIN series available at the Bacillus Genetic Stock Center.

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Abstract

We have constructed a set of plasmids that allow expression, from their native chromosomal loci, of *Staphylococcus aureus* proteins fused to one of four different fluorescent proteins (green fluorescent protein [GFP], cyan fluorescent protein [CFP], yellow fluorescent protein [YFP], and mCherry), using two different resistance markers (kanamycin and erythromycin). We have also constructed a plasmid that allows expression of proteins from the ectopic *spa* locus in the *S. aureus* chromosome. This toolbox can be used for studies of the localization of proteins in *S. aureus*, a prominent pathogen in both health care and community settings.

Introduction

Studies of the subcellular localization of proteins, which initially focused on the model organisms *Escherichia coli* and *Bacillus subtilis*, revolutionized the way we think about the bacterial cell by showing that many essential cellular processes, such as cell division or DNA replication, are precisely regulated not only in time but also in space. Knowledge regarding specific and dynamic localization of proteins in live bacterial cells was obtained mainly by looking at cells expressing fusions of proteins of interest to different fluorescent proteins, such as green fluorescent protein (GFP) (Tsien, 1998). There is an increasing interest in expanding cell biology studies to other bacterial species, with different morphologies, different developmental processes, or more clinical relevance, but this interest in many cases is impaired by the lack of availability of appropriate tools.

Staphylococcus aureus is a Gram-positive pathogen capable of causing diseases ranging from minor infections to lifethreatening ones with high morbidity and mortality rates (Tenover & Gorwitz, 2006). Besides its virulence, *S. aureus* is well known due to its increasing resistance to virtually all classes of antibiotics (Projan, 2000). Methicillin-resistant *S. aureus* (MRSA) strains, the most important cause of antibiotic-resistant nosocomial infections worldwide, have recently emerged in the community as well, emphasizing the growing importance of this pathogenic bacterium (Chambers, 2001). Besides its interest as a clinical pathogen, *S. aureus* is also a very interesting model for the study of cell division because it has a different (round) shape and mode of division (in three consecutive perpendicular division planes over three division cycles (Tzagoloff & Novick, 1977)) from those of the model organisms *E. coli* and *B. subtilis* (Zapun *et al.*, 2008).The availability of vectors which allow expression of staphylococcal proteins fused to fluorescent reporters would therefore be of great interest for *S. aureus* studies in different areas.

Over the last decade, there have been some reports of the use of GFP in *S. aureus*, either to fluorescently label whole cells, mainly for *in vivo* studies, or as a reporter

protein for transcriptional fusions (Bateman *et al.*, 2001, Cheung *et al.*, 1998, Schneider *et al.*, 2002). A recent report thoroughly describes a set of *S. aureus* vectors that allow cell labeling with different fluorescent proteins (Malone *et al.*, 2009). However, all of these vectors were designed to allow for the expression of fluorescent proteins which were not fused to staphylococcal proteins in the cytoplasm of *S. aureus* cells. In this report, we describe the construction of a toolkit of eight plasmids, containing either an erythromycin or a kanamycin resistance marker, which allow the expression, from their native chromosomal loci, of *S. aureus* proteins fused to GFP, yellow fluorescent protein (YFP), cyan fluorescent protein (CFP), or mCherry. Fusions of fluorescent proteins can be expressed either from replicative plasmids or from the chromosome. We chose to construct integrative plasmids that allow expression of fusion proteins from their native loci in the chromosome, under the control of their native promoters, so that the biology of the cell is altered as little as possible during localization studies. We have constructed an additional plasmid which enables the expression of proteins from the ectopic *spa* locus in the *S. aureus* chromosome, which can be used when introduction of a construct in the native chromosomal locus of a specific gene causes polar effects on downstream genes of the same operon.

Experimental procedures

Bacterial strains and growth conditions

All strains used and constructed during this study are listed in Table 3.1. *E. coli* strain DH5α was grown on Luria-Bertani agar or broth (Difco), supplemented with ampicillin (100µg/ml) as required. *S. aureus* strains were grown at 37°C on tryptic soy agar or tryptic soy broth (TSA; TSB; Difco) with aeration. The medium was supplemented, when necessary, with erythromycin (10µg/ml; Sigma), kanamycin and neomycin (50 µg/ml each; Sigma), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 100µg/ml), or isopropyl-β-D-thiogalactopyranoside (IPTG; various concentrations [indicated below]).

Table 3.1. Plasmids and strains used in this study

Plasmids and strains	Relevant characteristics	Source or reference
Plasmids		
pMutinCFP	*B. subtilis* integrative vector for C-terminal CFP fusions, Amp^r, Ery^r	(Kaltwasser *et al.*, 2002)
pMutinGFP+	*B. subtilis* integrative vector for C-terminal GFP fusions, Amp^r, Ery^r	(Kaltwasser *et al.*, 2002)
pMutinYFP	*B. subtilis* integrative vector for C-terminal YFP fusions, Amp^r, Ery^r	(Kaltwasser *et al.*, 2002)
pROD17	Plasmid containing the *mCherry* gene	D. Sherratt
pDG792	Plasmid containing a kanamycin resistance gene	(Guerout-Fleury *et al.*, 1995)
pBCB1-GE	*S. aureus* integrative vector for N- and C-terminal GFP fusions, Amp^r, Ery^r	This study
pBCB2-YE	*S. aureus* integrative vector for N- and C-terminal YFP fusions, Amp^r, Ery^r	This study
pBCB3-CE	*S. aureus* integrative vector for N- and C-terminal CFP fusions, Amp^r, Ery^r	This study
pBCB4-ChE	*S. aureus* integrative vector for N- and C-terminal mCherry fusions, Amp^r, Ery^r	This study
pBCB5-YK	*S. aureus* integrative vector for N- and C-termini YFP fusions, Amp^r, Kan^r	This study
pBCB6-CK	*S. aureus* integrative vector for N- and C-terminal CFP fusions, Amp^r, Kan^r	This study
pBCB7-ChK	*S. aureus* integrative vector for N- and C-terminal mCherry fusions, Amp^r, Kan^r	This study
pBCB8-GK	*S. aureus* integrative vector for N- and C-terminal GFP fusions, Amp^r, Kan^r	This study
pMAD	*E. coli – S. aureus* shuttle vector with a thermosensitive *oriC* for Gram-positive bacteria; Amp^r, Ery^r; *lacZ*	(Arnaud *et al.*, 2004)
pDH88	Plasmid containing the P_{spac} promoter and the *lacI* gene	(Henner, 1990)
pMAD-spa	pMAD derivative with the up- and downstream regions of *spa*	This study
pBCB13	pMAD-spa derivative with P_{spac}-*lacI* region from pDH88; Amp^r, Ery^r ; *lacZ*	This study
pGEreporter	pBCB1-GE with an intergenic region between *pbpB* and *SACOL1491* cloned downstream of the *gfp* gene, Amp^r, Ery^r	This study
pPBP4t-YFP	pBCB5-YK encoding a YFP fusion to the C-termini of truncated PBP4, Amp^r, Kan^r	This study

Plasmids and strains	Relevant characteristics	Source or reference
pPBP4-mCh	pBCB7-ChK encoding a mCherry fusion to the C-terminal of PBP4, Amp^r, Kan^r	This study
pEzrA-CFP	pBCB3-CE encoding a CFP fusion to the C-terminal of EzrA, Amp^r, Ery^r	This study
pHM232	Plasmid containing the *tetR-mCherry* fusion	(Murray & Errington, 2008)
pBCB13-TetRmCh	pBCB13 derivative with *tetR-mCherry;* Amp^r, Ery^r ; *lacZ*	This study
E. coli		
DH5α	*recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1* φ80 Δ*lacZ*ΔM15	Gibco-BRL
S. aureus		
RN4220	Restriction deficient derivative of NCTC8325-4	R. Novick
NCTC8325-4	MSSA strain	R. Novick
COL	MRSA strain	(Gill *et al.*, 2005)
RNpGEreporter	RN4220 with integrated pGEreporter plasmid expressing GFP in the cytoplasm, under the control of P_{spac}, Ery^r	This study
RNpPBP4t-YFP	RN4220 with integrated pBCB4-YK*pbpDt* plasmid encoding C-terminal PBP4-YFP fusion, Kan^r	This study
RNpPBP4-mCh	RN4220 with integrated pBCB7-ChKPBP4 plasmid encoding C-terminal PBP4-mCherry fusion, Kan^r	This study
RNpEzrA-CFP	RN4220 with integrated pEzrA-CFP plasmid encoding C-terminal EzrA-CFP fusion, Ery^r	This study
RNpPBP4t-YFP pEzrA-CFP	RNpPBP4t-YFP with integrated pEzrA-CFP plasmid encoding C-terminal EzrA-CFP fusion, Ery^r, Kan^r	This study
RNpBCB13-TetRmCh	RN4220 with integrated pBCB13-TetRmCh; Ery^r *lacZ*	This study
NCTCΔspaTetRmCh	NCTC8325-4 derivative with *spa* gene replaced by P_{spac}-*tetRmCherry-lacI*	This study

General procedures

S. aureus RN4220 cells were transformed by electroporation as previously described (Veiga & Pinho, 2009). Constructs were transduced from RN4220 strains into *S. aureus* NCTC8325-4 by using the phage 80α, as previously described (Oshida & Tomasz, 1992). Restriction enzymes were purchased from New England Biolabs, PCR was performed using Phusion high-fidelity DNA polymerase (Finnzymes), and sequencing reactions were carried out at Macrogen. Sequences of primers used are listed in Table 3.2.

Table 3.2. Primers used in this study

Primer Name	Primer Sequence (5'-3')*
BCBP1	ctcgaggcggccgcgccggctgagcactagtgcagcccgcc
BCBP2	tggttgagcatgcatcaggcctcttgtacagctcgtccatgccg
BCBP3	gactacgccatgggttcatgtaatcactccttc
BCBP4	gcgagatctggaaataattctatgagtcgc
BCBP5	cctaacagcacaagagcgg
BCBP6	gcatcaggccttttgtagagctcatccatg
BCBP7	gcatcaggcctcttgtacagccgtccatgcc
BCBP8	ctcgaggcggccgcgccggctgagcactagtgcagcccgcc
BCBP9	cgatggtaccggcgcgccgctagcatggctatcattaaagagttc
BCBP10	tgaggatcctggttcagttgtaaataacaatac
BCBP11	gtattaatgcagcgctagcgacgagaattcacgttcaaacaaacaatacacaacg
BCBP12	gtttgaacgtgaattctcgtcgctagcgctgcattaataccccctgtatg
BCBP13	tgcagtccatgggttagagctctcaataatttaaaaaagc
BCBP14	gctgaattcttctacacagcccagtccagac
BCBP15	caaatcgcctagctgcggccgcctcggagcgactcgagctaggctctagaaacccgggaaaagc
BCBP16	ctagagcctagctcgagtcgctccgaggcggccgcagctaggcgatttgttaacttagatctttatc
BCBP17	gcatggctagcgacctaactcacattaattgcg
BCBP18	gcgcccgaggccttaatccactcaacataaaatc
BCBP19	cgtgacactagtacaagattgatcatttcc
BCBP20	cgcggtaccgttacgtattacacattcaac
BCBP21	gctgcggtaccggaggcgccgcaggatttttctttttctaaataaacg
BCBP22	cgcggtaccggaaaagggaagattaacgc
BCBP23	cgccggtaccgggaggcgccgcaggattttctttttctaaataaacg
BCBP24	cgggtacccgatatagcgaggttcagg
BCBP25	ggcggtaccttgcttaataacttcttcttc
BCBP26	tactccccgggcttacaatatgacataaaatgc
BCBP27	tactccccgggcttacttgtacagctcgtccatg
BCBP28	tgaggatccccaggcttgttgttgtctt
BCBP29	tgcagtccatggttgaaaaagaaaaacatttattc

* Underlined sequences correspond to restriction sites

Construction of pBCB1-8 plasmids

A set of eight integrative plasmids for N and C-terminal fusions of fluorescent proteins to staphylococcal proteins was constructed using a series of pMUTIN plasmids as backbones (Kaltwasser *et al.*, 2002).

We first amplified plasmid pMutinCFP by using primers BCBP1 and BCBP2 in order to remove the *cfp* stop codon and to introduce a multiple cloning site (MCS), containing StuI, NsiI, SphI, XhoI, NotI, NaeI, and SpeI restriction sites, at the 3' end of the *cfp* gene. The SpeI restriction site carries a stop codon in frame with the *cfp* gene. The resulting PCR fragment was autoligated and used to transform DH5α. The P_{spac} promoter region, the *cfp* gene, and the new MCS were sequenced, and the resulting plasmid was named pBCB3-CE.

To allow for the use of more than one resistance marker, the erythromycin resistance cassette of pBCB3-CE was replaced by a kanamycin resistance cassette obtained from the pDG792 plasmid (Guerout-Fleury *et al.*, 1995). For that purpose, we used primers BCBP3 and BCBP4 to amplify the entire pBCB3-CE plasmid by PCR, excluding the erythromycin marker, and the resulting product was digested with NcoI and BglII. Plasmid pDG792 was also digested with NcoI and BglII, and a 1.5kb band containing the kanamycin resistance marker was isolated, ligated with the previous PCR product, and used to transform DH5α, resulting in plasmid pBCB6-CK.

Plasmids pBCB3-CE and pBCB6-CK were digested with KpnI and StuI to remove the *cfp* gene, which was replaced by the *gfp*, *yfp*, or *mCherry* gene without a stop codon, obtained from pMUTINGFP+ (Kaltwasser *et al.*, 2002) (using primers BCBP5 and BCBP6), pMUTINYFP (Kaltwasser *et al.*, 2002) (using primers BCBP5 and BCBP7), or pROD17 (a gift of D. Sherratt) (using primers BCBP8 and BCBP9), respectively, by PCR amplification. The resulting plasmids were named pBCB1-GE, pBCB8-GK, pBCB2-YE, pBCB5-YK, pBCB4-ChE, and pBCB7-ChK, for *gfp* (G), *yfp* (Y), or *mCherry* (Ch) insertion and for erythromycin (E) or kanamycin (K) resistance (Figure 3. 1). The primer BCBP9, which was used to amplify the *mCherry* gene, contained an extra NheI site that was added to the MCS upstream of the

gene. The integrity of the P_{spac} promoter region, the gene encoding the fluorescent protein, and the MCS was confirmed by sequencing each plasmid.

Construction of pBCB13 plasmid

The pBCB13 plasmid was constructed by cloning a fragment containing the P_{spac} promoter, an MCS, and the *lacI* gene between the downstream and upstream regions of the *spa* gene into the thermosensitive plasmid pMAD (Arnaud *et al.*, 2004). For that purpose, the downstream and upstream regions of *spa*, each of approximately 0.6kb, were amplified from NCTC8325-4 genomic DNA by using the primer pairs BCBP10/BCBP11 and BCBP12/BCBP13, respectively. The two PCR products were joined in a second PCR, using primers BCBP10 and BCBP13, which also introduced two restriction sites (EcoRI and NheI) between the downstream and upstream regions. The resulting fragment was cloned into the pMAD vector by using BamHI and NcoI restriction enzymes, giving plasmid pMAD-spa, which was subjected to sequencing to confirm its integrity. Subsequently, a 1.7kb DNA fragment containing the P_{spac} promoter followed by the MCS (with a new XhoI restriction site) and the *lacI* gene under the control of the constitutive P_{pcn} promoter was obtained from the pDH88 plasmid (Henner, 1990) by overlap PCR, firstly using primer pairs BCBP14/BCBP15 and BCBP16/BCBP17 and secondly using primer pair BCBP14/BCBP17. The final fragment was cloned into pMAD-spa by using the EcoRI and NheI restriction enzymes, giving rise to the vector pBCB13 (Figure 3.2A). The correct sequence of the cloned fragment was confirmed. It should be noted that the upstream and downstream regions of *spa* may vary among different strains. For example, the upstream region of *spa* in strain COL has a 63bp insertion compared to that of the gene in NCTC8325-4.

Construction of RNpGEreporter

A fragment of 0.6 kb containing an intergenic region of the *S. aureus* chromosome between the *pbpB* and *SACOL1491* genes was amplified from *S. aureus* strain NCTC8325-4 by a PCR using primers BCBP18 and BCBP19. The PCR product was

cloned into the pBCB1-GE plasmid by using the StuI and SpeI restriction enzymes. The resulting plasmid, which is nonreplicative in *S. aureus*, was named pGEreporter and was electroporated into *S. aureus* strain RN4220, where it integrated into the chromosome, generating strain RNpGEreporter, which expressed GFP not fused to any staphylococcal protein from the chromosome, under the control of the P_{spac} promoter.

Construction of RNpPBP4t-YFP

A 3' 0.7kb fragment of the *pbpD* gene, which encodes penicillin-binding protein 4 (PBP4), was amplified from *S. aureus* strain COL by a PCR using primers BCBP20 and BCBP21 and then cloned into KpnI-digested pBCB5-YK, upstream of and in frame with the *yfp* gene. After confirmation of the correct orientation of the insert, the resulting plasmid, named pPBP4t-YFP, was sequenced and electroporated into *S. aureus* strain RN4220, where it integrated into the *pbpD* locus of the chromosome. The resulting strain, RNpPBP4t-YFP, expressed a full copy of *pbpD* fused to *yfp* under the control of the *pbpD* native promoter, while a truncated copy of the *pbpD* gene was placed under the control of the P_{spac} promoter.

Construction of RNpPBP4-mCh

A fragment of 1.3 kb containing a full copy of the *pbpD* gene was amplified from *S. aureus* strain COL by a PCR using primers BCBP22 and BCBP23 and then cloned into KpnI-digested pBCB7-CHK, upstream of and in frame with the *mCherry* gene. The resulting plasmid, with a correctly oriented insert, was named pPBP4-mCh, sequenced, and electroporated into RN4220, where it integrated into the *pbpD* locus of the chromosome. The resulting strain, RNpPBP4-mCh, expressed the *pbpD-mCherry* fusion under the control of the native *pbpD* promoter, while the native *pbpD* gene was placed under the control of the P_{spac} promoter.

Construction of RNpEzrA-CFP

A 3' 0.56kb fragment of the *ezrA* gene was amplified from *S. aureus* strain COL by a PCR using primers BCBP24 and BCBP25 and then cloned into KpnI-digested pBCB3-CE, upstream of and in frame with the *cfp* gene. After confirmation of the correct orientation of the insert, the resulting plasmid, named pEzrA-CFP, was sequenced and electroporated into RN4220, where it integrated into the *ezrA* locus of the chromosome. The resulting strain, RNpEzrA-CFP, expressed the *ezrA-cfp* fusion under the control of the native *ezrA* promoter, while a truncated copy of the *ezrA* gene was placed under the control of the P_{spac} promoter.

Construction of RNpPBP4t-YFP pEzrA-CFP

The genomic region containing *ezrA-cfp* was transduced from *S. aureus* RNpEzrA-CFP into *S. aureus* RNpPBP4t-YFP by using phage 80α. Colonies were selected using erythromycin, kanamycin, and neomycin. The resulting strain, RNpPBP4t-YFP pEzrA-CFP, simultaneously expressed EzrA-CFP and PBP4-YFP, both from their native loci and under the control of their native promoters.

Construction of NCTCΔspaTetRmCh

A 1.395kb DNA fragment containing *tetR-mCherry* was amplified from plasmid pHM232 (Murray & Errington, 2008) by using primers BCBP26 and BCBP27, digested with XmaI, and cloned into pBCB13. After the correct orientation of the insert was confirmed, the resulting vector, pBCB13-TetRmCh, was sequenced and introduced into RN4220 by electroporation. The plasmid was then transferred to *S. aureus* NCTC8325-4 by transduction (selection at 30°C with 10μg/ml of erythromycin). The exchange of the *spa* gene for the *tetR-mCherry* construct was obtained after a two-step homologous recombination process. In the first step, recombinants in which the pBCB13-TetRmCh plasmid had integrated into the chromosome were selected at the nonpermissive temperature of 43°C, using erythromycin (10μg/ml). In the second step, cells were incubated at the permissive temperature of 30°C in the absence of antibiotic selection,

and white, erythromycin-sensitive colonies in which the vector (and consequently the *lacZ* and *erm* genes) had been excised were selected for further screening (Figure 3.2B). Screening for replacement of the *spa* gene by the *tetRmCherry* construct was performed by PCR, using primer pairs BCBP28/BCBP29 and BCBP10/BCBP27.

Fluorescence microscopy

S. aureus strains were grown overnight in TSB at 37°C with appropriate antibiotic selection. Cells were diluted 1:200 in fresh TSB (without antibiotic). For analysis of the RNpGEreporter strain, increasing concentrations of IPTG (ranging from 0 mM to 1 mM) were added to the culture. NCTCΔspaTetRmCh cultures were supplemented with 0.5 mM IPTG. After reaching an optical density at 600 nm (OD600) of 0.8, 1 ml of culture was pelleted and resuspended in 20 µl of phosphate-buffered saline (PBS). Cell suspension (1 µl) was placed on a thin layer of 1% agarose in PBS mounted on a microscope slide. Phase-contrast and fluorescence visualization of the live cells was performed using a Zeiss Axio Observer.Z1 microscope equipped with a Plan-Apochromat objective (100x/1.4 Oil Ph3; Zeiss). Semrock GFP, CFP, YFP and Texas Red filters were used. Image acquisition was done using a Photometrics CoolSNAP HQ2 camera (Roper Scientific) attached to the microscope and Metamorph v. 7.5 software (Molecular Devices). ImageJ software (Abramoff *et al.*, 2004) was used for the quantification of total fluorescence per cell in strain RNpGEreporter grown in the presence of different concentrations of IPTG. For this purpose, the mean fluorescence per pixel of each cell was measured. This value was corrected by subtracting the mean fluorescence of the background per pixel. The average for at least 400 cells was calculated for cells grown at each IPTG concentration used.

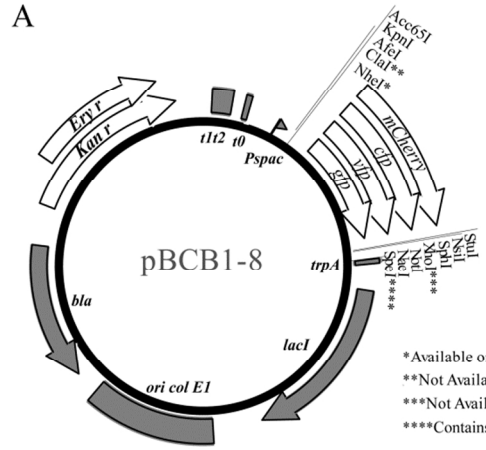
Results and discussion

Construction of integration vectors for chromosomal expression of fluorescent derivatives of staphylococcal proteins

A set of eight integrative plasmids for expression of staphylococcal proteins from their native chromosomal loci was constructed, using a series of pMUTIN plasmids constructed by M. Kaltwasser and colleagues as backbones (Kaltwasser *et al.*, 2002). We first introduced a multiple cloning site (with StuI, NsiI, SphI, XhoI, NotI, NaeI, and SpeI restriction sites) downstream of the *cfp* gene in plasmid pMUTIN-CFP, generating plasmid pBCB3-CE (Table 3.1 and Figure 3.1). Introduction of this multiple cloning site resulted in the deletion of the stop codon from the *cfp* gene and the introduction of a new, in-frame stop codon, included in the SpeI restriction site. The pMUTIN-CFP plasmid already had a multiple cloning site (with Acc65I, KpnI, AfeI, and ClaI restriction sites) upstream of *cfp* to allow fusion of the fluorescent protein to the C terminus of a protein of interest. The additional multiple cloning site which we introduced in pBCB3-CE makes it possible to fuse the fluorescent protein to the N terminus of a protein of interest. We then replaced the erythromycin resistance marker present in pBCB3-CE with a kanamycin resistance marker, which was excised from plasmid pDG792 (Guerout-Fleury *et al.*, 1995), generating plasmid pBCB6-CK (Table 3.1 and Figure 3.1). The availability of two different resistance markers allows for the possibility of coexpressing fluorescent derivatives of two different proteins in the same staphylococcal cell for colocalization studies. The *cfp* gene in plasmids pBCB3-CE and pBCB6-CK was subsequently replaced with *gfp* (from pMUTIN-GFP+(Kaltwasser *et al.*, 2002)), *yfp* (from pMUTIN-YFP (Kaltwasser *et al.*, 2002)), and *mCherry* (from pROD17 [a gift of D. Sherratt]), generating the set of plasmids described in Table 3.1 and Figure 3.1. An additional NheI restriction site was introduced into the multiple cloning site upstream of *mCherry* during the cloning process.

Genes encoding proteins of interest can be cloned downstream or upstream of the genes encoding the fluorescent proteins on plasmids pBCB1 to -8. When the resulting plasmids are introduced into *S. aureus* and integrated into the chromosome, fusions to

the N termini of proteins of interest are placed under the control of the P_{spac} promoter, while fusions to the C termini of proteins of interest remain under the control of their native promoters. In the latter case, if the gene of interest is part of an operon, the downstream genes will be placed under the control of the IPTG-inducible P_{spac} promoter.



B

	GFP	YFP	CFP	mCherry
Ery^r	pBCB1-GE	pBCB2-YE	pBCB3-CE	pBCB4-ChE
Kan^r	pBCB8-GK	pBCB5-YK	pBCB6-CK	pBCB7-ChK

Figure 3.1. Map and nomenclature of pBCB series of plasmids. (A) Map of pBCB1 to -8 plasmids. Genes that vary among plasmids, namely, those encoding the fluorescent proteins GFP, YFP, CFP, and mCherry and erythromycin and kanamycin resistance markers, are indicated by white arrows. Unique restriction sites in the multiple cloning sites are shown; asterisks indicate restriction sites which are not available/unique in all plasmids. Features common to all plasmids are indicated by gray arrows and boxes and include the inducible P_{spac} promoter (P_{spac}), the LacI repressor gene (*lacI*), the origin of replication in *E. coli* (*ori*ColE1), an ampicillin resistance gene (*bla*), and terminators (*t1t2*, *t0*, and *trpA*). (B) Table indicating the fluorescent protein and resistance marker encoded by each pBCB plasmid (pBCB1 to pBCB8).

Interruption of an operon by integration of a plasmid can often disrupt normal expression of different proteins encoded by that operon. For that reason, it may be useful to express a fluorescent derivative of a protein of interest from an ectopic locus in the chromosome. We constructed plasmid pBCB13 to allow insertion of DNA fragments into the *spa* locus of the *S. aureus* chromosome. The *spa* gene encodes the nonessential, cell-wall-attached protein A (Navarre & Schneewind, 1999). Although protein A has a role in *S. aureus* virulence (Foster, 2005), *in vitro* studies are often done using *spa* mutants, as

protein A is an IgG binding protein (Navarre & Schneewind, 1999) and thus can interfere in assays that require the use of antibodies.

Plasmid pBCB13 was constructed based on the backbone of the pMAD plasmid (Arnaud *et al.*, 2004) by cloning the P_{spac} promoter, followed by an MCS and the *lacI* gene, between the down- and upstream regions of the *spa* gene (Table 3.1 and Figure 3.2A).

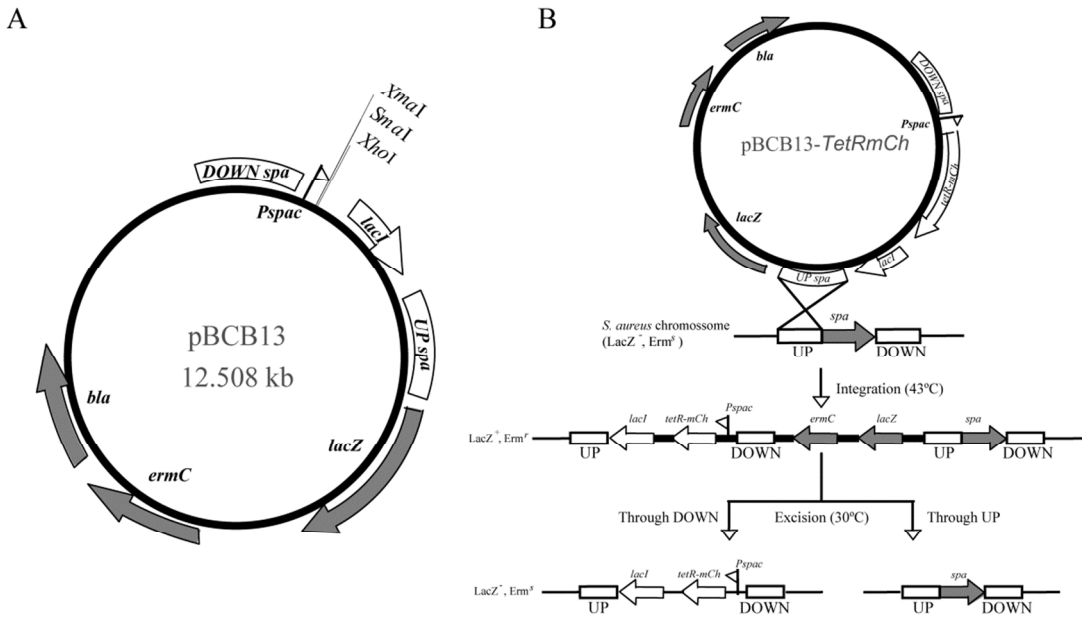


Figure 3.2. Map and mode of use of pBCB13. (A) Map of the pBCB13 plasmid showing the upstream (*UPspa*) and downstream (*DOWNspa*) regions of the *spa* gene locus; the P*spac* inducible promoter; the *lacI* gene under the control of a constitutive promoter; the *lacZ* gene, encoding a thermostable β-galactosidase; the erythromycin resistance marker (*ermC*); and an ampicillin resistance gene expressed in *E. coli* (*bla*). (B) Schematic representation of a two-step procedure for gene replacement using pBCB13. Integration of pBCB13 via homologous recombination can take place via the upstream (example shown in the figure) or downstream region. After a second recombination event, the *spa* gene will either remain in the chromosome (if the second recombination occurs through the upstream region, as in the given example) or be excised along with the plasmid (if the second recombination occurs through the downstream region) and replaced, in the example shown, by the *tetR-mCherry* construct.

DNA fragments encoding fusions of fluorescent proteins with a protein of interest can be subcloned from the pBCB1 to -8 plasmids into pBCB13, downstream of the P_{spac} promoter. The chromosomal *spa* gene can then be exchanged, by homologous recombination, for the fluorescent fusion present in the plasmid (Figure 3.2B). Plasmid pBCB13 can also be used to place genes encoding wild-type (nonfluorescent) proteins in the *spa* locus, namely, for complementation of mutants which require a low copy number of the gene and therefore cannot be complemented successfully from replicative plasmid vectors.

Expression of fluorescent proteins by use of the pBCB series of plasmids

We tested the constructed pBCB plasmids by expressing the four fluorescent proteins, GFP, YFP, CFP, and mCherry, using either the erythromycin or the kanamycin resistance marker. We chose to express two of the fluorescent proteins in the cytoplasm of *S. aureus*, either alone (GFP) or fused to the heterologous protein TetR (mCherry). We also fused mCherry, YFP, and CFP to native staphylococcal proteins (PBP4 and EzrA) expected to have a specific cellular localization, namely, at the division septum. In order to express free GFP in the cytoplasm of *S. aureus* under the control of the IPTG-inducible promoter P_{spac}, we cloned the intergenic region downstream of *pbpB* into pBCB1-GE. The resulting plasmid, pGEreporter, was introduced into RN4220, where it recombined into the chromosome through the intergenic region, leading to the expression of GFP not fused to any protein, under the control of P_{spac}. Figure 3.3A shows that green fluorescence was present in the cytoplasm of the RNpGEreporter strain and was indeed dependent on the concentration of IPTG added to the growth medium (Figure 3.3B). This fluorescence varied between 20 fold (0.1 mM IPTG) and 40 fold (1 mM IPTG) higher than the autofluorescence of wild-type cells not expressing GFP (data not shown).

Plasmids pBCB3-CE, pBCB5-YK, and pBCB7-ChK were used to clone the genes encoding the *S. aureus* homologue of the *B. subtilis* EzrA protein (a protein involved in cell division (Levin *et al.*, 1999)), a truncated version of PBP4 (a cell wall synthetic enzyme (Wyke *et al.*, 1981)), and a full version of PBP4 (resulting in the expression of the

fluorescent derivative of PBP4 under the control of the native promoter and the wild-type copy of PBP4 under the control of P_{spac}, respectively. Figures 3.3C, D, and E show that the three vectors led to the expression of fluorescent derivatives of the staphylococcal proteins that localized at the division septum.

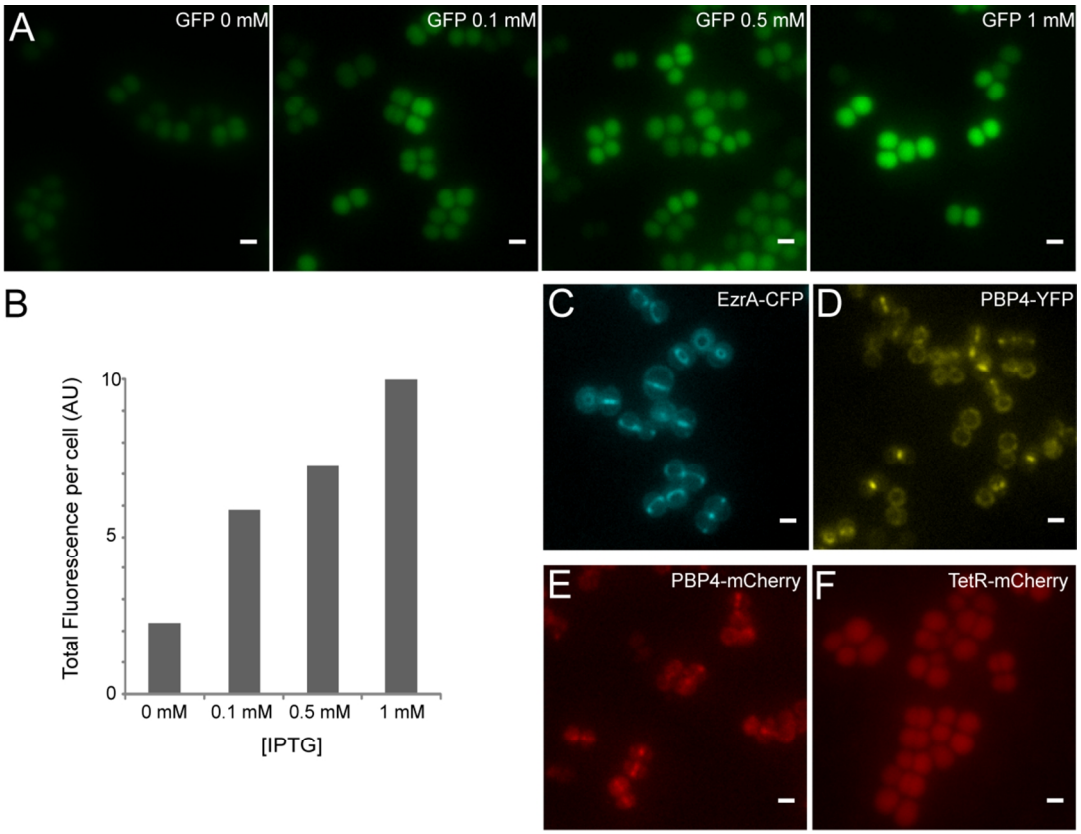


Figure 3.3. Expression of fluorescent proteins from the pBCB series of plasmids. (A) *S. aureus* RNpGEreporter strain (constructed using pBCB1-GE) expressing free GFP in the cytoplasm under the control of the IPTG-inducible P*spac* promoter, grown in the presence of increasing concentrations of IPTG (from 0 to 1 mM). (B) Quantification of GFP fluorescence in at least 400 cells for each IPTG concentration used showed that expression of *gfp* increases with increasing IPTG concentrations. (C) *S. aureus* RNpEzrA-CFP strain (constructed using pBCB3-CE) expressing EzrA-CFP, which localized to the division septum. (D) *S. aureus* RNpPBP4t-YFP strain (constructed using pBCB5-YK) expressing PBP4-YFP, which localized to the septum. (E) *S. aureus* RNpPBP4-mCh strain (constructed using pBCB7-ChK) expressing PBP4-mCherry, which localized to the septum. (F) *S. aureus* NCTCΔspaTetRmCh strain (constructed using pBCB13) expressing TetR-mCherry in the cytoplasm under the control of the IPTG-inducible P_{spac} promoter, grown in the presence of 0.5mM IPTG. All images are false-coloured. Bars, 1µm.

These results corresponded to the expected localization for both EzrA and PBP4, as the first interacts with the division ring in *B. subtilis* (Haeusser *et al.*, 2004) and the latter is involved in cell wall synthesis, a process that takes place at the septum in *S. aureus* (Pinho & Errington, 2003).

In order to test plasmid pBCB13, we cloned *tetR*, encoding the repressor of the tetracycline-inducible promoter, fused to *mCherry* downstream of the P_{spac} promoter, between the down- and upstream regions of the *spa* gene. The resulting plasmid, pBCB13-TetRmCh, was introduced into NCTC8325-4 and used to promote the allelic exchange of the *spa* gene for the *terR-mCherry* construct. The resulting strain, NCTCΔspaTetRmCh, does not have any resistance marker left in the chromosome, allowing the easy introduction of additional constructs encoding other mutations or fluorescent derivatives in other chromosomal loci. In the absence of operators of the tetracycline-inducible promoter, TetR is expected to localize in the cytoplasm, as observed (Figure 3.3F). The fluorescence obtained was 50 fold higher than the autofluorescence of RN4220 cells not expressing mCherry (data not shown).

Covisualization of different fluorescent proteins expressed in *S. aureus* cells

The availability of different fluorescent proteins that can be expressed in *S. aureus*, together with the possibility of inserting genes for protein fusions into the chromosome by use of either a kanamycin or erythromycin resistance marker (using pBCBs 1 to 8), or even leaving no residual antibiotic resistance marker in the chromosome (using pBCB13), allows the construction of *S. aureus* strains simultaneously expressing protein fusions to more than one fluorescent protein. In order to show that different fluorescent proteins could be covisualized in the same microscopy image, without overlap of the fluorescence emitted from one fluorescent protein with the channel used to detect a second fluorescent protein, we mixed cultures of *S. aureus* cells expressing TetR-mCherry, PBP4-YFP, and EzrA-CFP (Figure 3.4A) or TerRmCherry and GFP (Figure 3.4B). In both cases, the autofluorescence from *S. aureus* cells, as well as the overlap between different channels, was negligible, showing that mCherry, YFP, and CFP can be used in a

single experiment for colocalization studies, and the same applies to mCherry and GFP. GFP fluorescence overlaps with the channels used to detect CFP and YFP (data not shown) and therefore is not ideal for use in combination with these two proteins.

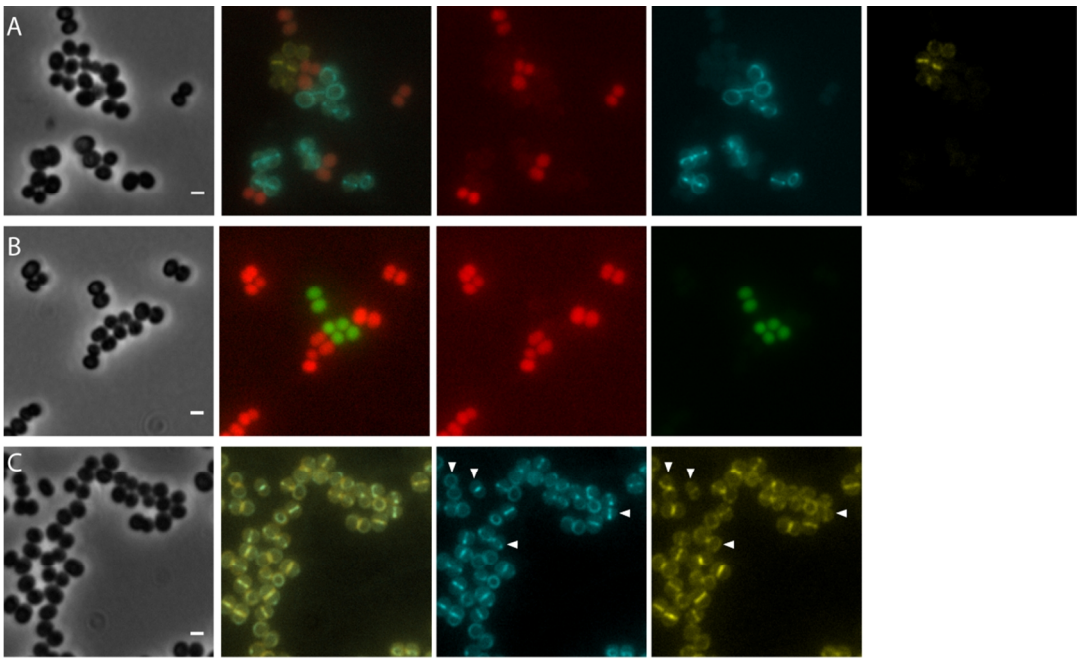


Figure 3.4. Covisualization of *S. aureus* cells labeled with different fluorescent reporters. Exponentially growing cultures of cells expressing different fluorescent proteins were mixed and placed on the same microscope slide. (A) (Left to right) Phase-contrast image of a mixed culture of *S. aureus* strains expressing TetR-mCherry (NCTCΔspaTetRmCh), EzrA-CFP (RNpEzrA-CFP), and PBP4-YFP (RNpPBP4t-YFP) fusion proteins; overlay of mCherry, CFP, and YFP fluorescence from the same strains; mCherry fluorescence from NCTCΔspaTetRmCh cells; CFP fluorescence from RNpEzrA-CFP cells; and YFP fluorescence from RNpPBP4t-YFP cells. (B) (Left to right) Phase-contrast image of a mixed culture of *S. aureus* strains expressing free GFP (RNpGEreporter) or TetR-mCherry (NCTCΔspaTetRmCh) in the cytoplasm; overlay of mCherry and GFP fluorescence from the same strains; mCherry fluorescence from NCTCΔspaTetRmCh cells; and GFP fluorescence from RNpGEreporter cells. (C) (Left to right) Phase-contrast image of *S. aureus* RNpPBP4t-YFP pEzrA-CFP, which expresses both PBP4t-YFP and EzrA-CFP fusion proteins; overlay of YFP and CFP fluorescence; YFP fluorescence from PBP4-YFP; and CFP fluorescence from EzrA-CFP. Arrowheads point to examples of cells in which PBP4 and EzrA do not colocalize. All images are false-coloured. Scale bars, 1 µm.

We also generated strain RNpPBP4t-YFPpEzrA-CFP, which simultaneously expresses both PBP4-YFP and EzrA-CFP fusions (Figure 3. 4C). This strain exemplifies the advantage of visualizing different proteins in the same cell. Although both EzrA-CFP and PBP4-YFP localized to the division septa, EzrA (an early cell division protein) was seen arriving at the septum before PBP4 (involved in the last stages of cell wall synthesis).

Final remarks

The fluorescent reporter systems described in this work allow studies of the cellular localization of *S. aureus* proteins fused to one of four different fluorescent proteins (GFP, CFP, YFP, or mCherry), using one of two different resistance markers (kanamycin or erythromycin), and expressed either from their native chromosomal locus or from the ectopic *spa* locus. We have shown that GFP and mCherry or CFP, YFP, and mCherry can be covisualized in a single image and that these fluorescent proteins can be used for studies of the colocalization of at least two proteins in a single staphylococcal cell.

It is worth mentioning that plasmid pBCB13 can be used not only for the ectopic expression of fluorescent derivatives of proteins of interest, as described above, but also to complement different mutants of *S. aureus*. Complementation of various mutants with the corresponding genes expressed from a replicative plasmid may not lead to full recovery of the affected phenotypes. The lack of success in complementation experiments using plasmid-borne genes may be due to the fact that the genes are not under the control of their native promoters or that the gene dosage is too high. Having a single copy of the complementing gene in the chromosome under the control of an inducible promoter should enable us to more accurately reproduce the wild-type gene dosage.

The toolbox described in this paper should facilitate future cell biology studies of the pathogenic bacterium *S. aureus.*

References

Abramoff, M. D., P. J. Magelhaes & S. J. Ram, (2004) Image Processing with ImageJ. *Biophoton Int* **11**: 36-42.

Arnaud, M., A. Chastanet & M. Debarbouille, (2004) New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, Gram-positive bacteria. *Appl Environ Microbiol* **70**: 6887-6891.

Bateman, B. T., N. P. Donegan, T. M. Jarry, M. Palma & A. L. Cheung, (2001) Evaluation of a tetracycline-inducible promoter in *Staphylococcus aureus in vitro* and *in vivo* and its application in demonstrating the role of *sigB* in microcolony formation. *Infect Immun* **69**: 7851-7857.

Chambers, H. F., (2001) The changing epidemiology of *Staphylococcus aureus*? *Emerg Infect Dis* **7**: 178-182.

Cheung, A. L., C. C. Nast & A. S. Bayer, (1998) Selective activation of sar promoters with the use of green fluorescent protein transcriptional fusions as the detection system in the rabbit endocarditis model. *Infect Immun* **66**: 5988-5993.

Foster, T. J., (2005) Immune evasion by *Staphylococci*. *Nat Rev Microbiol* **3**: 948-958.

Gill, S. R., D. E. Fouts, G. L. Archer, E. F. Mongodin, R. T. Deboy, J. Ravel, I. T. Paulsen, J. F. Kolonay, L. Brinkac, M. Beanan, R. J. Dodson, S. C. Daugherty, R. Madupu, S. V. Angiuoli, A. S. Durkin, D. H. Haft, J. Vamathevan, H. Khouri, T. Utterback, C. Lee, G. Dimitrov, L. Jiang, H. Qin, J. Weidman, K. Tran, K. Kang, I. R. Hance, K. E. Nelson & C. M. Fraser, (2005) Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *J Bacteriol* **187**: 2426-2438.

Guerout-Fleury, A. M., K. Shazand, N. Frandsen & P. Stragier, (1995) Antibiotic-resistance cassettes for *Bacillus subtilis*. *Gene* **167**: 335-336.

Haeusser, D. P., R. L. Schwartz, A. M. Smith, M. E. Oates & P. A. Levin, (2004) EzrA prevents aberrant cell division by modulating assembly of the cytoskeletal protein FtsZ. *Mol Microbiol* **52**: 801-814.

Henner, D. J., (1990) Inducible expression of regulatory genes in *Bacillus subtilis*. *Methods Enzymol* **185**: 223-228.

Kaltwasser, M., T. Wiegert & W. Schumann, (2002) Construction and application of epitope- and green fluorescent protein-tagging integration vectors for *Bacillus subtilis*. *Appl Environ Microbiol* **68**: 2624-2628.

Levin, P. A., I. G. Kurtser & A. D. Grossman, (1999) Identification and characterization of a negative regulator of FtsZ ring formation in *Bacillus subtilis*. *Proc Natl Acad Sci U S A* **96**: 9642-9647.

Malone, C. L., B. R. Boles, K. J. Lauderdale, M. Thoendel, J. S. Kavanaugh & A. R. Horswill, (2009) Fluorescent reporters for *Staphylococcus aureus*. *J Microbiol Methods* **77**: 251-260.

Murray, H. & J. Errington, (2008) Dynamic control of the DNA replication initiation protein DnaA by Soj/ParA. *Cell* **135**: 74-84.

Navarre, W. W. & O. Schneewind, (1999) Surface proteins of Gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. *Microbiol Mol Biol Rev* **63**: 174-229.

Oshida, T. & A. Tomasz, (1992) Isolation and characterization of a Tn*551*-autolysis mutant of *Staphylococcus aureus*. *J Bacteriol* **174**: 4952-4959.

Pinho, M. G. & J. Errington, (2003) Dispersed mode of *Staphylococcus aureus* cell wall synthesis in the absence of the division machinery. *Mol Microbiol* **50**: 871-881.

Projan, S., (2000) Antibiotic resistance in the *Staphylococci*. In: Gram-positive pathogens. V. A. Fischetti, R. P. Novick, J. J. Ferretti, D. A. Portnoy & J. I. Rood (eds). Washington, D.C.: ASM Press, pp. 463-470.

Schneider, W. P., S. K. Ho, J. Christine, M. Yao, A. Marra & A. E. Hromockyj, (2002) Virulence gene identification by differential fluorescence induction analysis of *Staphylococcus aureus* gene expression during infection-simulating culture. *Infect Immun* **70**: 1326-1333.

Tenover, F. C. & R. J. Gorwitz, (2006) The epidemiology of *Staphylococcus* infections. In: Gram-positive pathogens. V. A. Fischetti, R. P. Novick, J. J. Ferretti, D. A. Portnoy & J. I. Rood (eds). Washington, D.C.: ASM Press, pp. 526-534.

Tsien, R. Y., (1998) The green fluorescent protein. *Annu Rev Biochem* **67**: 509-544.

Tzagoloff, H. & R. Novick, (1977) Geometry of cell division in *Staphylococcus aureus*. *J Bacteriol* **129**: 343-350.

Veiga, H. & M. G. Pinho, (2009) Inactivation of the SauI type I restriction-modification system is not sufficient to generate *Staphylococcus aureus* strains capable of efficiently accepting foreign DNA. *Appl Environ Microbiol* **75**: 3034-3038.

Wyke, A. W., J. B. Ward, M. V. Hayes & N. A. Curtis, (1981) A role *in vivo* for penicillin-binding protein-4 of *Staphylococcus aureus*. *Eur J Biochem* **119**: 389-393.

Zapun, A., T. Vernet & M. G. Pinho, (2008) The different shapes of cocci. *FEMS Microbiol Rev* **32**: 345-360.

Chapter 4

Absence of nucleoid occlusion effector Noc impairs formation of orthogonal FtsZ rings during *Staphylococcus aureus* cell division

AUTHOR CONTRIBUTIONS

All experiments described in this chapter were performed by H. Veiga. A. M. Jorge constructed plasmid pBCBAJ002 and strains BCBAJ025 and BCBAJ029.

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Abstract

The Gram-positive pathogen *Staphylococcus aureus* divides by synthesizing the septum in three orthogonal planes over three consecutive division cycles. This process has to be tightly coordinated with chromosome segregation to avoid bisection of the nucleoid by the septum. Here we show that deletion of the nucleoid occlusion effector Noc in *S. aureus* results in the formation of Z-rings over the nucleoid, as well as in DNA breaks, indicating that Noc has an important role as an antiguillotine checkpoint that prevents septa from forming over the DNA. Furthermore, Noc deleted cells show multiple Z-rings which are no longer placed in perpendicular planes. We propose that the axis of chromosome segregation has a role in determining the placement of the division septum. This is achieved via the action of Noc which restricts the placement of the division septum to one of an infinite number of potential division planes that exist in *S. aureus.*

Introduction

Bacterial cell division requires coordination between cytokinesis and chromosome segregation to ensure placement of the division septum at the right place and time, avoiding bisection of the chromosome by the division apparatus.

Proper temporal and spatial control of the assembly of the division septum largely depends on the regulation of FtsZ localization, as polymerization of this tubulin homologue in a ring-like structure (Z-ring) constitutes the earliest known step in the process of cell division (Lutkenhaus, 2007, Adams & Errington, 2009). The Z-ring then serves as scaffold for the recruitment of the other components of the division apparatus, to form a highly complex protein machinery called the divisome, responsible for the synthesis of a new cell wall and for the constriction of the cell membrane (Errington *et al.*, 2003, Adams & Errington, 2009). Many bacteria seem to use a dual mechanism to ensure that FtsZ polymerization occurs at the middle of the cell, between the two segregated chromosomes: the Min system and nucleoid occlusion (Barak & Wilkinson, 2007, Lutkenhaus, 2007, Bramkamp & van Baarle, 2009).

The Min system inhibits FtsZ polymerization, and thus inappropriate septation, at the cell poles, through the action of a complex formed by MinC and MinD (de Boer *et al.*, 1989, Rothfield *et al.*, 2005, Lutkenhaus, 2007). MinC is an inhibitor of division that is activated by the membrane-associated ATPase MinD (Lutkenhaus, 2007, Rothfield *et al.*, 2005). In *Escherichia coli*, the inhibitory action of MinCD is retained at the vicinity of the cell poles as a result of a rapid pole-to-pole oscillation of this complex driven by the topological factor MinE (Raskin & De Boer, 1999), while in *Bacillus subtilis* it is achieved through the tethering action of the polar protein DivIVA (Cha & Stewart, 1997, Edwards & Errington, 1997).

Septation in mutants lacking the Min system is not totally unregulated, as these mutants form division septa randomly within DNA-free regions of the cell, but not on top of the nucleoid (Levin *et al.*, 1998). This is due to the nucleoid occlusion effect, which results from a negative effect of the chromosome on FtsZ polymerization (Mulder &

Woldringh, 1989, Woldringh *et al.*, 1991, Wu & Errington, 2004, Bernhardt & de Boer, 2005). Nucleoid occlusion therefore prevents division through areas occupied by the nucleoid and protects the DNA against guillotining by the division septum. The mechanistic base for nucleoid occlusion was elucidated with the identification of two DNA-binding proteins, Noc in *B. subtilis* (Wu & Errington, 2004) and SlmA in *E. coli* (Bernhardt & de Boer, 2005), that act as inhibitors of FtsZ polymerization and inhibit Z-ring formation until DNA segregation has properly cleared chromosomal DNA from midcell. In the absence of a functional Min system, *noc* or *slmA* mutants form numerous abortive FtsZ structures that overlap the nucleoid and may lead to bisection of the chromosome (Bernhardt & de Boer, 2005, Wu & Errington, 2004). In fact, under different conditions that perturb DNA replication or segregation, these nucleoid occlusion effectors have a critical role in the antiguillotine checkpoint that prevents formation of the septum over the DNA (Bernhardt & de Boer, 2005, Wu & Errington, 2004). Although evolutionary unrelated, both Noc and SlmA display similar localization patterns, binding to the nucleoid with highest concentration at the pole-proximal regions (Bernhardt & de Boer, 2005, Wu & Errington, 2004). Recently, Wu *et al*. showed that this uneven distribution of Noc results from the binding of this protein to about 70 copies of a conserved 14 bp inverted repeat, which are scattered throughout the chromosome but essentially absent from a large region around the replication terminus (Wu *et al.*, 2009). This strict localization of Noc activity, absent from the terminus region, suggests that this protein may not only be a spatial, but also a temporal regulator of cell division, inhibiting FtsZ polymerization during early stages of chromosome replication and segregation. This would allow the division machinery to start assembling at a defined moment, late in the DNA replication cycle, when the majority of Noc has been displaced from the cell center (Wu *et al.*, 2009).

Importantly, mutants devoid of both the Min system and nucleoid occlusion still show preferential localization of FtsZ rings in regions between the nucleoids, indicating that other(s), yet unknown factors may also contribute to the nucleoid occlusion effect (Bernhardt & de Boer, 2005, Wu & Errington, 2004).

Division site selection has been studied in detail in the two rod-shaped model organisms *B. subtilis* and *E. coli*, both of which divide by placing the division septum always at the same medial plane, equidistant from the two cell poles and perpendicular to the long axis of the cell, the only division plane that generates two identical daughter cells. The problem becomes more complex in cocci, which have an infinite number of theoretical division planes (all circles with maximum diameter within the cell) that can generate two identical daughter cells. *Staphylococcus aureus*, an important clinical pathogen, divides by switching the division plane in three consecutive perpendicular orientations in successive division cycles (Koyama *et al.*, 1977, Tzagoloff & Novick, 1977), akin to the initial series of division planes in a fertilized egg. This implies a novel mechanism for the definition of the division plane and septum placement in bacteria, for which virtually no information is available. S. Foster and colleagues have recently proposed a model in which *S. aureus* uses epigenetic information contained in the cell wall, in the form of a large belt of peptidoglycan material that labels the division sites, to define the sequential orthogonal planes (Turner *et al.*, 2010). As for the known systems that regulate Z-ring placement, *S. aureus* does not have a Min system (based on homology searches) but, although the existence of nucleoid occlusion has never been shown, a *noc* homologue is present in its genome, as well as in the genomes of other spherical bacteria that divide in either two or three planes. Importantly, most studies regarding choice of division planes in round cells used either *Neisseria* or *E. coli* round cells as models (Westling-Haggstrom *et al.*, 1977, Begg & Donachie, 1998, Zaritsky *et al.*, 1999, Pas *et al.*, 2001, Ramirez-Arcos *et al.*, 2001, Corbin *et al.*, 2002). These organisms, however, have a Min system which is required for normal growth and division, and therefore are not the most appropriate models for the mode of division in three orthogonal planes used by *S. aureus.*

The fact that cocci have multiple potential division planes, but only a few that will not lead to bisection of the DNA, raises the possibility that chromosome segregation may have a crucial role in the selection of division planes in spherical cells devoid of Min. Here we show that initiation of chromosome segregation in *S. aureus* precedes assembly of the

FtsZ ring. We also show that the nucleoid occlusion effector Noc has a vital role in determining the placement of the FtsZ ring in orthogonal planes that correspond to Noc-free regions, generated between the two segregated chromosomes, in each division cycle.

Experimental procedures

Bacterial strains and growth conditions

All plasmids and strains used in this study are listed, respectively, in Table 4.1 and Table 4.2. Sequences of primers used are listed in Table 4.3. *S. aureus* strains were grown in tryptic soy agar (TSA, Difco) at 37°C or in tryptic soy broth (TSB, Difco) at 37°C with aeration. The medium was supplemented, when necessary, with erythromycin (10 μg/ml, Sigma), chloramphenicol (10 μg/ml, Sigma), kanamycin and neomycin (50 μg/ml each, Sigma), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, 100 μg/ml, BDH Prolabo) or isopropyl-β-D-thiogalactopyranoside (IPTG, at various concentrations as indicated below, BDH Prolabo).

Construction of the Noc null mutant

The Noc null mutant was constructed in the background of *S. aureus* NCTC8325-4 strain using the thermosensitive plasmid pMAD (Arnaud *et al.*, 2004). PCR fragments containing the upstream and downstream flanking regions of the *noc* gene were amplified from chromosomal DNA of NCTC8325-4, using primer pairs NocP1/NocP2 and NocP3/NocP4. These two PCR products were joined in a second PCR reaction, using primers NocP1/NocP4. The resulting fragment was restricted with BamHI and EcoRI and cloned into pMAD, giving rise to pBCBHV001. The insert was sequenced and the plasmid was electroporated as previously described (Veiga & Pinho, 2009) into the transformable strain RN4220 at 30°C (using erythromycin selection) and subsequently transduced to NCTC8325-4 using phage 80α (Oshida & Tomasz, 1992). The deletion of *noc* was

completed after a two-step homologous recombination process. In the first step, recombinants in which pBCBHV001 was integrated into the chromosome were selected at the nonpermissive temperature of 43°C. In the second step, recombinants in which the integrated plasmid (and consequently the *lacZ* and *erm* genes) had been excised were selected at the permissive temperature (30°C) in the absence of antibiotic selection. The absence of *noc* gene was confirmed by PCR screening of the obtained erythromycin sensitive, white colonies, using the primers NocP5/NocP6 and the strain was named BCBHV001.

Complementation of Noc mutation

To complement the *noc* null mutant strain BCBHV001, a full copy of *noc* gene was placed in the *spa* locus of *S. aureus* chromosome, under the control of the IPTG-inducible P_{spac} promoter, using pBCB13 plasmid (Pereira *et al.*, 2010). For that purpose, an 865 bp DNA sequence, containing the ribosomal binding site (RBS) and the *noc* gene, was amplified by PCR using the primers NocP7 and NocP8. After restriction with XmaI and XhoI, the insert was cloned into pBCB13, downstream of the P_{spac} promoter, and sequenced. The resulting pBCBHV002 plasmid was electroporated into RN4220 at 30°C (using erythromycin selection) and subsequently transduced to a Noc mutant strain containing an extra, plasmid-encoded, copy of the *lacI* gene (BCBHV003). To exchange the *spa* gene for the P_{spac}-*noc* DNA fragment and obtain strain BCBHV010, the transductants were incubated at the non-permissive temperature of 43°C, in the presence of erythromycin (to select for plasmid integration) and then incubated at the permissive temperature of 30°C, in the absence of antibiotic selection, to select for white colonies in which the vector had been excised. As a control, the pBCB13 vector was also transduced from RN4220 into BCBHV002 (NCTC8325-4 strain containing an extra, plasmid-encoded, copy of the *lacI* gene) and BCBHV003 strains, which went through the two-steps recombination process, resulting in strains BCBHV008 and BCBHV009 in which the *spa* gene was deleted.

Table 4.1. Plasmids used and constructed in this study

Plasmids	Relevant characteristics	Source or reference
pMAD	*E. coli – S. aureus* shuttle vector with a thermosensitive origin of replication for Gram-positive bacteria; Amp^r, Ery^r	(Arnaud *et al.*, 2004)
pBCB13	pMAD derivative with up- and downsteam regions of *spa* and P_{spac}-*lacI;*Amp^r, Ery^r	(Pereira *et al.*, 2010)
pMUTINCFP	*B. subtilis* integrative vector for C-terminal CFP fusions; Amp^r, Ery^r	(Kaltwasser *et al.*, 2002)
pMUTINYFP	*B. subtilis* integrative vector for C-terminal YFP fusions; Amp^r, Ery^r	(Kaltwasser *et al.*, 2002)
pMUTINCFPKan	*B. subtilis* integrative vector for C-terminal CFP fusions; Amp^r, Kan^r	This study
pMUTINYFPKan	*B. subtilis* integrative vector for C-terminal YFP fusions; Amp^r, Kan^r	(Atilano *et al.*, 2010)
pMUTINCFPΔermB	pMUTINCFP with *ermB* gene deleted	This study
pDG792	Plasmid containing a kanamycin resistance gene	(Guerout-Fleury *et al.*, 1995)
pMGPII	Plasmid encoding *lacI* gene; Cm^r	(Pinho *et al.*, 2001)
pBCB4-ChE	*S. aureus* integrative vector for N- and C-terminal mCherry fusions; Amp^r, Ery^r	(Pereira *et al.*, 2010)
pBCBHV001	pMAD containing up- and downsteam regions of *noc*; Amp^r, Ery^r	This study
pBCBHV002	pBCB13 containing P_{spac}-*noc-lacI*; Amp^r, Ery^r	This study
pBCBHV003	pBCB13 containing P_{spac}-*ftsZ-CFP-lacI*; Amp^r, Ery^r	This study
pBCBHV004	pMUTINYFP containing *spo0J- yfp*; Amp^r, Ery^r	This study
pBCBHV005	pMUTINYFPKan containing *noc -yfp*; Amp^r, Kan^r	This study
pBCBHV006	pMUTINCFPKan containing *ftsZ-cfp*; Amp^r, Kan^r	This study
pBCBAJ001	pMAD containing the 3' end and the downstream region of *ezrA;* Amp^r, Ery^r	This study
pBCBAJ002	pBCBAJ001 containing *ezrA-mCherry;* Amp^r, Ery^r	This study

Table 4.2. Strains used and constructed in this study

Strains	Relevant characteristics	Source or reference
E. coli		
DH5α	*recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1* φ80Δ*lacZ*ΔM15	Lab strain
S. aureus		
RN4220	MSSA strain. Mutagenized strain derived from NCTC8325-4, that accepts foreign DNA	R. Novick
NCTC8325-4	MSSA strain	R. Novick
COL	MRSA strain	(Gill *et al.*, 2005)
BCBHV001	NCTC8325-4 Δ*noc*	This study
BCBHV002	NCTC8325-4 *lacI* ^{mc}; Cm^r	This study
BCBHV003	NCTC8325-4 ΔNoc *lacI* ^{mc}; Cm^r	This study
BCBHV004	RN4220 *ftsZ*::pBCBHV006*,* expressing *ftsZcfp* fusion; Kan^r	This study
BCBHV005	RN4220 *ftsZ*::pBCBHV006 *spo0J*:: pBCBHV004, expressing *ftsZcfp* and *spo0Jyfp* fusions; Kan^r , Ery^r	This study
BCBHV006	NCTC8325-4 *noc*::pBCBHV005, expressing *nocyfp* fusion; Kan^r	This study
BCBHV007	NCTC8325-4 *noc*::pBCBHV005 *lacI* ^{mc}, expressing *nocyfp* fusion; Kan^r, Cm^r	This study
BCBHV008	NCTC8325-4 Δ*spa*::P_{spac}-*lacI lacI* ^{mc}; Cm^r	This study
BCBHV009	NCTC8325-4 Δ*noc* Δ*spa*::P_{spac}-*lacI lacI* ^{mc}; Cm^r	This study
BCBHV010	NCTC8325-4 Δ*noc* Δ*spa*::*Pspa-noc-lacI lacI* ^{mc}; Cm^r	This study
BCBHV011	NCTC8325-4 Δ*spa*::P_{spac}-*ftsZ-cfp-lacI lacI* ^{mc}; Cm^r	This study
BCBHV012	NCTC8325-4 Δ*noc* Δ*spa*::P_{spac}-*ftsZ-cfp-lacI lacI* ^{mc}; Cm^r	This study
BCBHV013	NCTC8325-4 Δ*spa*::P_{spac}-*ftsZ-cfp-lacI*	This study
BCBHV014	NCTC8325-4 Δ*spa*::P_{spac}-*ftsZ-cfp-lacI lacI* ^{mc} *noc*:: pBCBHV005, expressing *ftsZcfp* and *nocyfp* fusions; Kan^r; Cm^r	This study
RNpEzrACFP	RN4220 *ezrA*::pEzrA-CFP, expressing *ezrAcfp* fusion; Ery^r	(Pereira *et al.*, 2010)
BCBHV015	NCTC8325-4 *ezrA*::pEzrA-CFP, expressing *ezrAcfp* fusion; Ery^r	This study
BCBHV016	NCTC8325-4 Δ*noc ezrA*::pEzrA-CFP, expressing *ezrAcfp* fusion; Ery^r	This study
BCBAJ025	NCTC8325-4 Δ*ezrA::ezrA-mCherry*	This study
BCBAJ029	NCTC8325-4 Δ*ezrA::ezrA-mCherry* Δ*spa*::P_{spac}-*ftsZ-cfp-lacI*	This study

lacI ^{mc} – cells expressing multiple copies of the *lacI* gene (encoded by pMGPII)

Construction of fluorescent derivatives of *S. aureus* proteins

For the construction of YFP fusions to the C-terminal of Spo0J and Noc we used, respectively, the pMUTINYFP vector (Kaltwasser *et al.*, 2002) and its derivative pMUTINYFPKan in which the erythromycin-resistance marker was replaced by a kanamycin-resistance marker (Atilano *et al.*, 2010).

DNA fragments of *spo0J* and *noc* genes without the stop codon and connected to a 5-codon linker (Table 4.3, highlighted in bold) were amplified by PCR using, respectively, primer pairs Spo0JP1 /Spo0JP2 and NocP9/NocP10.

The *spo0J* insert was restricted with HindIII and EagI and cloned into pMUTINYFP, generating pBCBHV004. The insert was sequenced and the plasmid was electroporated into RN4220 (selection with 10 µg/ml erythromycin and 0.1 mM IPTG) and transduced to BCBHV004 strain (see below) using phage 80α (selection with 50 µg/ml kanamycin, 50 µg/ml Neomycin, 10 µg/ml erythromycin and 0.1 mM IPTG) to obtain BCBHV005 strain. In this strain, the expression of *spo0J* and *ftsZ* fusions from their native loci, is regulated, respectively, by *spo0J* and *ftsZ* native promoters while an extra un-fused copy of each gene is expressed under control of P_{spac} by the addition of 0.1 mM IPTG.

The *noc* insert was restricted with KpnI, cloned into pMUTINYFPKan and sequenced. The resulting vector, pBCBHV005, was electroporated into RN4220 (selected with 50 µg/ml of kanamycin, 50 µg/ml of Neomycin and 0.1 mM IPTG) and transduced into NCTC8325-4. The integration of pBCBHV005 in the *noc* locus originates *S. aureus* strain BCBHV006, in which the *noc–yfp* fusion is controlled by its native promoter and an extra copy of *noc* (controlled by P_{spac}) is expressed by addition of 0.1 mM IPTG to the culture medium.

To fully repress the expression of the Noc un-tagged protein in BCBHV006 strain and test the functionality of Noc–YFP, we introduced plasmid pMGPII (encoding *lacI* gene) into strain BCBHV006 to originate strain BCBHV007 that was grown in the absence of IPTG.

For co-visualization of FtsZ–CFP and Noc–YFP, pBCBHV005 was also transduced into the background of strain BCBHV011 to originate BCBHV014. This strain, expresses at

the same time, an FtsZ–CFP fusion from the ectopic *spa* locus and a Noc–YFP protein from *noc* native locus.

To construct a *S. aureus* strain expressing an FtsZ–CFP C-terminal fusion from the *ftsZ* native locus, a PCR fragment encompassing the *ftsZ* gene (without the stop codon) and a 5 codon linker (Table 4.3, highlighted in bold) was amplified using primers FtsZP1 and FtsZP2, digested with KpnI, cloned into pMUTINCFPKan plasmid (see below) and sequenced. The resulting plasmid, pBCBHV006, was electroporated into RN4220 competent cells (selected with 50 µg/ml kanamycin, 50 µg/ml Neomycin and 0.1 mM IPTG) giving rise to BCBHV004. This strain expresses both an *ftsZ–cfp* fusion from the *ftsZ* native promoter and *ftsZ* from the P_{spac} promoter by addition of 0.1 mM IPTG to the culture medium.

The pMUTINCFPKan vector is a derivative of pMUTINCFP (Kaltwasser *et al.*, 2002) in which the erythromycin resistance marker was replaced by a kanamycin-resistance marker from pDG792 vector (Guerout-Fleury *et al.*, 1995). For this replacement, primers KanP1 and KanP2 were used to amplify the entire pMUTINCFP, except the ermB cassette, which was then auto-ligated to generate pMUTINCFPΔermB. The kanamycin marker was excised from pDG792 by restriction with NcoI and BglII and cloned into pMUTINCFPΔermB, generating the pMUTINCFPKan plasmid.

To construct a strain in which the FtsZ–CFP fusion is ectopically expressed, under the control of the P_{spac} promoter, from the *spa* locus of *S. aureus* chromosome, the pBCB13 vector was used. For that purpose, a *ftsZ–cfp* DNA fragment was amplified from pBCBHV006 plasmid, using primers FtsZP3 and FtsZP4. The fragment was restricted with XmaI and XhoI, cloned into previously digested pBCB13, downstream of the P_{spac} promoter, and sequenced. The resulting pBCBHV003 plasmid was electroporated into RN4220 at 30°C (selected with erythromycin) and subsequently transduced to NCTC8325-4, BCBAJ025 (see below; selection at 30°C with erythromycin) and BCBHV002, BCBHV003 (selection at 30°C with erythromycin and chloramphenicol). The replacement of the *spa* gene for the *P_{spac}-ftsZ–cfp* DNA fragment was completed after a two-step homologous recombination process as described above and confirmed by PCR. The resulting strains

expressing *ftsZ– cfp* from the *spa* locus were named BCBHV013, BCBAJ029, BCBHV011 and BCBHV012 respectively.

To construct *S. aureus* strains expressing an EzrA–CFP fusion protein, the genomic region containing *ezrA-cfp* was transduced from RNpEzrA–CFP (Pereira *et al.*, 2010) into NCTC8325-4 and BCBHV001 (using erythromycin selection). In the resulting strains, named, respectively, BCBHV015 and BCBHV016, EzrA–CFP fusion is expressed under the control of *ezrA* native promoter from its native chromosomal locus.

To allow the co-visualization of EzrA and FtsZ in the same cells, a functional *ezrA–mCherry* fusion, was expressed from its native locus. For that purpose, two PCR fragments containing the truncated 3' end of *ezrA* gene (1099bp) without its stop codon and the 1107bp sequence downstream of *ezrA* gene, were amplified from *S. aureus* COL genome using primer pairs EzrAP4/EzrAP5 and EzrAP1/EzrAP2 respectively. The two fragments were joined by overlap PCR using primers EzrAP4 and EzrAP2, which resulted in the introduction of a 5 codon linker at the 3' end of *ezrA* gene. The resulting fragment was digested and cloned into the EcoRI and BamHI restriction sites of pMAD vector, generating plasmid pBCBAJ001. The mCherry coding sequence (711bp) was amplified from pBCB4-ChE (Pereira *et al.*, 2010) using primers mCherryP3 and mCherryP4. The PCR product was cloned into pBCBAJ001 using enzymes NheI and XhoI, generating plasmid pBCBAJ002. This plasmid was introduced into RN4220 cells by electroporation and transduced to NCTC8325-4 using phage 80α. Integration of pBCBAJ002 into the *ezrA* locus was selected for by growing cells at 43°C with erythromycin. After a second recombination event leading to plasmid excision, cells in which *ezrA* was replaced by an *ezrA–mCherry* fusion, were identified by PCR and the resulting strain was named BCBAJ025.

Table 4.3. Primers used in this study

Primer Name	Primer Sequence (5'- 3')*
NocP1	tgaggatccgttatatcaataaatcactacaacaag
NocP2	cctactactatatccattcgctccttttatgttagttg
NocP3	cgaatggatatagtagtaggatgtcgtatacatgatg
NocP4	gctgaattccactaaaaatactgccggaaacgttgc
NocP5	gatgctggagagcgccagatg
NocP6	cttcgtagagcatagaacattg
NocP7	tactccccgggctaacataaaaggagcgaatgg
NocP8	gctgcctcgagctaacgtttatatattcg
KanP1	gactacgccatgggttcatgtaatcactccttc
KanP2	gcgagatctggaaataattctatgagtcgc
Spo0JP1	gctgcaagcttctgttgttatcaaataaaaag
Spo0JP2	gctggcacggccg**ggaggcgccgcagga**tttaccatacctacgatttaattg
NocP9	gctgcggtaccctaacataaaaggagcgaatgg
NocP10	gctgcggtacc**ggaggcgccgcagga**acgtttatatattcgaa
FtsZP1	gctgcggtaccggccaataaaactaggagg
FtsZP2	gctgcggtacc**ggaggcgccgcagga**acgtcttgttcttcttgaacg
FtsZP3	tactccccgggggccaataaaactaggagg
FtsZP4	gctgcctcgagggaaaaaagcccgctcattaggcgggc
EzrAP1	caa**tctgcaagtgctgca**gctagctctgcaagtctcgagaaactagtatgtagttatac
EzrAP2	ccgggatccccattgcaatatcatttggc
EzrAP4	tcagaattccatatagctgccttgaatg
EzrAP5	ctcgagacttgcagagctagc**tgcagcacttgcaga**ttgcttaataacttcttcttc
mCherryP3	taagctagcatgattgtgagcaagggcga
mCherryP4	ttactcgagttacttgtacagctcgtcc

* Underlined sequences correspond to restriction sites;

Bold sequences correspond to the five-codon linker.

TUNEL assay for the detection of DNA breaks

Staphylococcus aureus cells were prepared for TUNEL labeling of DNA breaks using a fixation and permeabilization protocol adapted from a previously described method for immunofluorescence (Pinho & Errington, 2003). NCTC8325-4 and BCBHV001 cultures were grown to mid exponential phase (OD_{600nm} 0.5), fixed with Histochoice (Amresco) washed three times with PBS and resuspended in GTE buffer (50 mM glucose, 20 mM Tris-HCl pH = 7.5, 10 mM EDTA). The fixed cells were then gently lysed with 2.5–5 µg/ml lysostaphin for 40s on a poly-L-lysine-treated slide, washed with PBS, air dried and rehydrated with PBS (Phosphate Buffer Saline). An aliquot of fixed and permeabilized

NCTC8325-4 cells was treated at room temperature, for 20 min, with 100 U/ml DNaseI, to obtain cells with DNA breaks, used as positive control.

The DNA breaks were detected by catalytic incorporation of dUTPs fluorescein conjugates at the 3'-hydroxyl ends of the fragmented DNA using an *In Situ* Cell Death Detection Kit (Roche). Fixed cells were incubated for 1h at room temperature in the dark, with a TUNEL reaction mixture containing the terminal deoxynucleotidyl transferase (TdT) diluted 1/1000 in the dUTPs mixture labeling solution. After washing 8 times with PBS, Vecta shield mounting medium (Vector Laboratories) was added and the cells were visualized by fluorescence microscopy.

Fluorescence microscopy

Strains were grown overnight, in TSB at 37°C, with appropriate antibiotic selection. Cells were then diluted 1/1000 into fresh TSB, supplemented when necessary with 0.1 mM IPTG. When required, cells were stained with the cell wall dye Van-FL (1 μg/ml), the DNA dye Hoechst 33342 (1 μg/ml) and the membrane dyes Nile Red (5 μg/ml) or FM5-65 (1 μg/ml).

To inhibit DNA replication, BCBHV013 cells were grown with 0.1 mM of IPTG until OD_{600nm} 0.3. At that time, HPUra (40 μg/ml) was added to the culture, which was then incubated for 1 h, prior to FtsZ–CFP visualization.

Chloramphenicol was used to condense the DNA. For that purpose, NCTC8325-4, BCBHV006 and BCBHV013 cells were grown until OD = 0.4 (BCBHV013 cells were grown in the presence of 0.1 mM IPTG). At that point, chloramphenicol (2μg/ml) was added to the culture, which was then incubated for 30 min. BCBHV013 cells were stained with DNA Hoechst 33342. NCTC8325-4 and BCBHV006 cells were stained with Hoechst 33342 and, respectively, with the membrane dyes Nile Red and FM5-65.

Cells were mounted on a thin film of 1% agarose in PBS and observed by fluorescence microscopy using a Zeiss Axio observer.Z1 microscope. Image acquisition was performed using a Photometrics CoolSNAP HQ2 camera (Roper Scientific) and Metamorph 7.5 software (Molecular Devices).

The measurement of the angles established between the multiple FtsZ–CFP ring/arc structures in BCBHV012 strain was performed using the ImageJ angle tool. ImageJ software was also used to quantify the cell diameter of approximately one thousand NCTC8325-4 and BCBHV001 cells.

Results

Chromosome segregation initiates prior to septum assembly in actively dividing *S. aureus* cells

The volume of an *S. aureus* spherical cell is almost entirely filled by the nucleoid (see example cells in Figure 4.5A). Therefore, if the chromosome is not segregated, there is no circular plane with a diameter equal to that of the cell, which can be used to generate two identical daughter cells, without bisecting the nucleoid. When chromosome segregation occurs, a nucleoid-free area "competent" for Z-ring assembly is generated. To visualize chromosome position as well as FtsZ localization we labeled FtsZ and the chromosome origin of replication in *S. aureus* cells. For that purpose we constructed *S. aureus* strain BCBHV005 coexpressing a fluorescent derivative of FtsZ (FtsZ–CFP) and a fluorescent derivative of SpoOJ (SpoOJ–YFP), a member of the ParB family of DNA binding proteins, which colocalizes with the *oriC* region (Lewis & Errington, 1997, Pinho & Errington, 2004). In this strain, *ftsZ–cfp* and *spo0J–yfp* are expressed from their native loci, under the control of their native promoters. An extra, un-tagged, copy of each gene, controlled by P_{spac}, is expressed in the presence of 0.1 mM IPTG. The extra FtsZ copy is required because, as observed for other organisms (Levin *et al.*, 1999), the fluorescent derivative of FtsZ is only partially functional (data not shown).

Visualization of BCBHV005 cells by fluorescence microscopy showed that the two origins of replication (marked by Spo0J) were already segregated at a time when FtsZ had not yet started to polymerize at the future division site (Figure 4.1A), confirming that chromosome segregation initiates prior to Z-ring formation in staphylococcal cells. We

can hypothesize that once chromosome segregation has proceeded to generate an area of low DNA concentration at mid cell, the FtsZ ring can only assemble between the nucleoids, in the only possible division plane which does not bisect the DNA and which is perpendicular to the division plane from the previous division cycle. Therefore, theoretically, the axis of chromosome segregation could be the only spatial cue required for specifying the division plane. We thus raised the possibility that the nucleoid occlusion effect may have an important role in determining the placement of the division plane in *S. aureus* spherical cells, which led us to study the role of Noc in this organism.

S. aureus Noc colocalizes with the origin proximal region of the chromosome

A good candidate for the role of nucleoid occlusion effector in *S. aureus* is the product of the gene identified in the NCBI database as SAOUHSC_03049 in the NCTC8325 genome. This gene encodes a 279 amino acid protein, with a predicted molecular mass of 32 kDa, which shares 48.4% identity with the *B. subtilis* protein Noc.

To determine the subcellular localization of Noc, we visualized by fluorescence microscopy BCBHV006 cells, which express two copies of *noc* gene: a native copy controlled by the IPTG-induced P_{spac} promoter and a *noc–yfp* fusion, under the control of *noc* native promoter. Importantly, we have introduced a plasmid-encoded copy of *lacI* into BCBVH006 (resulting in strain BCBHV007) to allow stringent repression of the P_{spac} promoter. When BCBHV007 cells were grown in the absence of IPTG, therefore expressing Noc–YFP but not native Noc, they did not exhibit the characteristic Noc null mutant phenotype (data not shown), indicating that the Noc–YFP fusion is functional.

Noc–YFP colocalized with the nucleoid but, similarly to its *B. subtilis* counterpart (Wu & Errington, 2004), it seems absent from the mid cell region in elongated, bilobed chromosomes, most likely corresponding to the final segregating section of the chromosome, near the replication terminus region (Figure 4.1B). Noc–YFP localization is reminiscent of Spo0J characteristic pattern of origin localization. However, Noc–YFP signal is more diffuse than that of Spo0J–YFP (comparison between Figures 4.1A and 4.1B). In agreement with the findings in *B. subtilis*, Noc recognizes DNA sequences more

distributed throughout the chromosome than Spo0J (Wu *et al.*, 2009, Gruber & Errington, 2009).

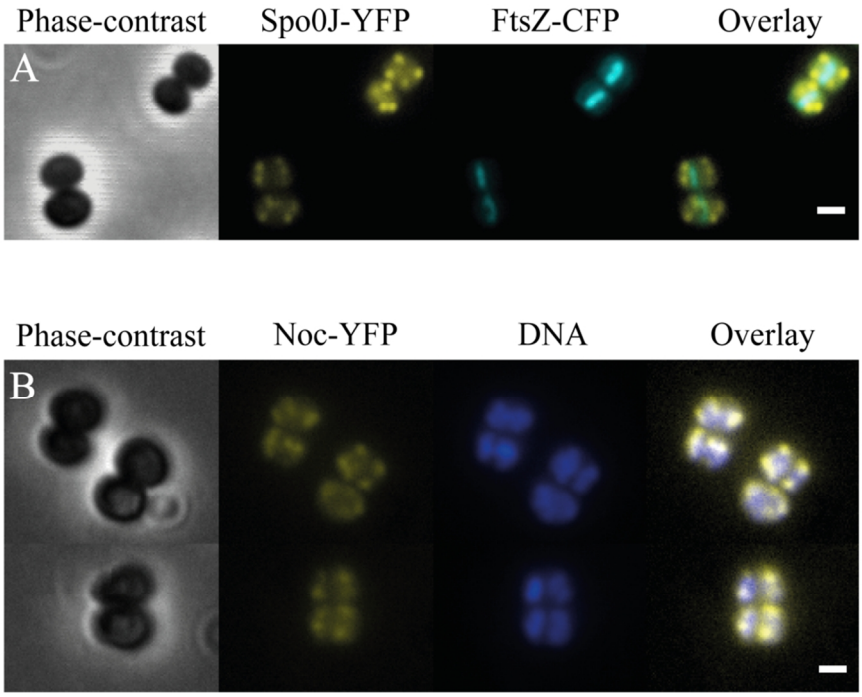


Figure 4.1. (A) Chromosome segregation initiates prior to FtsZ assembly. Localization of the *oriC* marker Spo0J–YFP and of FtsZ–CFP in BCBHV005 cells. The overlay image (right) shows that the two new origins of replication in each daughter cell, segregate before FtsZ polymerization at the future division site, between the segregating nucleoids (note that the Z-ring shown corresponds to the division cycle being finished). **(B) Noc–YFP colocalizes with the nucleoid.** Localization of Noc–YFP in BCBHV006 cells shows this protein colocalizing with the nucleoid, often as diffuse spot at places compatible with the origin of replication. The panels show, from left to right, phage-contrast images, Noc–YFP fluorescence, DNA (stained with Hoechst 33342 dye) and a merge of the two last channels. All images are false-coloured. Scale bars, 1 µm.

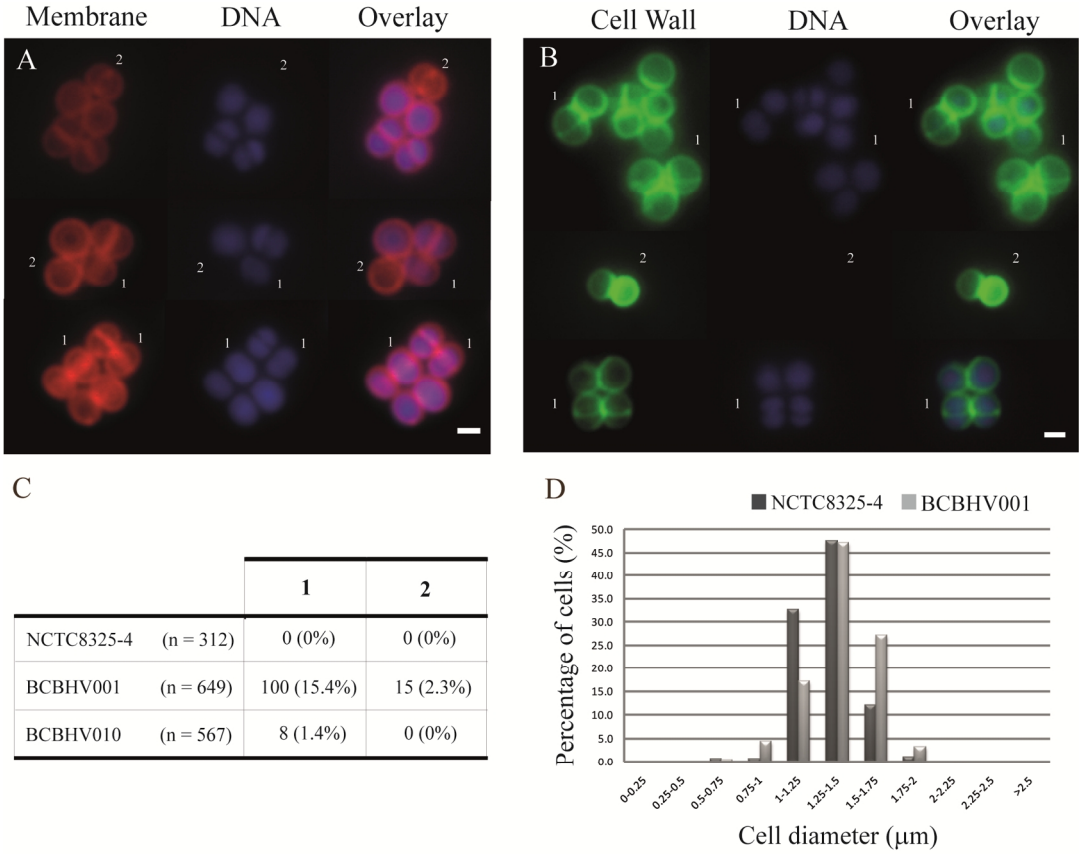
Noc mutants fail to avoid bisection of the chromosome, which results in DNA breaks

To functionally characterize the *S. aureus* Noc homologue, we analysed the phenotypic effects of its absence. For that, a *noc* null mutant was constructed in the background of *S. aureus* strain NCTC8325-4 by removing the entire gene from the

chromosome, leaving no resistance marker. Fluorescence microscopy analysis of the *noc* null mutant BCBHV001 cells showed that 18.5% (*n*=649) of the cells show division defects due to the absence of Noc (Figure 4.2). The most striking phenotype present, in 15.4% of the BCBHV001 cells, and not observed in WT cells (Figure 4.5A), was the presence of septa over the DNA. We were also able to observe 2.3% of anucleate cells, most likely resulting from the degradation of DNA due to DNA breaks (see below) and a small percentage of cells (0.8%) with condensed DNA (data not shown). Moreover, the BCBHV001 Noc mutant cells have an increased average diameter, when compared with wild-type cells (Figure 4.2D).

The phenotype observed in *noc* null mutant BCBHV001 cells could be rescued by ectopic expression of the *noc* gene, under the control of P_{spac}, at the distant *spa* locus in the chromosome, in strain BCBHV010, which results in a 10 fold reduction in the percentage of cells with bisected nucleoid (Figure 4.2C).

While both in *B. subtilis* and in *E. coli* Noc and SlmA have a role in preventing septum formation over the nucleoid only under conditions of DNA replication/ segregation perturbation (Wu & Errington, 2004, Bernhardt & de Boer, 2005), our data indicate that in *S. aureus* the presence of Noc is critical to avoid bisection of the chromosome under normal growth conditions. A possible consequence of the formation of the septum over the nucleoid is the occurrence of DNA breaks with the consequent loss of genomic integrity and/or cell death. Using a TUNEL (Terminal deoxynucleotidyl transferase mediated X-dUTP nick end labeling) assay, which allows the visualization of DNA breaks, we showed that deletion of Noc eventually results in the formation of DNA breaks, which could be detected in 16% of the BCBHV001 *noc* mutant cells (Figure 4.3A). In contrast, wild-type NCTC8325-4 cells were not labeled in the TUNEL assay (data not shown), while 100% of NCTC8325-4 cells pre-treated with the DNA cleavage protein DNaseI were labeled (Figure 4.3B).



		1	2
NCTC8325-4	(n = 312)	0 (0%)	0 (0%)
BCBHV001	(n = 649)	100 (15.4%)	15 (2.3%)
BCBHV010	(n = 567)	8 (1.4%)	0 (0%)

Figure 4.2. Effects of Noc deletion in *S. aureus* cells and rescue by *noc* ectopic expression. Images of BCBHV001 Δ*noc* cells stained with membrane dye Nile Red and DNA dye Hoechst 33342 (A) or with the cell wall dye Van-FL and Hoechst 33342 (B) showing cells with the septum closing over the DNA (labeled '1') and anucleate cells (labeled '2'). Images are false-coloured. Scale bars, 1 μm. (C) Quantitative analysis of the observed Noc deletion phenotypes in wild-type (NCTC8325-4) and Δ*noc* mutant (BCBHV001) strains, as well as in the Noc mutant complemented by ectopic expression of *noc* (induced with 0.1 mM IPTG) from the *spa* locus of the *S. aureus* chromosome (BCBHV010); n = total number of cells analysed. Phenotypes "1" and "2" as described above. (D) Quantification of cells diameter in NCTC8325-4 and Δ*noc* BCBHV001 strains, showing that, in average, Noc mutant cells are bigger than wild-type cells.

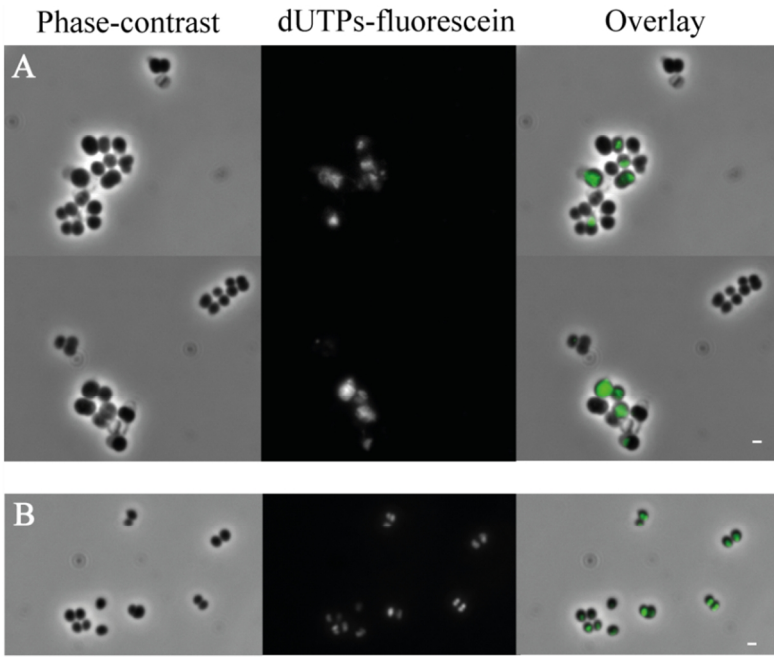


Figure 4.3. Noc deletion results in DNA breaks. DNA breaks detected by catalytic incorporation of dUTPs-fluorescein (TUNEL assay) in Δ*noc* BCBHV001 cells (A) and NCTC8325-4 wild-type cells treated with DNaseI (positive control, B). Left panels: phase-contrast images. Middle panels: fluorescence of dUTPs-fluorescein incorporated in DNA breaks. Right panels: Merge of the two last fields with dUTPs-fluorescein fluorescence false coloured in green. Scale bars, 1 µm.

FtsZ polymerizes in multiple ring/arc structures in the absence of Noc

The results shown above indicate that the presence of Noc is important to prevent Z-ring assembly over the DNA. To further evaluate the role of Noc in the localization of FtsZ, we examined the subcellular localization of this protein using a strain expressing an FtsZ–CFP fusion protein. For that purpose, we ectopically expressed *ftsZ–cfp* under the control of the P_{spac} promoter, from the *spa* region of the chromosome, leaving the native *ftsZ* locus intact. In the parental strain BCBHV011, the FtsZ–CFP fusion protein assembles, as expected, as a ring at the future division site (Figure 4.4A). However, in 137 out of 906 (15%) of the BCBHV012 Noc mutant cells, FtsZ polymerized in multiple ring/arc structures, usually with weak fluorescence, disposed at different angles

in the spherical *S. aureus* cells (Figure 4.4B). These aberrant FtsZ structures polymerized over the nucleoid (Figure 4.4C).

It should be noted that in 119 (87%) of the BCBHV012 cells with accumulation of FtsZ in multiple sites (*n* = 137), the Z-ring structures formed are not perpendicular to each other (forming angles less than 80°). These results suggest an important role of Noc in defining the orthogonal planes in *S. aureus*, as one could argue that if a second factor would have a major role in promoting the assembly of FtsZ rings in orthogonal planes, then, in the absence of Noc, Z-rings would be assembled over the nucleoid, but they would still be mostly perpendicular to each other.

Importantly, Western blotting showed that the intracellular levels of FtsZ were not significantly altered as a consequence of Noc deletion (Figure 4.4D), indicating that the formation of multiple FtsZ rings does not result from an excess of FtsZ, but most likely from a lack of the spatial confinement that is usually imposed by the presence of Noc on FtsZ polymerization. The FtsZ multiple ring/arc structures observed in Noc null mutants usually have weaker fluorescence than FtsZ ring in wild-type cells, presumably because similar amounts of FtsZ protein are distributed throughout a larger number of structures.

As the FtsZ multiple ring/arc structures were difficult to visualize by immunofluorescence (data not shown) we used EzrA as a surrogate marker for FtsZ localization, to confirm the presence of these structures in the absence of Noc, using an independent strain. EzrA is an early component of the division apparatus and a regulator of FtsZ polymerization (Levin *et al.*, 1999), which colocalizes with FtsZ during *S. aureus* cell division (Figure 4.4E). Localization of an EzrA–CFP fusion in *noc* deleted BCBHV016 strain showed the formation of structures containing two or more rings/arcs in 12% (*n* = 480) of the cells (Figure 4.4G), similar to those observed for FtsZ (Figure 4.4B).

These results constitute strong evidence of the negative regulatory role of Noc in preventing the polymerization of FtsZ in random planes, by confining the formation of the Z-ring to the only free space that does not bisect the nucleoid, the future division site.

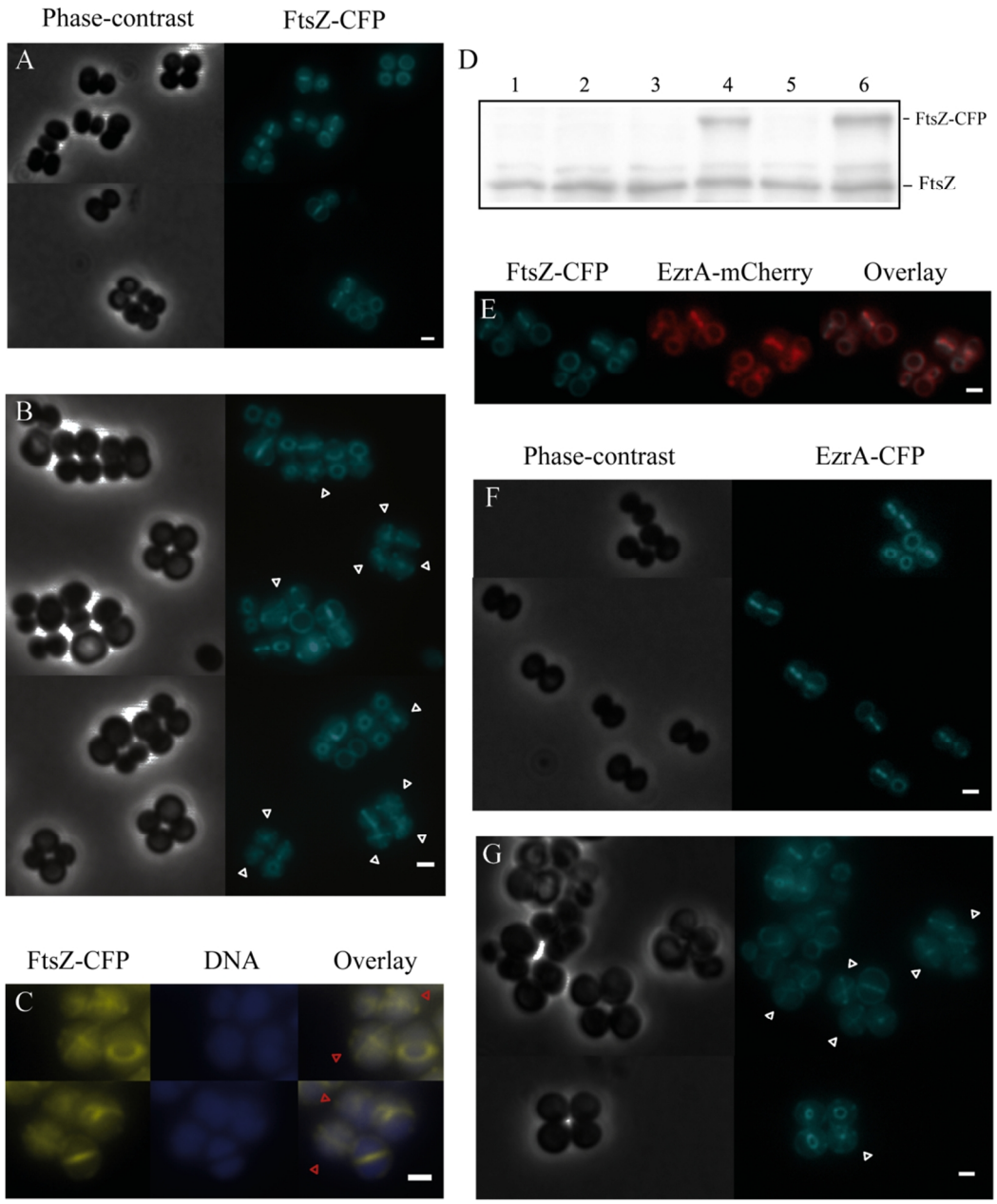


Figure 4.4. In the absence of Noc, FtsZ forms multiple ring/arc structures on top of the nucleoid. (A)FtsZ–CFP localized in mid-cell rings (seen as rings or lines across the cell) in BCBHV011 cells that produce Noc. (B) FtsZ–CFP localized in multiple rings/arcs (white arrowheads) in BCBHV012 Δ*noc* cells. In both cases *ftsZ–cfp* was expressed from the *spa* locus of *S. aureus* chromosome by induction with 0.1 mM IPTG. (C) Localization FtsZ–CFP in BCBHV012 Δ*noc* cells with labeled DNA (using Hoechst 33342) showing FtsZ–CFP ring/arc structures polymerized over the nucleoid (red arrowheads). **(Continued on next page)**

Figure 4.4 (continuation) (D) Western Blot analysis of FtsZ cellular levels in *noc* deletion mutants and parental strains. 1: NCTC8325-4; 2: BCBHV001; 3: BCBHV011 grown without IPTG; 4: BCBHV011 grown in the presence of 0.1 mM IPTG; 5: BCBHV012 grown without IPTG; 6: BCBHV012 grown with 0.1 mM IPTG. Comparison of lanes 1 and 2, and 4 and 6 shows that deletion of *noc* does not result in significant alteration of FtsZ levels. (E) FtsZ–CFP and EzrA–mCherry localization in BCBAJ029 cells showing that EzrA colocalizes with FtsZ during *S. aureus* cell division. (F) Localization of EzrA–CFP at mid-cell in BCBHV015 cells that produce Noc. (G) Localization of EzrA–CFP in the Noc null mutant BCBHV016 showing cells with multiple non-orthogonal FtsZ rings/arcs (arrowheads). All images are false-coloured. Scale bars, 1 µm.

Perturbation of DNA replication/condensation results in the assembly of non-orthogonal Z-rings

If proper chromosomal segregation is determinant for the correct assembly of FtsZ rings in orthogonal planes, in a Noc-dependent manner, then inhibition of chromosome segregation/replication should have an effect on the correct placement of the Z-rings. To test this hypothesis we incubated BCBHV013 cells, which express FtsZ–CFP, in the presence of the replication elongation inhibitor HPUra. As predicted, we were able to observe multiple non-orthogonal Z-rings in 32% (*n* = 613) of HPUra treated cells (Figure 4.6A). Furthermore, one could expect that condensation of the nucleoid would result in the availability of extra space within the cell, which could allow for the formation of Z-rings in places other than at midcell. Addition of chloramphenicol to wild-type NCTC8325-4 *S. aureus* cells results in the condensation of the nucleoid (Figure 4.5), an effect previously described for *E. coli* cells (Zimmerman, 2006). When chloramphenicol was added to BCBHV013 cells, we could observe FtsZ rings around condensed chromosomes, which do not present a bilobed morphology (Figure 4.6B, cells labeled “2”), possibly because the distance between the condensed nucleoid and the membrane was too large for Noc to have an inhibitory effect on FtsZ polymerization. Even more surprisingly, some cells showed FtsZ rings with smaller diameters than that of the cell, which were not located at mid cell (Figure 4.6B, cells labeled “1”). Importantly, after 30 min treatment with chloramphenicol, the growth rate of BCBHV013 culture was not altered (data not shown) and examination of BCBHV006 strain showed that Noc–YFP was still expressed and colocalized with the condensed nucleoids (Figure 4.5C).

These results described above support the idea that the position occupied by the nucleoid is determinant for the positioning of the Z-ring in *S. aureus.*

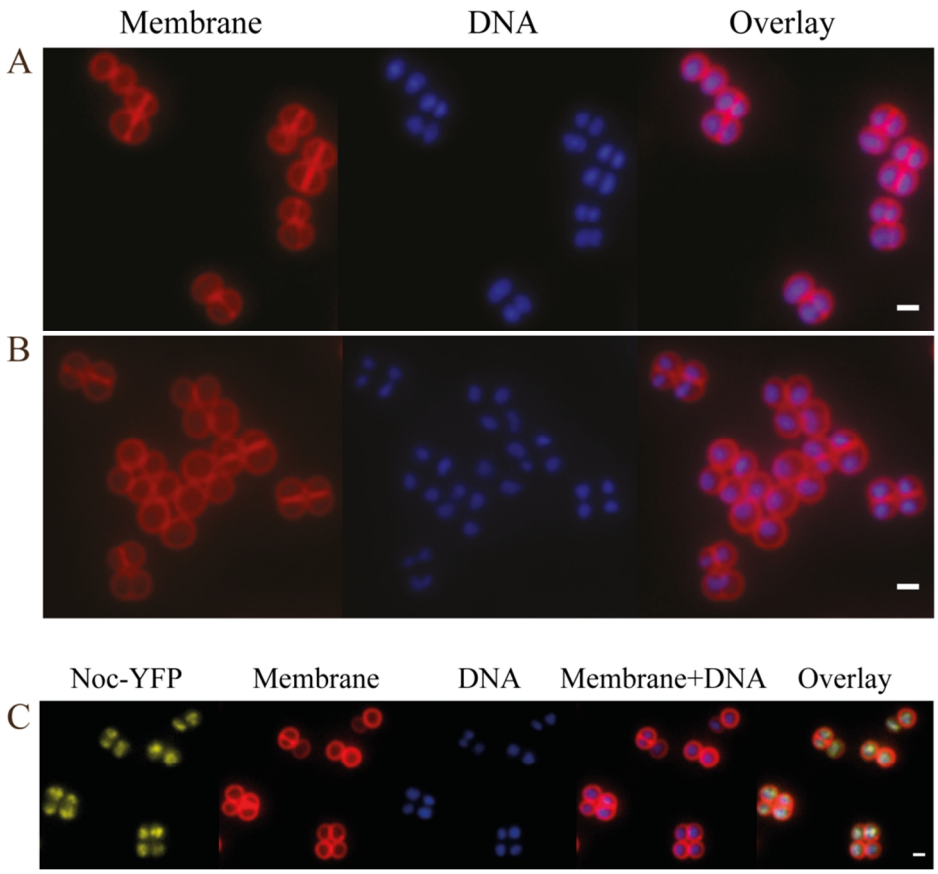


Figure 4.5. Incubation of *S. aureus* cells with chloramphenicol results in DNA condensation. (A) NCTC8325-4 wild-type cells in the absence of chloramphenicol treatment. (B) NCTC8325-4 cells showing condensed nucleoids as a result of the addition of chloramphenicol for 30 min. (C) BCBHV006 cells, expressing Noc-YFP, incubated for 30 min with chloramphenicol, showing that the antibiotic treatment does not result in absence or delocalization of Noc. Cells were stained with the DNA dye Hoechst 33342 and the membrane dyes Nile Red (A,B) or FM5-65 (C). Images are false-coloured. Scale bars, 1μm.

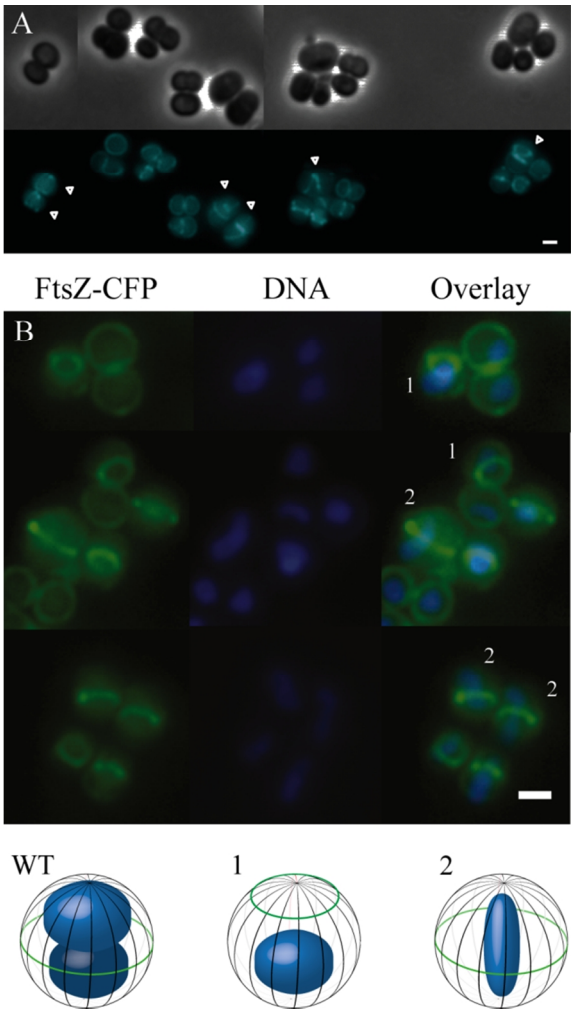


Figure 4.6. FtsZ forms abnormal, non-orthogonal structures when DNA replication/condensation is impaired. (A) FtsZ–CFP localization in BCBHV013 cells that produce Noc, grown for 1h in the presence of the DNA replication inhibitor HPUra. Top panels: phase-contrast images. Bottom panels: FtsZ–CFP fluorescence. Arrowheads point to examples of cells with multiple non-orthogonal FtsZ structures. (B) FtsZ–CFP localization in BCBHV013 cells that produce Noc presenting a condensed nucleoid as a result of a 30 min treatment with chloramphenicol. FtsZ forms abnormal structures in cells with condensed DNA. Cells labeled "1" show FtsZ rings of small diameters which are not located at mid cell; cells labeled "2" show FtsZ rings forming in the DNA free area around condensed, non-bilobed nucleoids presumably because the large distance between the nucleoid and the membrane impairs the action of Noc. DNA was visualized using Hoechst 33342. *ftsZ–CFP* expression was induced by the addition of 0.1 mM IPTG. Images are false-coloured. Scale bars, 1 µm.; WT: Schematic representation of a wild-type cell with Z-ring (green) at mid cell, between the chromosomes (blue). 1 and 2: Schematic representation of cells labeled "1" and "2" described above.

Discussion

Similarly to what occurs in *S. aureus*, initiation of chromosome segregation in *B. subtilis* precedes FtsZ assembly. However, establishment of the axis of chromosome segregation does not constitute, on its own, sufficient information for definition of the division site in this rod-shaped bacteria. Accordingly, *B. subtilis* cells are able to form septa not only at mid-cell, but also near the poles, resulting in the formation of anucleate minicells, a phenotype prevented by the action of the Min system (Levin *et al.*, 1992, Levin *et al.*, 1998). In fact, the Min system seems sufficient to guarantee FtsZ assembly only at mid cell, even in the absence of nucleoid occlusion. For this reason the function of Noc in *B. subtilis* is apparently redundant under normal growth conditions. Only under conditions that perturb DNA replication/segregation or in the absence of the Min system was it possible to observe a strong phenotype of bisection of the nucleoid by the septum, in *B. subtilis noc* mutants (Wu & Errington, 2004). As discussed below, the situation is very different in *S. aureus*, despite the likelihood that the mechanism by which Noc inhibits FtsZ polymerization in *S. aureus* is similar to the mechanism in *B. subtilis.*

The localization of the nucleoid occlusion effector in *S. aureus* overlaps the nucleoid, with highest concentration at places compatible with the localization of the origins of replication. We therefore infer that, like in rod shaped bacteria, the progression of chromosome segregation in *S. aureus* leaves space at midcell where Noc inhibition of FtsZ polymerization is released, allowing the divisome to assemble at that position, even before replication/segregation is completed. However, contrary to rod-shaped bacteria, deletion of *noc* has dramatic consequences for the spherical staphylococcal cells, even under normal growth conditions, in the absence of any perturbations in DNA replication/segregation. We have observed Z-rings associated with membrane constriction assembled over the nucleoid in 15% of the BCBHV001 *noc* mutant cells (Figure 4.2) and multiple FtsZ rings/arcs, also polymerized on top of the DNA, in 15% of the cells (Figure 4.4B and C). The fact that the septum assembles over the nucleoid in BCBHV001 *noc* mutant cells does not necessarily imply that DNA breaks will occur, as cells

have means of clearing trapped DNA, mainly through the action of the DNA translocases SpoIIIE in *B. subtilis* (Wu & Errington, 1994) and FtsK in *E. coli* (Aussel et al., 2002). In fact, Bernhardt and de Boer (2005) have seen apparent movement of the nucleoid through the central septal pore in DnaA$^-$ SlmA$^-$ cells. We have now shown that lack of Noc in *S. aureus* does indeed lead to actual breakage of the chromosomal DNA in 16% of the *noc* null cells (Figure 4.3), implying that Noc acts as an important antiguillotine checkpoint during normal growth of *S. aureus*.

Staphylococcus aureus divides in three orthogonal planes over three successive division cycles leading to the formation of cubic packs of eight cells (Koyama *et al.*, 1977, Tzagoloff & Novick, 1977). The mechanism by which selection of orthogonal division planes occurs is an intriguing question. We have shown that in actively dividing *S. aureus* cells, the replicating chromosomes in each of the two daughter cells are seen elongated, with two segregated origins of replication, in cells that have a division septum but in which FtsZ assembly in the next division plane is not yet observed (Figure 4.1A). Therefore, the axis for chromosome segregation in actively dividing cells is established well before Z-ring placement. This is illustrated in more detail in Figure 4.7A, a schematic representation of a dividing *S. aureus* cell. The left panel represents a cell that has recently formed a septum at the equatorial plane. In theory, at this stage conformational entropy alone could determine the direction of chromosome segregation along an axis parallel to the equator: immediately after septum formation, each of the daughter cells is highly asymmetrical, having one short axis (perpendicular to the equator) and one long axis (parallel to the equator). It has been suggested that under strong confinement conditions, replicated circular chromosomes could partition spontaneously as a result of conformational entropy and excluded-volume interactions of the replicating nucleoid, and this segregation would always occur along the longer axis of a cell (Jun & Mulder, 2006, Jun & Wright, 2010). This is represented in the middle panel of Figure 4.7A, where the nucleoids start to segregate in both daughter cells along one of the longer axis of the cell, releasing the mid cell from the Noc action which inhibits FtsZ polymerization. At this stage, only one division plane becomes available for FtsZ-ring assembly without bisection

of the nucleoid (plane shown in green in Figure 4.7A, right panel), therefore explaining how round cells would divide in two (but not in three) orthogonal planes. However, the system in *S. aureus* has to be considerably more complex, as theoretically there is an infinite number of possible axis for chromosome segregation (all longer axis in daughter cells) and therefore an infinite number of possible division planes (all the meridians in Figure 4.7A). Although all these planes are perpendicular to the previous division plane, only one is chosen by the cell, a choice that cannot be explained by the sole action of entropy.

If chromosome segregation determines the division plane, as we suggest, then in order to understand the basis for the definition of the three orthogonal division planes characteristic of *S. aureus*, we first need to understand what determines the choice of one particular axis for chromosome segregation. We have observed that after complete segregation of the origins of replication, they localize parallel to the previous division plane (Figure 4.1A) near the points which we can infer (based on the geometry of the division planes) correspond to cross junctions of the two previous division planes. This is illustrated in Figure 4.7B, which represents one cell that has previously divided along the two perpendicular planes shown in red (n-1 and n-2) and, in the next division, is going to divide along the meridian plane shown in green (labeled n). It has been previously reported that *S. aureus* cells have "scars" of the two previous division cycles, with the major autolysin Atl, for example, localizing along two perfectly orthogonal rings around the cell (Yamada *et al.*, 1996). If an *oriC* binding protein would localize at the scars from the two previous division planes, then its concentration would be highest at the two poles where these planes cross each other (red circles in Figure 4.7B), where it could anchor the origins of replication. One plausible candidate for this function would be the DivIVA protein. In *B. subtilis*, the polar anchored DivIVA protein is involved in anchoring the origin of replication during sporulation (Ben-Yehuda *et al.*, 2003, Wu & Errington, 2003). However, we have previously deleted DivIVA in *S. aureus* and, under the tested conditions, we were unable to detect any phenotype in cell division or chromosome segregation in these mutants (Pinho & Errington, 2004). Recently, S. Foster and

colleagues have suggested that *S. aureus* uses epigenetic information, contained in a large belt of peptidoglycan with a 'piecrust' texture, to divide in orthogonal planes (Turner *et al.*, 2010). It is possible that this information is used to define the axis of chromosome segregation. An alternative explanation is the existence of a yet unknown and novel mechanism responsible for defining the axis of chromosome segregation in *S. aureus*.

We propose that the following conditions provide sufficient information to establish division in three orthogonal planes over successive division cycles, as illustrated in Figure 4.7B: (i) the origins of replication segregate towards the point where the two previous division planes (n-1 and n-2) cross each other (as we could infer), thereby establishing the axis of chromosome segregation; (ii) the division plane is placed (as we have shown) in the Noc free region generated upon chromosome segregation, and is therefore perpendicular to the axis of chromosome segregation.

Interestingly, the proposed model does not require asymmetry in daughter cells in order for the axis of chromosome segregation to be established. Therefore, although cells emerging from stationary phase may divide in a randomly chosen plane, it is also possible that the information regarding the localization of the two previous planes is maintained in these cells, and is used to determine the first division plane.

If the model we propose is correct, then one would predict that interfering with chromosome segregation would have an effect in Z-ring placement. In fact, addition of the replication elongation inhibitor HPUra to wild-type *S. aureus* cells expressing FtsZ–CFP resulted in the formation of multiple, non-orthogonal Z-rings (Figure 4.6A). Moreover, our model predicts that condensation of the nucleoid would result in free space in the cell, devoid of Noc-mediated inhibition of FtsZ polymerization. In theory, this space could be used for incorrect assembly of Z-rings, which is what we have observed in cells treated with chloramphenicol that have condensed nucleoids (Figure 4.6B).

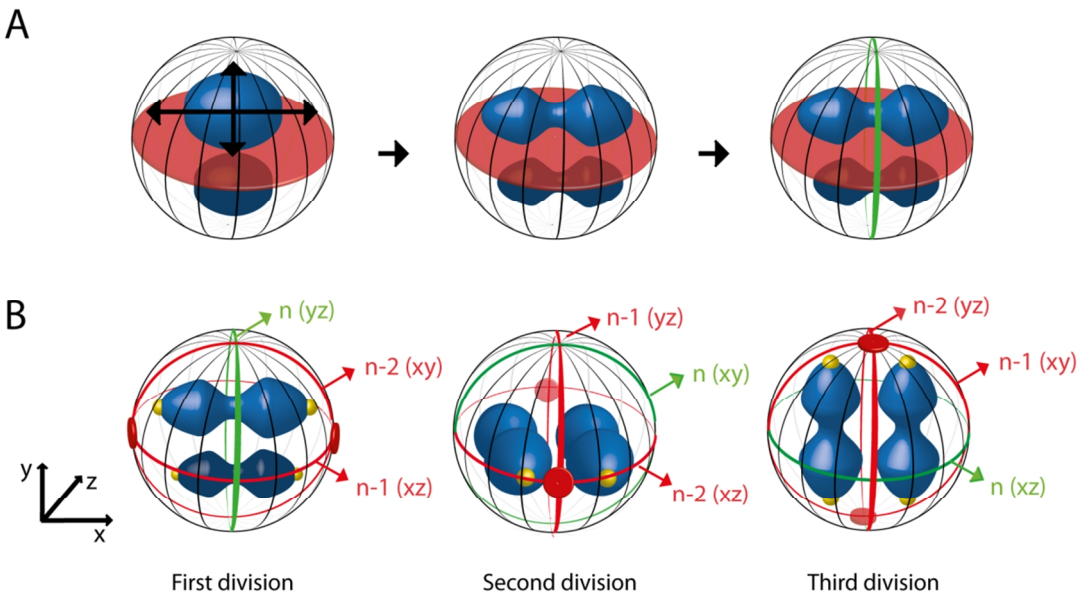


Figure 4.7. Proposed model for cell division in three orthogonal planes. (A) Schematic representation of a cell that has just formed the septum (red) at the equatorial plane of division (left panel). Each daughter cell is asymmetric and has a longer and a shorter axis (black arrows). All potential division planes (meridians) perpendicular to the equatorial plane would result in bisection of the nucleoid (blue). With the progression of the cell cycle, chromosomes segregate along the longer axis of each daughter cell (middle panel). The localization of the chromosome terminus region (free of Noc inhibition of FtsZ polymerization), defines the only division plane available for Z-ring formation, which is shown in green and is perpendicular to the previous division plane in red (right panel). (B) Schematic representation of a cell where the orientation of the current division plane (n, green), the previous division plane (n-1, red) and the division plane from two divisions ago (n-2, red) are represented. The chromosome origins of replication (yellow spheres) always segregate towards the intersection points (small red circles) of the last two planes of division, n-1 and n-2. This automatically defines the next division plane n, as the only plane that does not bisect the nucleoid and which is perpendicular to the two previous division planes. It is possible that an unknown origin-binding factor accumulates at the cross-junction between division planes n-1 and n-2. Notice that green and red lines are not intended to represent complete circles of material in the cell surface, but merely indicate the orientation of the division planes. As each cell divides, it generates daughter cells in which one hemisphere is constituted of new material. However, a T-junction between the two previous division planes remains present in each daughter cell.

If nucleoid occlusion is so important in determining division planes in *S. aureus* cells, why are not Z-rings formed in all cells? Several lines of evidence point to the likely existence of additional nucleoid occlusion effectors in *B. subtilis* and *E. coli*, besides Noc and SlmA. The original nucleoid occlusion model proposed by Woldringh suggested that active transcription/translation around the nucleoid exerts an inhibiting effect on cell division (Mulder & Woldringh, 1989, Woldringh *et al.*, 1991). In the first report of *B. subtilis* Noc, Wu and Errington suggest the existence of a Noc-independent pathway for nucleoid occlusion based on the bias of septum formation toward internucleoid regions in mutants lacking both Noc and the Min system (Wu & Errington, 2004). Similar observations were made in *E. coli* mutants lacking SlmA and the Min system (Bernhardt & de Boer, 2005). More recently, Harry and colleagues proposed an additional mechanism for Z-ring positioning in which the mid cell becomes increasingly "potentiated" for the formation of a Z-ring upon completion of initiation of replication (Moriya *et al.*, 2010), while Rudner and co-workers showed evidence for Noc-independent nucleoid occlusion in the prevention of inappropriate cell division during replication fork arrest (Bernard *et al.*, 2010). Therefore, it is not unlikely that *S. aureus* also uses other system(s) to ensure nucleoid occlusion, particularly because nucleoid occlusion has such a relevant role in cell division in this bacterium.

The fact that lack of a conserved protein, Noc, results in such different phenotypes in cocci and rods, highlights the importance of studying cell division mechanisms in different model organisms, particularly those with different shapes and modes of division from the more traditional model organisms *E. coli* and *B. subtilis.*

References

Adams, D. W. & J. Errington, (2009) Bacterial cell division: assembly, maintenance and disassembly of the Z ring. *Nat Rev Microbiol* **7**: 642-563.

Arnaud, M., A. Chastanet & M. Debarbouille, (2004) New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, Gram-positive bacteria. *Appl Environ Microbiol* **70**: 6887-6891.

Atilano, M. L., P. M. Pereira, J. Yates, P. Reed, H. Veiga, M. G. Pinho & S. R. Filipe, (2010) Teichoic acids are temporal and spatial regulators of peptidoglycan cross-linking in *Staphylococcus aureus*. *Proc Natl Acad Sci U S A* **107**: 18991-18996.

Aussel, L., F. X. Barre, M. Aroyo, A. Stasiak, A. Z. Stasiak & D. Sherratt, (2002) FtsK Is a DNA motor protein that activates chromosome dimer resolution by switching the catalytic state of the XerC and XerD recombinases. *Cell* **108**: 195-205.

Barak, I. & A. J. Wilkinson, (2007) Division site recognition in *Escherichia coli* and *Bacillus subtilis*. *FEMS Microbiol Rev* **31**: 311-326.

Begg, K. J. & W. D. Donachie, (1998) Division planes alternate in spherical cells of *Escherichia coli*. *J Bacteriol* **180**: 2564-2567.

Ben-Yehuda, S., D. Z. Rudner & R. Losick, (2003) RacA, a bacterial protein that anchors chromosomes to the cell poles. *Science* **299**: 532-536.

Bernard, R., K. A. Marquis & D. Z. Rudner, (2010) Nucleoid occlusion prevents cell division during replication fork arrest in *Bacillus subtilis*. *Mol Microbiol* **78:** 866-882.

Bernhardt, T. G. & P. A. de Boer, (2005) SlmA, a nucleoid-associated, FtsZ binding protein required for blocking septal ring assembly over chromosomes in *E. coli*. *Mol Cell* **18**: 555-564.

Bramkamp, M. & S. van Baarle, (2009) Division site selection in rod-shaped bacteria. *Curr Opin Microbiol* **12**: 683-688.

Cha, J.-H. & G. C. Stewart, (1997) The *divIVA* minicell locus of *Bacillus subtilis*. *J Bacteriol* **179**: 1671-1683.

Corbin, B. D., X. C. Yu & W. Margolin, (2002) Exploring intracellular space: function of the Min system in round-shaped *Escherichia coli*. *EMBO J* **21**: 1998-2008.

de Boer, P. A. J., R. E. Crossley & L. I. Rothfield, (1989) A division inhibitor and a topological specificity factor coded for by the minicell locus determine proper placement of the division septum in *E. coli*. *Cell* **56**: 641-649.

Edwards, D. H. & J. Errington, (1997) The *Bacillus subtilis* DivIVA protein targets to the division septum and controls the site specificity of cell division. *Mol Microbiol* **24**: 905-915.

Errington, J., R. A. Daniel & D. J. Scheffers, (2003) Cytokinesis in bacteria. *Microbiol Mol Biol Rev* **67**: 52-65, table of contents.

Gill, S. R., D. E. Fouts, G. L. Archer, E. F. Mongodin, R. T. Deboy, J. Ravel, I. T. Paulsen, J. F. Kolonay, L. Brinkac, M. Beanan, R. J. Dodson, S. C. Daugherty, R. Madupu, S. V. Angiuoli, A. S. Durkin, D. H. Haft, J. Vamathevan, H. Khouri, T. Utterback, C. Lee, G. Dimitrov, L. Jiang, H. Qin, J. Weidman, K. Tran, K. Kang, I. R. Hance, K. E. Nelson & C. M. Fraser, (2005) Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *J Bacteriol* **187**: 2426-2438.

Gruber, S. & J. Errington, (2009) Recruitment of condensin to replication origin regions by ParB/SpoOJ promotes chromosome segregation in *B. subtilis*. *Cell* **137**: 685-696.

Guerout-Fleury, A. M., K. Shazand, N. Frandsen & P. Stragier, (1995) Antibiotic-resistance cassettes for *Bacillus subtilis*. *Gene* **167**: 335-336.

Jun, S. & B. Mulder, (2006) Entropy-driven spatial organization of highly confined polymers: lessons for the bacterial chromosome. *Proc Natl Acad Sci U S A* **103**: 12388-12393.

Jun, S. & A. Wright, (2010) Entropy as the driver of chromosome segregation. *Nat Rev Microbiol* **8**: 600-607.

Kaltwasser, M., T. Wiegert & W. Schumann, (2002) Construction and application of epitope- and green fluorescent protein-tagging integration vectors for *Bacillus subtilis*. *Appl Environ Microbiol* **68**: 2624-2628.

Koyama, T., M. Yamada & M. Matsuhashi, (1977) Formation of regular packets of *Staphylococcus aureus* cells. *J Bacteriol* **129**: 1518-1523.

Levin, P. A., I. G. Kurtser & A. D. Grossman, (1999) Identification and characterization of a negative regulator of FtsZ ring formation in *Bacillus subtilis*. *Proc Natl Acad Sci U S A* **96**: 9642-9647.

Levin, P. A., P. S. Margolis, P. Setlow, R. Losick & D. Sun, (1992) Identification of *Bacillus subtilis* genes for septum placement and shape determination. *J Bacteriol* **174**: 6717-6728.

Levin, P. A., J. J. Shim & A. D. Grossman, (1998) Effect of *minCD* on FtsZ ring position and polar septation in *Bacillus subtilis*. *J Bacteriol* **180**: 6048-6051.

Lewis, P. J. & J. Errington, (1997) Direct evidence for active segregation of *oriC* regions of the *Bacillus subtilis* chromosome and co-localization with the SpoOJ partitioning protein. *Mol Microbiol* **25**: 945-954.

Lutkenhaus, J., (2007) Assembly dynamics of the bacterial MinCDE system and spatial regulation of the Z Ring. *Annu Rev Biochem* **76**: 539–562.

Moriya, S., R. A. Rashid, C. D. Rodrigues & E. J. Harry, (2010) Influence of the nucleoid and the early stages of DNA replication on positioning the division site in *Bacillus subtilis*. *Mol Microbiol* **76**: 634-647.

Mulder, E. & C. L. Woldringh, (1989) Actively replicating nucleoids influence positioning of division sites in *Escherichia coli* filaments forming cells lacking DNA. *J Bacteriol* **171**: 4303-4314.

Oshida, T. & A. Tomasz, (1992) Isolation and characterization of a Tn*551*-autolysis mutant of *Staphylococcus aureus*. *J Bacteriol* **174**: 4952-4959.

Pas, E., M. Einav, C. L. Woldringh & A. Zaritsky, (2001) Perpendicular planes of FtsZ arcs in spheroidal *Escherichia coli* cells. *Biochimie* **83**: 121-124.

Pereira, P. M., H. Veiga, A. M. Jorge & M. G. Pinho, (2010) Fluorescent reporters for studies of cellular localization of proteins in *Staphylococcus aureus*. *Appl Environ Microbiol* **76**: 4346-4353.

Pinho, M. G. & J. Errington, (2003) Dispersed mode of *Staphylococcus aureus* cell wall synthesis in the absence of the division machinery. *Mol Microbiol* **50**: 871-881.

Pinho, M. G. & J. Errington, (2004) A *divIVA* null mutant of *Staphylococcus aureus* undergoes normal cell division. *FEMS Microbiol Lett* **240**: 145-149.

Pinho, M. G., S. R. Filipe, H. de Lencastre & A. Tomasz, (2001) Complementation of the essential peptidoglycan transpeptidase function of penicillin-binding protein 2 (PBP2) by the drug resistance protein PBP2A in *Staphylococcus aureus*. *J Bacteriol* **183**: 6525-6531.

Ramirez-Arcos, S., J. Szeto, T. Beveridge, C. Victor, F. Francis & J. Dillon, (2001) Deletion of the cell-division inhibitor MinC results in lysis of *Neisseria gonorrhoeae*. *Microbiology* **147**: 225-237.

Raskin, D. M. & P. A. J. De Boer, (1999) Rapid pole-to-pole oscillation of a protein required for directing division to the middle of *Escherichia coli*. *Proc Natl Acad Sci U S A.* **96**: 4971-4976.

Rothfield, L., A. Taghbalout & Y. L. Shih, (2005) Spatial control of bacterial division-site placement. *Nat Rev Microbiol* **3**: 959-968.

Turner, R. D., E. C. Ratcliffe, R. Wheeler, R. Golestanian, J. K. Hobbs & S. J. Foster, (2010) Peptidoglycan architecture can specify division planes in *Staphylococcus aureus*. *Nat Commun* **1**: 1-9.

Tzagoloff, H. & R. Novick, (1977) Geometry of cell division in *Staphylococcus aureus*. *J Bacteriol* **129**: 343-350.

Veiga, H. & M. G. Pinho, (2009) Inactivation of the SauI type I restriction-modification system is not sufficient to generate *Staphylococcus aureus* strains capable of efficiently accepting foreign DNA. *Appl Environ Microbiol* **75**: 3034-3038.

Westling-Haggstrom, B., T. Elmros, S. Normark & B. Winblad, (1977) Growth pattern and cell division in *Neisseria gonorrhoeae*. *J Bacteriol* **129**: 333-342.

Woldringh, C. L., E. Mulder, P. G. Huls & N. Vischer, (1991) Toporegulation of bacterial division according to the nucleoid occlusion model. *Res Microbiol* **142**: 309-320.

Wu, L. J. & J. Errington, (1994) *Bacillus subtilis* SpoIIIE protein required for DNA segregation during asymmetric cell division. *Science* **264**: 572-575.

Wu, L. J. & J. Errington, (2003) RacA and the Soj-Spo0J system combine to effect polar chromosome segregation in sporulating *Bacillus subtilis*. *Mol Microbiol* **49**: 1463-1475.

Wu, L. J. & J. Errington, (2004) Coordination of cell division and chromosome segregation by a nucleoid occlusion protein in *Bacillus subtilis*. *Cell* **117**: 915-925.

Wu, L. J., S. Ishikawa, Y. Kawai, T. Oshima, N. Ogasawara & J. Errington, (2009) Noc protein binds to specific DNA sequences to coordinate cell division with chromosome segregation. *Embo J* **28**: 1940-1952.

Yamada, S., M. Sugai, H. Komatsuzawa, S. Nakashima, T. Oshida, A. Matsumoto & H. Suginaka, (1996) An autolysin ring associated with cell separation of *Staphylococcus aureus*. *J Bacteriol* **178**: 1565-1571.

Zaritsky, A., A. Van Geel, I. Fishov, E. Pas, M. Einav & C. L. Woldringh, (1999) Visualizing multiple constrictions in spheroidal *Escherichia coli* cells. *Biochimie* **81**: 897-900.

Zimmerman, S. B., (2006) Shape and compaction of *Escherichia coli* nucleoids. *J Struct Biol* **156**: 255-261.

Chapter 5

SpoIIIE and Slp mediate DNA translocation in *Staphylococcus aureus*

AUTHOR CONTRIBUTIONS

All experiments described in this chapter were performed by H. Veiga. P. M. Pereira constructed plasmid pBCBPM099.
J. Monteiro and Dr. M. P. DeLisa kindly provided pCNX and pTrc99A-P7 plasmids, respectively.

Abstract

The faithful coordination between bacterial cell division and chromosome segregation in rod-shaped bacteria, such as *Escherichia coli* and *Bacillus subtilis*, is known to be dependent on the DNA translocase activity of FtsK/SpoIIIE proteins, which move DNA away from the division site before cytokinesis. However, it is not known at which level these proteins are required for correct chromosome partioning in spherical bacteria like *Staphylococcus aureus*.

Here, we report our findings regarding the activity of the two *S. aureus* FtsK/SpoIIIE homologues, SpoIIIE and Slp (SpoIIIE-like protein). We showed that these proteins operate in independent, but synergistic, pathways to promote movement of the chromosomes, mainly under conditions that compromise their normal partioning. SpoIIIE forms foci in the center of the septum when its activity is required, which occurs even during normal growth. Slp is a multifunctional septal protein which has a C-terminal domain with DNA translocase activity, and N-terminal and linker domains that perform a second function, essential for a proper cell division.

Introduction

Generation of equal progeny through bacterial cell division depends on accurate replication and segregation of the genetic material and on strict coordination between these processes and cytokinesis. Among the several effectors required for successful completion of the bacterial division cycle, are the members of the conserved FtsK/SpoIIIE family of DNA translocases, which synchronize the late stages of cell division with chromosome segregation (Kaimer & Graumann, 2011). All known members of this family share a C-terminal ATPase domain, which forms homo-hexameric DNA motors responsible for the translocation of the DNA away from the division site, thus avoiding its catastrophic bisection by the septum (Kaimer & Graumann, 2011, Kaimer *et al.*, 2009, Massey *et al.*, 2006, Aussel *et al.*, 2002, Bath *et al.*, 2000). The C-terminal motor is connected, via a linker, to a poorly conserved N-terminal domain that often mediates functions unrelated to DNA translocation. The N-terminal domain is also responsible for the septal/membrane localization of FtsK/SpoIIIE proteins despite the absence, in some cases, of an obvious membrane-spanning region (Kaimer & Graumann, 2011).

Escherichia coli cells express only one septal DNA translocase, FtsK, a multifunctional protein that links three essential cell division steps: formation of the division septum, chromosome segregation and resolution of chromosome dimers. The essential FtsK N-terminal domain ($FtsK_N$), which has four transmembrane regions (Dorazi & Dewar, 2000), localizes the protein to the septum and is involved in the assembly of the division apparatus (Wang & Lutkenhaus, 1998, Yu *et al.*, 1998a, Draper *et al.*, 1998, Chen & Beckwith, 2001). This domain is linked to the C-terminal DNA motor ($FtsK_C$) by a long linker that is required for the establishment of important interactions between FtsK and other divisome proteins (Bigot *et al.*, 2004, Dubarry *et al.*, 2010). The $FtsK_C$ functions to clear DNA from the division site and promotes XerCD dependent site-specific recombination between *dif* sites, which is necessary to convert chromosome dimers (formed during replication as a result of crossover events) into two monomers (Yu *et al.*, 1998b, Liu *et al.*, 1998, Steiner *et al.*, 1999).

In *Bacillus subtilis,* two DNA translocases with distinct roles, SpoIIIE and SftA, ensure a proper coordination between cell division and chromosome segregation. SftA, like its *E. coli* homologue FtsK, moves DNA away from the division site before septum constriction, while SpoIIIE acts at a different stage of septation, to move DNA trapped by closed septa (Wu & Errington, 1994, Kaimer *et al.*, 2009, Biller & Burkholder, 2009, Wu *et al.*, 1995).

B. subtilis SpoIIIE was initially known for its essential activity during sporulation (Wu & Errington, 1994). One of the first steps of spore development is the formation of an asymmetric septum to separate the mother cell from the future spore. When this septum assembles, only the origin-proximal region of the chromosome is positioned at the forespore region. Approximately two-thirds of the DNA destined for the spore are still incorrectly located in the mother cell and have to be transported across the closed septum by SpoIIIE (Wu *et al.*, 1995, Bath *et al.*, 2000, Wu & Errington, 1997). This protein is usually distributed throughout the cytoplasmic membrane but, upon chromosome entrapment, it forms DNA translocation channels that cross the septum, visible by fluorescence microscopy as bright foci (Wu & Errington, 1997, Burton *et al.*, 2007). These channels are also formed during *B. subtilis* vegetative growth but only in the rare occasions in which a post-septational mechanism is required to rescue trapped DNA (Sharpe & Errington, 1995).

The rescuing activity of SpoIIIE during vegetative growth is complemented by SftA (Biller & Burkholder, 2009, Kaimer *et al.*, 2009), which localizes at the septum throughout the cell cycle to guarantee a proper segregation of the chromosome before septum constriction. The septal localization of SftA requires a properly assembled FtsZ-ring and is mediated by its N-terminal and linker domains, despite the absence of a predicted transmembrane domain (Kaimer *et al.*, 2009). Under normal growth conditions, the absence of SftA causes only a small increase in the number of chromosomes bisected by the septum. However, the important role of the protein becomes evident when the normal organization, replication and/or segregation of the DNA are perturbed. In these situations, the number of septa formed over unsegregated DNA increases, increasing the

need for a DNA translocase (Kaimer *et al.*, 2009, Biller & Burkholder, 2009). Importantly, the phenotypic defects that result from the absence of SftA C-terminal translocase domain are the same as those resulting from complete deletion of *sftA*, indicating that SftA is most likely a monofunctional protein (Biller & Burkholder, 2009, Kaimer *et al.*, 2009). In contrast, SpoIIIE has a second, independent, role in mediating membrane fusion/fission during sporulation (Sharp & Pogliano, 2003, Sharp & Pogliano, 1999, Liu *et al.*, 2006) .

Overall, SpoIIIE and SftA, have synergistic activities during *B. subtilis* vegetative growth, with SftA always localizing at the septum to regulate chromosome segregation and SpoIIIE acting as a back-up system whenever chromosomes become trapped by the septum (Kaimer *et al.*, 2009, Biller & Burkholder, 2009).

Cell division and chromosome segregation have been studied mostly in the rod-shaped organisms *E. coli* and *B. subtilis* and virtually nothing is known about the mechanism that ensures proper segregation of the chromosomes in bacteria with a spherical shape. *Staphylococcus aureus*, a pathogenic cocci well known for its virulence and ability to acquire resistance to virtually all known classes of antibiotics (Boucher & Corey, 2008, Klevens *et al.*, 2007), has a characteristic mode of division in three ordered orthogonal planes over successive division cycles (Koyama *et al.*, 1977, Tzagoloff & Novick, 1977, Zapun *et al.*, 2008). This mode of division implies that the *S. aureus* chromosomes have to segregate along three consecutive perpendicular axes, contrary to rod-shaped bacteria in which the chromosomes always segregate along the long axis of the cell. Additionally, due to the small size of a staphylococcal cell, not much bigger than a *B. subtilis* spore, chromosome segregation may be under high spatial constraints and require the activity of DNA translocases to move, in a timely manner, the sister chromosomes into the daughter cells compartments. In fact, *S. aureus* cells encode two putative DNA translocases belonging to the FtsK/SpoIIIE family, SpoIIIE, the close homologue of *B. subtilis* SpoIIIE and Slp (SpoIIIE-like protein) that shares a high degree of identity with *B. subtilis* SftA and *E. coli* FtsK proteins.

In this work, we show that the two *S. aureus* DNA translocases act through independent, but cooperative, pathways to ensure proper segregation of the chromosomes. *S. aureus* SpoIIIE assembles in foci at the division septum whenever its activity is needed, which occurs even during normal growth, while Slp always localizes to the septum, through its N-terminal and linker domains. The DNA translocase activity of Slp seems to be required only in situations of defective chromosome replication/ segregation. However, this protein has a second function, associated with its N-terminal and linker domains, which is essential for proper cell division.

Experimental procedures

Bacterial strains and growth conditions

The plasmids and bacterial strains used in this study are listed, respectively, in Tables 5.1 and 5.2. All the primers used are listed in Table 5.3. *S. aureus* strains were grown in tryptic soy broth (TSB, Difco) or tryptic soy agar (TSA, Difco) at 37°C with aeration. The growth medium was supplemented, when required, with: 100 µg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal; BDH Prolabo), 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG; VWR), 0.1µM Cadmium (Sigma-Aldrich) and/or the appropriated antibiotics (10 µg/ml erythromycin, 10 µg/ml chloramphenicol, 50 µg/ml kanamycin and neomycin [each]; Sigma-Aldrich).

Construction of the SpoIIIE mutants

Two SpoIIIE mutants were constructed by removing from the *S. aureus* genome, the entire *spoIIIE* gene (SpoIIIE knock-out mutant) or the sequence coding for its C-terminal domain (SpoIIIE C-terminal deletion mutant). However, since the Shine-Dalgarno sequence of the gene *ymfC*, downstream of *spoIIIE,* is most likely encoded within the last 64bp of *spoIIIE*, this region was maintained in both mutants to avoid polar effects. In the case of the SpoIIIE C-terminal deletion, a stop codon was introduced after codon 280,

between the 3' end of $spoIIIE_{NL}$ (region of *spoIIIE* that encodes for *spoIIIE* N-terminal and linker domains) and the 64bp fragment that was maintained in the chromosome.

Chromosomal deletions were performed using the pMAD plasmid (Arnaud *et al.*, 2004). PCR fragments containing the upstream and downstream flanking regions of the sequences to delete were amplified from chromosomal DNA of NCTC8325-4, using primer pairs SpoIIIEP7/SpoIIIEP8 and SpoIIIEP9/SpoIIIEP6 (for SpoIIIE knock-out mutant), SpoIIIEP10/SpoIIIEP11 and SpoIIIEP12/SpoIIIEP6 (for SpoIIIE C-terminal deletion mutant). In both cases, the two PCR products were joined in a second PCR reaction using primers SpoIIIEP7/SpoIIIEP6, for SpoIIIE knock-out, and SpoIIIEP10/SpoIIIEP6 for deletion of the region encoding the C-terminal domain. The resulting fragments were digested with BamHI and NcoI and cloned into the pMAD vector, giving rise to pBCBHV009 and pBCBHV010, respectively. The insert in each plasmid was sequenced, the vectors were electroporated into RN4220 at 30°C (using erythromycin selection) and subsequently transduced to NCTC8325-4 using phage 80α (Oshida & Tomasz, 1992). The SpoIIIE knock-out mutant strain BCBHV018 and the SpoIIIE C-terminal deletion mutant BCBHV019 were obtained after a two-step homologous recombination process. In the first step, recombinants in which the plasmids were integrated into the chromosome were selected at the non-permissive temperature of 43°C. In the second step, recombinants in which the integrated plasmids (and consequently the *lacZ* and *erm* genes) have been excised were selected at the permissive temperature (30°C) in the absence of antibiotic selection. Both deletions were confirmed by PCR.

Complementation of the SpoIIIE mutation

To complement the *spoIIIE* null mutant BCBHV018, a full copy of the *spoIIIE* gene was inserted in the *spa* locus of the *S. aureus* chromosome, under control of the *spoIIIE* native promoter. For this insertion pMAD-spa (Pereira *et al.*, 2010) was used, a plasmid designed to allow the substitution of *spa* gene for any DNA fragment of interest. Initially, a 2625 bp DNA fragment encompassing the intergenic region between *spoIIIE* and its upstream gene SAOUHSC_01252 (which likely contains the *spoIIIE* promoter) followed by

the *spoIIIE* entire coding sequence, was amplified by PCR, from the NCTC8325-4 genome, using primers SpoIIIEP13/SpoIIIEP14. This insert was then digested with NheI and cloned into pMAD-spa between the downstream and the upstream regions of the *spa* gene. The resulting plasmid pBCBHV011 was sequenced, electroporated into RN4220 at 30°C (with erythromycin selection) and subsequently transduced to the SpoIIIE knockout mutant BCBHV018. The exchange of *spa* for $P_{spoIIIE}$ -*spoIIIE* was obtained after an integration/ excision process, as described above, and confirmed by PCR. The final strain was named BCBHV020.

Construction of *S. aureus* strains expressing a SpoIIIE fluorescent derivative

To study SpoIIIE localization we constructed *S. aureus* strains expressing a C-terminal SpoIIIE-YFP fusion as the only *spoIIIE* copy in the cell. For that purpose, a DNA fragment encompassing a copy of the *spoIIIE* gene without its *stop* codon and encoding a five amino acid linker, at the 3' end, was amplified from NCTC8325-4 genomic DNA using primers SpoIIIEP1 and SpoIIIEP2, digested with KpnI and cloned, in frame with the *yfp* gene, into the pMUTINYFPKan plasmid (Atilano *et al.*, 2010), giving rise to pBCBHV007. The insert in pBCBHV007 was sequenced and this plasmid was used as a template to amplify a DNA fragment containing the 3'end of the *spoIIIE* gene (1065bp) connected to the linker and the *yfp* gene, using primers SpoIIIEP3 and SpoIIIEP4. This fragment was digested and cloned into the BamHI and XmaI restriction sites of the pMAD vector (Arnaud *et al.*, 2004), generating plasmid pMADspoIIIEyfp. A second PCR product, encompassing the last 64bp of *spoIIIE* reading frame (containing the Shine-Dalgarno sequence of the downstream gene) and the 1kb region downstream of *spoIIIE*, was amplified from NCTC8325-4 genome using primers SpoIIIEP5 and SpoIIIEP6. This PCR product was restricted with XmaI and NcoI and subsequently cloned in pMADspoIIIEyfp, downstream of *yfp*, generating plasmid pBCBHV008. The two inserts in pBCBHV008 were sequenced and this plasmid was electroporated into RN4220 and subsequently transduced, using phage 80α, to NCTC8345-4, to the Noc deletion mutant BCBHV001 and to strains BCBHV023, BCBHV024 and BCBHV027 described below (selection with

erythromycin and in the case of BCBHV027 also chloramphenicol and IPTG were used). Integration and excision of pBCBHV008 from the genome was performed as previously described (Arnaud *et al.*, 2004) and colonies in which *spoIIIE* was replaced by the *spoIIIE-yfp* (with the last 64bp of *spoIIIE* duplicated after the *yfp* gene), were selected by PCR. The NCTC8325-4, BCBHV001, BCBHV023, BCBHV024 and BCBHV027 strains expressing SpoIIIE-YFP were named, BCBHV021, BCBHV022, BCBHV028, BCBHV029 and BCBHV030, respectively.

Table 5.1. Plasmids used and constructed in this study

Plasmids	Relevant characteristics	Source or reference
pMAD	*E. coli – S. aureus* shuttle vector with a thermosensitive origin of replication for Gram-positive bacteria; Amp^r, Ery^r	(Arnaud *et al.*, 2004)
pMADspa	pMAD derivative with up- and downstream regions of *spa;* used for ectopic expression of any fragment of interest from the *spa* locus; Amp^r, Ery^r	(Pereira *et al.*, 2010)
pBCB13	pMAD derivative with up- and downstream regions of *spa* and P_{spac}-*lacI*; used for ectopic expression of genes of interest from the *spa* locus, under the control of P_{spac}; Amp^r, Ery^r	(Pereira *et al.*, 2010)
pMGPII	Plasmid encoding *lacI* gene; Cm^r	(Pinho *et al.*, 2001)
pUC18	*E. coli* replicative plasmid, Amp^r	Promega
pCNX	*E. coli – S. aureus* replicative plasmid with cadmium-inducible promoter P_{cad} and a kanamycin resistance cassette; Amp^r, Kan^r	(Charpentier *et al.*, 2004)
pTrc99A-P7	*E. coli* replicative plasmid containing *sfgfp*; Amp^r	(Fisher & DeLisa, 2008)
pMUTINCFP	*B. subtilis* integrative vector for C-terminal CFP fusions, Amp^r, Ery^r	(Kaltwasser *et al.*, 2002)
pMUTINYFPKan	*B. subtilis* integrative vector for C-terminal YFP fusions; Amp^r, Kan^r	(Atilano *et al.*, 2010)
pBCB7–CHKPBP4	pBCB7–CHK containing *pbpD-mcherry*; Amp^r, Kan^r	(Atilano *et al.*, 2010)
pBCBAJ002	pMAD containing *ezrA-mCherry*; Amp^r, Ery^r	(Veiga *et al.*, 2011)
pBCBHV007	pMUTINYFPKan containing *spoIIIE–yfp;* Amp^r, Kan^r	This study

Plasmids	Relevant characteristics	Source or reference
pMADspoIIIEyfp	pMAD derivative containing 3' end of *spoIIIE* –linker-*yfp*; Amp^r, Ery^r	This study
pBCBHV008	pMADspoIIIEyfp derivative containing 3' end of *spoIIIE* – linker-*yfp*-3'64bp of *spoIIIE*-downstream region; Amp^r Ery^r	This study
pBCBHV009	pMAD containing *spoIIIE* upstream region-3' 64bp of *spoIIIE*-downstream region of *spoIIIE* ; Amp^r Ery^r	This study
pBCBHV010	pMAD containing 1064bp upstream of $spoIIIE_C$ –stop codon-3'64bp of *spoIIIE*-downstream region of *spoIIIE*; Amp^r Ery^r	This study
pBCBHV011	pMADspa containing a 381bp fragment upstream of *spoIIIE- spoIIIE* gene; Amp^r Ery^r	This study
pBCBHV012	pMAD containing up- and downstream regions of *slp;* Amp^r Ery^r	This study
pBCBHV013	pBCB13 containing P_{spac}-*slp-lacI*; Amp^r, Ery^r	This study
pBCBHV014	pMAD containing a 1003bp fragment upstream of slp_C - *slp* downstream region ; Amp^r Ery^r	This study
pUC18-SplwalkerA	pUC18 containing a 351bp fragment of *slp* gene, here denominated *splwalkerA*; Amp^r	This study
pUC18-SplwalkerA^{K971A}	pUC18-SplwalkerA containing *splwalkerA* with a point mutation that codes for K971A; Amp^r	This study
pBCBHV015	pMAD containing *splwalkerA* upstream region-*splwalkerA*^{K971A}- *splwalkerA* downstream region; Amp^r Ery^r	This study
pBCB13-SalISlp	pBCB13 containing P_{spac}-SalI restriction site-*slp*- l*acI*; Amp^r Ery^r	This study
pBCBHV016	pBCB13-SalISlp containing P_{spac}-*ftsZ* RBS site– *sfgfp*–linker-*slp*-l*acI*; Amp^r Ery^r	This study
pBCBHV017	pMAD containing *slp* upstream region-*sfgfp*-linker-*slp* 3' end; Amp^r Ery^r	This study
pBCBHV018	pMUTINCFP containing *slp -cfp*; Amp^r Ery^r	This study
pBCBPM099	pCNX containing *pbpD-mcherry;* Amp^r, Kan^r	This study
pCNX-Slp	pCNX containing *slp;* Amp^r, Kan^r	This study
pCNX-Slp_N	pCNX containing slp_N; Amp^r, Kan^r	This study
pCNX-Slp_{NL}	pCNX containing slp_{NL}; Amp^r, Kan^r	This study
pCNX-sfGFP-Slp	pCNX containing *sfgfp- slp;* Amp^r, Kan^r	This study
pCNX-sfGFP-Slp_N	pCNX containing *sfgfp-* slp_N; Amp^r, Kan^r	This study
pCNX-sfGFP-Slp_{NL}	pCNX containing *sfgfp-* slp_{NL}; Amp^r, Kan^r	This study
pCNX-sfGFP-Slp_{LC}	pCNX containing *sfgfp-* slp_{LC}; Amp^r, Kan^r	This study
pCNX-sfGFP-Slp_C	pCNX containing *sfgfp-* slp_C; Amp^r, Kan^r	This study

Table 5.2. Bacterial strains used and constructed in this study

Name	Relevant characteristics	Source or reference
E. coli		
DH5α	*recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1* φ80Δ*lacZ*ΔM15	Lab strain
S. aureus		
RN4220	MSSA strain. Mutagenized strain derived from NCTC8325-4, that accepts foreign DNA	R. Novick
NCTC8325-4	MSSA strain	R. Novick
BCBHV018	NCTC8325-4 Δ*spoIIIE*	This study
BCBHV019	NCTC8325-4 Δ*spoIIIE::spoIIIE*$_{NL}$	This study
BCBHV020	NCTC8325-4 Δ*spoIIIE* Δ*spa::P*$_{spoIIIE}$*-spoIIIE*	This study
BCBHV021	NCTC8325-4 Δ*spoIIIE::spoIIIE-yfp*	This study
BCBHV022	NCTC8325-4 Δ*noc* Δ*spoIIIE::spoIIIE-yfp*	This study
BCBHV023	NCTC8325-4 Δ*slp::slp*$_{NL}$	This study
BCBHV024	NCTC8325-4 Δ*slp::slp*^{K971A}	This study
8325-4ΔSlp*	NCTC8325-4 Δ*slp* with phenotype A (see results)	This study
8325-4ΔSlp	NCTC8325-4 Δ*slp* with phenotype B (see results)	This study
BCBHV025	NCTC8325-4 Δ*spa::P*$_{spac}$*-slp-lacI*	This study
BCBHV026	NCTC8325-4 Δ*slp* Δ*spa::P*$_{spac}$*-slp-lacI*	This study
BCBHV027	NCTC8325-4 Δ*slp* Δ*spa::P*$_{spac}$*-slp-lacI lacI* ^{mc}; Cm^r	This study
BCBHV028	NCTC8325-4 Δ*spoIIIE::spoIIIE-yfp* Δ*slp::slp*$_{NL}$	This study
BCBHV029	NCTC8325-4 Δ*spoIIIE::spoIIIE-yfp* Δ*slp::slp*^{K971A}	This study
BCBHV030	NCTC8325-4 Δ*spoIIIE::spoIIIE-yfp* Δ*slp* Δ*spa::P*$_{spac}$*-slp-lacI lacI* ^{mc}; Cm^r	This study
BCBHV031	NCTC8325-4 Δ*spoIIIE* Δ*slp::slp*$_{NL}$	This study
BCBHV032	NCTC8325-4 Δ*spoIIIE* Δ*slp::slp*^{K971A}	This study
BCBHV033	NCTC8325-4 Δ*slp::sfgfp-slp*	This study
BCBHV034	NCTC8325-4 Δ*spoIIIE* Δ*slp::sfgfp-slp*	This study
BCBHV035	NCTC8325-4 Δ*slp::sfgfp-slp*$_{NL}$	This study
BCBHV036	NCTC8325-4 Δ*slp::sfgfp-slp* Δ*ezrA::ezrA-mCherry*	This study
BCBHV037	NCTC8325-4 Δ*slp::sfgfp-slp* pBCBPM099, expressing sfGFP-Slp and PBP4-mCherry; Kan^r	This study
BCBHV038	NCTC8325-4 Δ*spoIIIE::spoIIIE-yfp slp::*pBCBHV018, expressing SpoIIIE-YFP and Slp-CFP; Ery^r	This study
BCBHV039	8325-4ΔSlp pCNX; Kan^r	This study
BCBHV040	8325-4ΔSlp pCNX-Slp; Kan^r	This study
BCBHV041	8325-4ΔSlp pCNX-Slp$_N$; Kan^r	This study
BCBHV042	8325-4ΔSlp pCNX-Slp$_{NL}$; Kan^r	This study
BCBHV043	8325-4ΔSlp pCNX-sfGFP-Slp; Kan^r	This study
BCBHV044	8325-4ΔSlp pCNX-sfGFP-Slp$_N$; Kan^r	This study
BCBHV045	8325-4ΔSlp pCNX-sfGFP-Slp$_{NL}$; Kan^r	This study
BCBHV046	8325-4ΔSlp pCNX-sfGFP-Slp$_{LC}$; Kan^r	This study
BCBHV047	8325-4ΔSlp pCNX-sfGFP-Slp$_C$; Kan^r	This study

lacI ^{mc} – cells expressing multiple copies of the *lacI* gene (encoded by pMGPII)

Construction of a Slp knockout mutant

To investigate the function of *S. aureus* Slp, we constructed a knockout mutant of this gene in the background of NCTC8325-4. For that purpose, the majority of the *slp* open reading frame was removed from the *S. aureus* genome, except for the first 9bp at 5' end and the last 10 bp at 3' end, which were kept to avoid possible polar effects, resulting from alterations in the expression of genes in the *slp* operon. A PCR fragment containing the upstream and downstream regions of the sequence to be deleted were amplified from NCTC8325-4 genomic DNA, in two sequential PCR steps. First, the upstream and downstream regions were amplified individually using the primer pairs SlpP1/SlpP2 and SlpP3/SlpP4, respectively. These two amplified products were then purified and joined in a second PCR reaction, using primers SlpP1 and SlpP4. The final PCR product was digested with BamHI and NcoI and cloned into pMAD, giving rise to pBCBHV012. The insert in pBCBHV012 was sequenced and the vector was electroporated into RN4220 (selection with erythromycin) and subsequently transduced to NCTC8325-4 by phage transduction, using phage 80α. The NCTC8325-4 strain containing the pBCBHV012 plasmid was incubated at the non-permissive temperature of 43°C, in the presence of erythromycin, to select for recombinants in which the plasmid had integrated into the chromosome and then incubated at the permissive temperature of 30°C, in the absence of antibiotic selection, to select for white colonies in which the vector (and consequently *lacZ* and *erm* genes) had been excised. The absence of the *slp* gene was confirmed by PCR screening of the white, erythromycin sensitive colonies obtained, using primers SlpP5 and SlpP6. As described in the results section, the integration/excision procedure was repeated several times and 10 independent Slp knock-out mutants were obtained, they were named 8325-4ΔSlp* and 8325-4ΔSlp as described in the results section.

The complementation studies of 8325-4ΔSlp* and 8325-4ΔSlp strains were performed by expressing Slp from the ectopic *spa* locus in the *S. aureus* chromosome, under the control of the IPTG-inducible P_{spac} promoter. For that purpose, the entire *slp* coding sequence was amplified by PCR, from NCTC8325-4 genomic DNA, using primers

SlpP7 and SlpP8, digested with XmaI and XhoI, and cloned into pBCB13 (Pereira *et al.*, 2010), generating pBCBHV013. The insert in pBCBHV013 was sequenced and the vector electroporated into RN4220 and transduced to 8325-4ΔSlp* and 8325-4ΔSlp strains (selection with erythromycin). The substitution of the *spa* gene for the P_{spac}-*slp* sequence was completed after a two-step homologous recombination process, as described above.

Generation of an *S. aureus* Slp inducible mutant

To construct an inducible Slp mutant, a full copy of the *slp* gene was first placed in the *spa* locus under the control of the P_{spac} promoter and, subsequently, while in the presence of IPTG, *slp* was deleted from its native chromosomal locus.

The substitution of the *spa* gene for P_{spac}-*slp,* was performed as described above, using the pBCBHV013 plasmid. This vector was transduced from RN4220 to NCTC8325-4 strain and then, after an integration and excision events, strain BCBHV025 was generated, which contains two copies of *slp* gene in the chromosome, one in the native locus and another in the *spa* locus. Subsequently, to delete *slp* from its normal locus in the BCBHV025 background, the pBCBHV012 plasmid was transduced into this strain and, after an integration and excision events, strain BCBHV026 was generated expressing a single *slp* copy from the *spa* locus, under the control of P_{spac}. The plasmid pMGPII, which expresses the P_{spac} repressor LacI, was also transduced into BCBHV026 strain, to ensure tight regulation of *slp* expression. The resultant *slp* inducible mutant was named BCBHV027.

Construction of Slp mutants in the DNA translocase domain

In order to study Slp DNA translocase activity, a Slp C-terminal deletion mutant and a Slp Walker A mutant were constructed, in the background of NCTC8325-4.

The Slp C-terminal deletion mutant was generated by removing from the *S. aureus* genome the sequence that codes for the Slp C-terminal domain (after codon 816). For that purpose, the upstream and downstream regions of the sequence to delete were amplified by PCR, using the primer pairs SlpP9/SlpP10 and SlpP11/SlpP4, respectively. The

two PCR products were purified, joined in a second PCR reaction using primers SlpP9 and SlpP4 and the resulting fragment was digested with BamHI and NcoI and cloned into pMAD. The resulting plasmid pBCBHV014 was sequenced, electroporated into RN4220 (selection with erythromycin) and subsequently transduced to NCTC8325-4, using phage 80α. The BCBHV023 Slp C-terminal deletion mutant was obtained after integration and excision of pBCBHV014.

The Slp Walker A mutant was constructed by introducing a point mutation into the *slp* gene that causes the substitution of a conserved lysine, K971, into an alanine. Firstly, a 351bp DNA fragment, containing the coding sequence for the Slp Walker A ATP motif (here named *splwalkerA* fragment), was amplified by PCR, from NCTC8325-4 genomic DNA, using primers SlpP12 and SlpP13. After restriction with KpnI, this fragment was cloned into pUC18, giving rise to pUC18-SplwalkerA. The insert in pUC18-splwalkerA was sequenced and this vector was used as DNA template for the site directed mutagenesis PCR, using primers SlpP14 and SlpP15. The resultant PCR product was treated with DpnI, transformed into *E. coli* strain DH5α, and the positive colonies, harboring a pUC18-splwalkerA^{K971A} plasmid with the point mutation, were selected by sequencing. To substitute the chromosomal *splwalkerA* sequence for *splwalkerA*^{K971A}, this fragment was amplified from pUC18-splwalkerA^{K971A} using the primers Slp18 and Slp19 and joined, by PCR, to its chromosomal upstream and downstream regions amplified from NCTC8325-4 genomic DNA, using the primer pairs Slp16/Slp17 and Slp20/Slp21, respectively. The upstream and *splwalkerA*^{K971A} sequences were first joined by PCR using primers SlpP16 and SlpP19 and the resulting fragment was then linked to the downstream sequence in a second PCR reaction, using primers SlpP16 and SlpP21. The final PCR product was digested with EcoRI and NcoI, cloned into pMAD and sequenced. The pMAD vector containing the upstream-*splwalkerA*^{K971A}-downstream fragment, named pBCBHV015, was electroporated into RN4220 and transduced to NCTC8325-4 (selection with erythromycin). The substitution of *splwalkerA* for *splwalkerA*^{K971A}, in the *spl* gene of NCTC8325-4 chromosome, was obtained after a two-step homologous recombination

process and was confirmed by sequencing. The Slp Walker A mutant was named BCBHV024.

Construction of *S. aureus* mutants lacking the DNA translocase activities from SpoIIIE and Slp proteins

In order to construct *S. aureus* strains lacking both SpoIIIE and Slp DNA translocase motors, we deleted the *spoIIIE* gene in the background of BCBHV023 Slp C-terminal mutant and BCBHV024 Slp Walker A mutant. For that, plasmid pBCBHV009 was transduced into BCBHV023 and BCBHV024, using phage 80α, and the *spoIIIE* gene was deleted, in these backgrounds, as described previously for NCTC8325-4. The double mutants SpoIIIE/Slp C-terminal domain and SpoIIIE/Walker A were named, BCBHV031 and BCBHV032, respectively.

Construction of Slp fluorescent derivatives

To localize Slp, we constructed *S. aureus* strains expressing a super-fast folding GFP (sfGFP) (Fisher & DeLisa, 2008) N-terminal fusion to Slp, from the native locus, as the only Slp copy in the cell. For that, we inserted *sfgfp* and the coding sequence for a 7 amino acids linker, upstream of *slp* gene, in the *S. aureus* chromosome.

Initially, a DNA fragment containing a copy of *slp* gene, preceded by a SalI restriction site, was amplified from NCTC8325-4 genomic DNA using primers SlpP22 and SlpP8, digested with XmaI and XhoI and cloned into pBCB13, giving rise to pBCB13-SalISlp. A second DNA sequence encompassing the *ftsZ* RBS site, an ATG codon, *sfgfp* without the stop codon and a five amino acids linker was amplified from the pTrc99A-P7 vector (Fisher & DeLisa, 2008) using primers sfGFPP1 and sfGFPP2. This fragment was subsequently digested with XmaI and SalI and cloned upstream of the first fragment in pBCB13-SalISlp plasmid, generating pBCBHV016. Both inserts in pBCBHV016 were sequenced and this plasmid was used as a template for a PCR reaction using primers SlpP25 and SlpP26, to amplify a 1441bp fragment encompassing *sfgfp*, a 7 amino acids linker (the linker became longer after insertion of the SalI restriction site) and the first

706bp of the *slp* gene. The 707bp region upstream of the *slp* gene was also amplified by PCR, from the NCTC8325-4 genome, using primers slpP23 and slpP24. These two fragments were then joined in a second PCR reaction, with primers slpP23 and slpP26, and the final PCR product was digested and cloned between the BamHI and XmaI sites of pMAD, generating pBCBHV017. The insert in pBCBHV017 was sequenced and the plasmid was electroporated into RN4220 at 30°C (selection with erythromycin) and subsequently transduced to NCTC8325-4 and BCBHV018 strains. The introduction of *sfgfp*-7 aminoacids linker between *slp* upstream region and the 5' end of *slp* gene was obtained after integration and excision of pBCBHV017 from the *slp* locus. NCTC8325-4 and BCBHV018 strains expressing sfGFP-Slp were named BCBHV033 and BCBHV034, respectively.

In order to observe the localization of Slp_{NL} (Slp protein without its C-terminal domain, after amino acid 816), pBCBHV017 was also introduced into BCBHV023 Slp C-terminal deletion mutant, by phage transduction using phage 80α. The BCBHV023 strain expressing sfGFP-Slp_{NL} was selected after integration/excision events and was named BCBHV035.

To visualize, simultaneously, the position of Slp and the early divisome protein EzrA, we constructed an *S. aureus* strain expressing sfGFP-Slp and EzrA-mcherry. For that, plasmid pBCBAJ002 (Veiga *et al.*, 2011) was introduced into strain BCBHV033 by phage transduction and, through an integration and excision process, the *ezrA* gene was replaced by *ezrA–mCherry*, resulting in strain BCBHV036. To co-localize Slp with the late divisome protein PBP4, a *S. aureus* strain expressing sfGFP-Slp and PBP4-mcherry was also constructed. The *pbp4-mCherry* sequence was amplified from pBCB7–CHKPBP4 plasmid (Atilano *et al.*, 2010), using primers PBP4mChP1 and PBP4mChP1, digested with BamHI and cloned into pCNX (pCN plasmid from (Charpentier *et al.*, 2004) harboring a kanamycin resistance cassette; Monteiro J. unpublished) downstream of P_{cad} promoter, generating pBCBPM099. The insert was sequenced and pBCBPM099 was electroporated in RN4220 and subsequently transduced to strain BCBHV033, generating the strain BCBHV037, which expresses sfGFP-Slp, as well as two copies of PBP4, a native copy from *pbpD* locus and PBP4-mCherry from the pCNX plasmid by induction with 0.1 µM of Cadmium.

Construction of a *S. aureus* strain co-expressing fluorescent derivatives of SpoIIIE and Slp

In order to colocalize SpoIIIE and Slp, a *S. aureus* strain expressing SpoIIIE-YFP and Slp-CFP was constructed. A DNA fragment encompassing a complete copy of the *slp* gene was amplified from NCTC8325-4 genomic DNA, using the primer pair SlpP27/SlP28, digested with HindIII and EagI and cloned into pMUTINCFP (Kaltwasser *et al.*, 2002), giving rise to pBCBHV018. After sequencing of the insert, pBCBHV018 was electroporated into RN4220 (selection with erythromycin) and transduced to BCBHV021 strain, which expresses SpoIIIE-YFP. The integration of pBCBHV018 into the *slp* locus originated strain BCBHV038, in which the *slp–cfp* fusion was controlled by the *slp* native promoter. An extra copy of Slp was controlled by P_{spac} and was induced with 0.5 mM IPTG, and the SpoIIIE-YFP fusion was expressed from the *spoIIIE* locus as the only copy in the cell.

Construction of 8325-4ΔSlp strains expressing truncated derivatives of Slp and sfGFP-Slp

To investigate the function and localization of Slp domains, different truncated forms of Slp and sfGFP-Slp proteins were expressed from the replicative plasmid pCNX in the Slp knock-mutant strain 8325-4ΔSlp.

The entire *slp* gene sequence and the coding sequences for Slp N-terminal domain (slp_N, between codons 1 and 174) and Slp N-terminal and linker domains (slp_{NL}, between codons 1 and 815) were amplified by PCR, from NCTC8325-4 genomic DNA, using the primer pairs Slp7/Slp29, Slp7/Slp30 and Slp7/SlpP31 (SlpP30 and SlpP31 introduce a STOP codon at 3' end of the PCR products), respectively. The three fragments were then digested with XmaI and cloned into the pCNX plasmid, downstream of the P_{cad} promoter, to generate plasmids pCNX-Slp, pCNX-Slp_N and pCNX-Slp_{NL}, respectively. The inserts were sequenced and these plasmids, as well as the empty pCNX plasmid, were electroporated into RN4220 (selection with Kanamycin and Neomycin) and subsequently transduced to strain 8325-4ΔSlp, using phage 80α. The *S. aureus* 8325-4ΔSlp strains containing pCNX-Slp, pCNX-Slp_N, pCNX-Slp_{NL} and pCNX were named BCBHV039, BCBHV040, BCBHV041 and BCBHV042, respectively.

The N-terminal sfGFP fusions to the full-length Slp protein, Slp_N and Slp_{NL} were obtained by first amplifying, from the pBCBHV016 plasmid, the coding sequences for *sfgfp-slp*, *sfgfp-slp*$_N$ and *sfgfp-slp*$_{NL}$ using the primer pairs sfGFPP1/SlpP29, sfGFPP1/SlpP30 and sfGFPP1 /SlpP31, respectively. The *sfgfp-slp*$_{LC}$ and *sfgfp-slp*$_C$ DNA fragments were obtained in sequential PCR steps. First, *slp*$_{LC}$ and *slp*$_C$ coding sequences were amplified by PCR, from NCTC8325-4 genomic DNA, using respectively the primer pairs SlpP32/SlpP29 and SlpP33/SlpP29. In parallel, two DNA sequences encompassing *sfgfp*, a 5 amino acids linker and a 3' end "tail" that overlaps with the 5' end of *slp*$_{LC}$ or *slp*$_c$ were also amplified, using pTrc99A-P7 plasmid as a template and the primer pairs sfGFPP1/sfGFPP3 and sfGFPP1/sfGFPP4. Finally, the amplified DNA fragments were joined in second PCR reactions to obtain *sfgfp*-5aa linker-*slp*$_{LC}$ and *sfgfp*-5aa linker-*slp*$_C$ sequences, using primers sfGFPP1 and SlpP29. The amplified fragments were digested with XmaI, cloned into pCNX and sequenced. The resulting plasmids pCNX-sfGFP-Slp, pCNX-sfGFP-Slp_N, pCNX-sfGFP-Slp_{NL}, pCNX-sfGFP-Slp_{LC} and pCNX-sfGFP-Slp_C were electroporated into RN4220 and then transduced to 8325-4ΔSlp strain, generating respectively, the strains BCBHV043, BCBHV044, BCBHV045, BCBHV046 and BCBHV047.

Importantly, all of the pCNX-encoded proteins in strains BCBHV039 to BCBHV047 were expressed without the addition of Cadmium to the growth medium. This occurs because the P_{cad} promoter is leaky and we observed that the basal expression level of the proteins was sufficient to complement lack of the *slp* gene.

Analysis of growth of *S. aureus* strains

Overnight liquid cultures of *S. aureus* strains NCTC8325-4, BCBHV018, BCBHV023, BCBHV024, BCBHV031 and BCBHV032, were diluted (1:1000) into fresh TSB media and incubated at 37°C with aeration. The Slp inducible mutant BCBHV027 was grown overnight in TSB medium supplemented with 10 µg/ml of chloramphenicol and 0.5mM of IPTG. The following day, the BCBHV027 culture was harvested, washed with fresh TSB and re-inoculated in media with and without IPTG. Growth was analyzed by measuring, at regular intervals, the optical density at 600nm (OD_{600nm}) of the cultures.

Table 5.3. Primers used in this study

Primer Name	Primer Sequence (5'- 3')*
SpoIIIEP1	gctgcggtaccgtcatagctattttagtagttg
SpoIIIEP2	gctgcggtacc**ggaggcgccgcagga**cacctcgtcattattaagatc
SpoIIIEP3	tgaggatccgatgaaaaattcccgtct
SpoIIIEP4	tactccccgggttacttgtacagctcgtcc
SpoIIIEP5	tactccccgggcggtccacaaaaaggaag
SpoIIIEP6	tgcattccatgggacatgctgatctttgaattttgaaattg
SpoIIIEP7	tgaggatcccgtgctggcgcacatattattccaaataac
SpoIIIEP8	ccttttttgtggaccgtaatacaactactaaaatagc
SpoIIIEP9	gttgtattacggtccacaaaaaggaagcaagcctag
SpoIIIEP10	tgaggatcccctttcatcgtctgtttggac
SpoIIIEP11	ggaccgttaaacatacgatacattcgtaac
SpoIIIEP12	cgtatgtttaacggtccacaaaaaggaagc
SpoIIIEP13	ggtcatgctagctcaaaaagtcattaacataaaagagg
SpoIIIEP14	ggtcatgctagcttacacctcgtcattattaagatc
SlpP1	tgaggatccggtaatatttttttattttattaactc
SlpP2	cataattattctttaccagctcatattttcactacc
SlpP3	gagctggtaaagaataattatgagtaaggag
SlpP4	tgcattccatgggtttcaattgtagcactaatttctcttgg
SlpP5	ggtgttgcctcaagcggtatg
SlpP6	gagcaacttctgtaaatacg
SlpP7	tactccccggggcatttttttgaataattaaggaagg
SlpP8	gctgcctcgagttattctttatttaaatctgc
SlpP9	tgaggatccggatactacttcaaatatc
SlpP10	ccttactcataattatttaatatttgggcctttacg
SlpP11	cccaaatattaaataattatgagtaaggagttttat
SlpP12	gctgcggtaccgatgacattaaaatggc
SlpP13	gctgcggtaccaaccatttttggatcgataag
SlpP14	agacatcaaaatactattgatacaaactgatGCccctgatccagttgcacctgcaattagtgc
SlpP15	gcactaattgcaggtgcaactggatcagggGCatcagtttgtatcaatagtattttgatgtct
SlpP16	gctgaattcgataaaaagcgtatgatgg
SlpP17	cattttaatgtcatcttgtaatgccgtaattc
SlpP18	ggcattacaagatgacattaaaatggcattggc
SlpP19	ggagctaattcaaccatttttggatcgataag
SlpP20	ccaaaaatggttgaattagctccttataatgg
SlpP21	tgcattccatggcgtaacataaacatcccttgg
SlpP22	tactccccggggcctccgtcgacatgagctggtttgataaat
SlpP23	tgaggatccgagatccaaacgtgattcagcg
SlpP24	ctcctttactcatattttcactaccttccttaa
SlpP25	ggtagtgaaaatatgagtaaaggagaagaacttttc
SlpP26	tactccccgggctaattgtttattttgagacg
SlpP27	gctgcaagcttgcatttttttgaataattaaggaagg
SlpP28	gctggcacggccgttctttatttaaatctgcttccg
SlpP29	tactccccgggttattctttatttaaatctgc
SlpP30	tactccccgggttatggagttgaatctttagcacg
SlpP31	tactccccgggttatttaatatttgggcctttacgaatc

Primer Name	Primer Sequence (5'- 3')*
SlpP32	gaactatacaaatcctgcggcgcctccgattatcacaaagaaagtttc
SlpP33	gaactatacaaatcctgcggcgcctccttgccaagtgtttcattactag
sfGFPP1	tactcccgggggccaataaaactaggaggaaatttaaatgagtaaaggagaagaacttttc
sfGFPP2	gctgcgctgtcgac**ggaggcgccgcagga**tttgtatagttcatccatgcc
sfGFPP3	gtgataatcggaggcgccgcaggatttgtatagttcatccatgcc
sfGFPP4	gaaacacttggcaaggaggcgccgcaggatttgtatagttcatccatgcc
PBP4mChP1	cgcaggatccaggaggtaccttatgaaaaatttaatatctattatc
PBP4mChP2	Gctggatcctacttgtacagctcgtccat

* Underlined sequences correspond to restriction sites; Bold sequences correspond to the five-codon linker; Capital letters in primers SlpP14 and SlpP25 correspond to the point mutations introduced

Determination of the minimal inhibitory concentration (MIC)

The MIC of Nalidixic acid, HPUra and Oxacillin in the SpoIIIE and Slp single and double mutant strains were determined in liquid TSB medium by a micro-dilution method. Overnight cultures were added, at a final cell density of $5x10^5$ CFU/ml, to wells in a 96-well plate containing 2-fold dilutions of each antibiotic and plates were incubated for 24 hours, or 48h for Oxacillin, at 37°C. The MIC was recorded as the lowest antibiotic concentration that inhibited bacterial growth. All MIC determinations were performed in triplicate.

Analysis of the expression of fluorescent proteins in *S. aureus*

To compare the expression levels of sfGFP-Slp in strains BCBHV033 and BCBHV034 and SpoIIIE-YFP in strains BCBHV021, BCBHV022, BCBHV028 and BCBHV021 grown in the presence of Nalidixic acid, total protein extracts from each strain were prepared. For that purpose, cells were harvested from mid-exponential phase cultures, re-suspended in 1X Phosphate Buffered Saline (PBS) and disrupted with glass beads in a Fast Prep FP120 (Thermo Electro Corporation). The total protein content of the extracts was quantified by the Bradford method, using bovine serum albumin as a standard (BCA protein assay kit, Pierce) and equal amounts of protein, from each sample, were loaded onto an 8% SDS-PAGE gel and separated at 120V. Gel images were acquired on a Fuji FLA 5100 laser scanner (Fuji Photo Film) using 473 nm laser for YFP and GFP fusions and 635 nm laser for detection of protein molecular weight marker.

Fluorescence microscopy

S. aureus strains were grown overnight, in TSB at 37°C, with appropriate antibiotic selection and, the next day, were diluted (1:1000) in fresh TSB supplemented with 0.5mM IPTG when necessary. To visualize the DNA and the membrane, 1ml of the mid-exponential phase culture was incubated with the DNA dye Hoechst 33342 (1 µg/ml, Invitrogen) and the membrane dye Nile Red (5µg/ml, Invitrogen) at room temperature for 5 minutes. The cultures were then centrifuged, re-suspended in 20 µl of PBS and 1 µl was placed on a thin film of 1% agarose in PBS. Fluorescence microscopy was performed using a Zeiss Axio Observer.Z1 microscope equipped with a Photometrics CoolSNAP HQ2 camera (Roper Scientific), using Metamorph software (Molecular devices). Analysis of fluorescence images was performed using Metamorph and ImageJ software.

To determine SpoIIIE-YFP localization in the presence of Nalidixic acid, HPUra and Oxacillin, BCBHV021 cells were grow until OD_{600nm} 0.5 and, at that point, the antibiotic of interest was added to the medium (800 µg/ml Nalidixic acid, 20 µg/ml HPUra or 0.8 µg/ml Oxacillin). One aliquot was kept without antibiotic as a control. The cultures were then incubated for 30 min at 37ºC, prior to SpoIIIE-YFP visualization.

The effect of Nalidixic acid on Slp C-terminal deletion mutant and Slp Walker A mutant was tested by growing NCTC8325-4, BCBHV023 and BCBHV024 cells until OD_{600nm} 0.5 and then adding 800 µg/ml Nalidixic acid to the medium. After antibiotic addition, the cultures were incubated for 30 min at 37ºC, the cells were then labeled with Hoechst 33342 and Nile Red and visualized by fluorescence microscopy.

Results

Staphylococcus aureus expresses two putative DNA translocases of the FtsK/SpoIIIE family

The *S. aureus* chromosome encodes two genes, identified in NCTC8325 NCBI genome database as SAOUHSC_01253 and SAOUHSC_01857, which encode for homologues of the two already identified *B. subtilis* DNA translocases, SpoIIIE and SftA, respectively.

The product of the gene SAOUHSC_01253, which we named SpoIIIE, is a close homologue of the *B. subtilis* SpoIIIE protein (45.9% identity, LALIGN), with 747 amino acids and a calculated molecular mass of 83.4 kDa. The protein encoded by SAOUHSC_01857 is the *S. aureus* homologue of the *E. coli* FtsK and *B. subtilis* SftA proteins, and was named Slp (SpoIIIE-like protein). This is a 1274-residue-long protein with a mass of 144.7 kDa that, like its SftA counterpart, is predicted to be soluble (TMHMM Server v. 2.0). The putative role of Slp as a DNA translocase is evidenced by the high degree of homology between its C-terminal domain and the DNA translocase motors of FtsK and SftA (44.4% and 60.6% amino acid identity respectively).

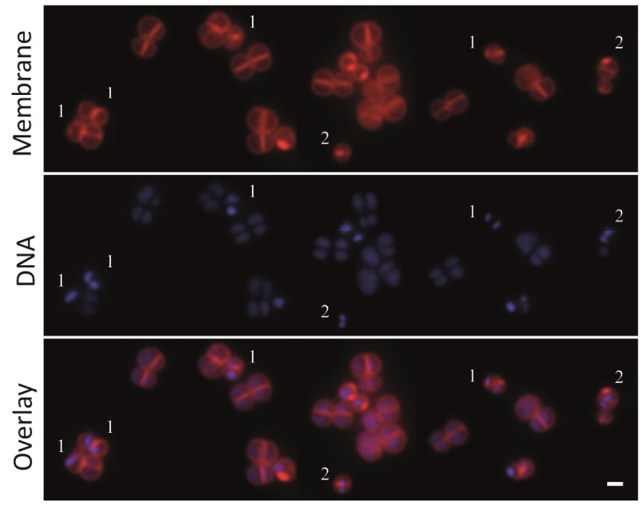
Lack of *S. aureus* SpoIIIE causes chromosome condensation/segregation defects

To investigate the function of *S. aureus* SpoIIIE we constructed a knockout mutant of this protein, in the background of NCTC8325-4, named BCBHV018. The *spoIIIE* gene sequence was almost entirely removed from the *S. aureus* chromosome except for 64bp at the 3' end which most likely contain the RBS site for the *ymfC* gene, located downstream of *spoIIIE*.

Deletion of *spoIIIE* did not affect the growth rate at 37°C in rich medium (duplication time of BCBHV018 and NCTC8325-4 strains = 27 min) but it caused a mild chromosome condensation/segregation defect that was visible by fluorescence microscopy analysis of the mutant cells labelled with membrane and DNA dyes. 6.7% of SpoIIIE knockout mutant cells (n=920) had condensed nucleoids and an additional 0.9%

had the condensed nucleoid bisected by the septum (Figure 5.1). This phenotype could be partially rescued by the ectopic expression of SpoIIIE under the control of its native promoter, at the distant *spa* locus (strain BCBHV020) (Figure 5.1B).

A



B

	1	2
NCTC8325-4 (n = 969)	3 (0.3%)	0 (0%)
BCBHV018 (n = 920)	62 (6.7%)	8 (0.9%)
BCBHV020 (n = 1278)	23 (1.8%)	2 (0.2%)
BCBHV019 (n = 828)	50 (6.0%)	14 (1.7%)

Figure 5.1. The absence of SpoIIIE causes nucleoid condensation and bisection by the septum. (A) Images of BCBHV018 SpoIIIE knock-out mutant, stained with membrane dye Nile Red and DNA dye Hoechst 33342, showing cells with condensed DNA (labelled "1") and cells with condensed DNA bisected by the septum (labelled "2"). Images are false-coloured. For simplification, only some cells of each phenotype are labelled. Scale bar, 1µm. (B) Quantitative analysis of the number of cells with phenotypes "1" and "2" in wild-type NCTC8325-4 strain, in SpoIIIE knock-out mutant BCBHV018 and in BCBHV020 SpoIIIE mutant complemented by ectopic expression of *spoIIIE*, under the control of *spoIIIE* native promoter, at the *spa* locus of *S. aureus* chromosome. The quantification for BCBHV019 SpoIIIE C-terminal deletion mutant is also shown. n=total number of cells analysed. Phenotypes "1" and "2" as described above.

Importantly, the defects caused by the lack of a complete SpoIIIE protein were equivalent to those obtained after deletion of only the SpoIIIE C-terminal domain, in strain BCBHV019 (Figure 5.1B). This suggests that, contrarily to other members of the FtsK/SpoIIIE family, *S. aureus* SpoIIIE is a monofunctional protein whose activity is dependent only in its C-terminal domain.

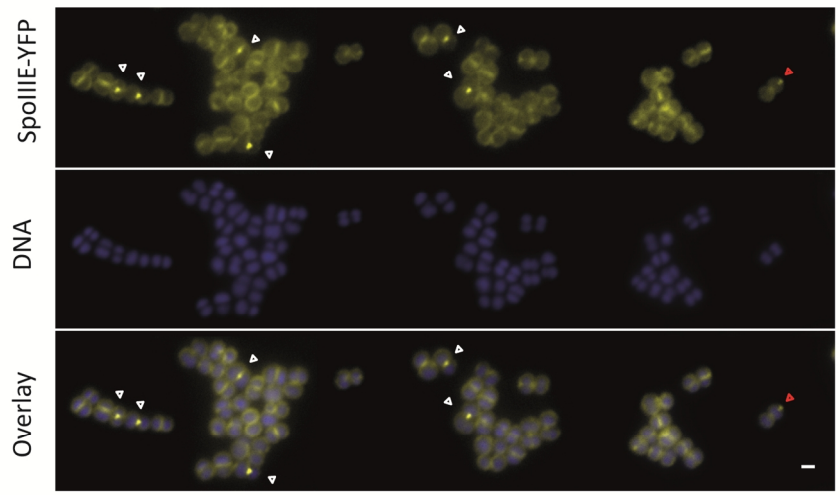
S. aureus SpoIIIE can be found throughout the membrane or assembled in foci

Using BCBHV021, a NCTC8325-4 strain expressing a SpoIIIE-YFP fusion as the only SpoIIIE copy in the cell, expressed from its native locus and under the control of its native promoter, we were able to monitor the localization of this protein in live cells. The fact that strain BCBHV021 did not show increased frequency of cells with condensed DNA (0.4%, n=965) or nucleoids bisected by the septum (0%), compared to the parental strain NCTC8325-4 (see Figure 5.1B), indicated that the SpoIIIE-YFP fusion was functional.

Similarly to its *B. subtilis* homologue, SpoIIIE was localized to the membrane. In rich media, the majority of the cells had SpoIIIE-YFP distributed uniformly throughout the cell membrane (Figure 5.2), while in 8.9% of the cells (n=1135) SpoIIIE-YFP appeared as a bright fluorescent spot (arrowheads in Figure 5.2A) that, in the majority of the cases (6.2% of the total population), was present at the middle of the septum (white arrowheads in Figure 5.2A).

It is known that, in *B. subtlis*, when the chromosome gets trapped by the septum, several SpoIIIE molecules are recruited to this place to form DNA-conducting channels that are visible, by fluorescent microscopy, as bright fluorescent foci (Wu & Errington, 1997, Burton *et al.*, 2007). It is likely that the observed *S. aureus* SpoIIIE-YFP foci also result from the accumulation of SpoIIIE molecules at the site where a DNA pump/rescuer activity is needed. Unfortunately, it was not possible to identify, among the cells with a septum-localized SpoIIIE-YFP focus, cases in which the focus is clearly placed on top of the DNA. It is possible that the DNA transfer process is so fast that we cannot visualize DNA being translocated. Alternatively, the amount of DNA to be rescued may be too small to be detected by Hoescht staining.

A



B

	BCBHV021	BCBHV022 (ΔNoc)	BCBHV021 + Nalidixic acid	BCBHV021 + HPUra	BCBHV021 + Oxacillin
Number of cells quantified	1135	967	675	606	1561
Percentage of SpoIIIE-YFP foci	8.9%	31.3%	24.4%	38.0%	10.4%

C

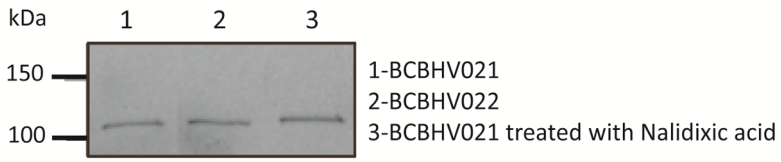


Figure 5.2. SpoIIIE molecules are usually distributed throughout the cell membrane and, in specific cases, assemble in foci. (A) Images of SpoIIIE-YFP localization in BCBHV021 cells. The panels show, from top to bottom, SpoIIIE-YFP fluorescence, DNA (stained with Hoechst 33342 dye) and a merge of the two channels. White arrowheads indicate SpoIIIE foci localizing at the centre of the septum. Red arrowheads point to a SpoIIIE focus present at the "lateral" membrane. Images are false-coloured. Scale bar, 1 µm. (B) Quantification analysis of the frequency of SpoIIIE foci in BCBHV021, in the Noc deletion mutant BCBHV022 and in BCBHV021 cells grown for 30 min in the presence of Nalidixic acid, HPUra and Oxacillin. (C) Analysis of SpoIIIE-YFP (111kDa) expression levels in the parental strain BCBHV021 (1), in BCBHV022 ΔNoc strain (2) and in BCBHV021 cells incubated for 30 min with Nalidixic acid (3), showing that SpoIIIE is not produced at higher levels in the absence of Noc or under impaired chromosome replication/segregation conditions.

In 2.7% of the cells, SpoIIIE-YFP foci were not present in the middle of the septum, either because cells did not have a division septum (2.0%, red arrowhead in figure 5.2A) or because the foci did not co-localize with the existing septum (0.7%, data not shown). It is likely that these SpoIIIE foci correspond to complexes assembled during the previous cell cycle, which remain associated with the membrane of the daughter cells.

To confirm that SpoIIIE foci assemble under circumstances in which the chromosome is trapped by the septum, we determined the localization of SpoIIIE-YFP in the absence of Noc and after treatment with antibiotics that affect chromosome replication/segregation.

In the absence of the nucleoid occlusion effector Noc, FtsZ can polymerize on top of the chromosomes and in 15% of the cells was possible to observe the septum bisecting the DNA (Veiga *et al.*, 2011). In this situation, it is expected that more cells assemble SpoIIIE pumps to resolve chromosome bisection and, indeed, the number of cells with SpoIIIE foci increased by more than 3 fold in the Noc deletion BCBHV022 mutant expressing SpoIIIE-YFP, compared to the parental strain BCBHV021 (Figure 5.2B).

The number of cells presenting a SpoIIIE bright focus also increased after treatment with antibiotics that affect chromosome replication/segregation and cause bisection of the DNA, but not in the presence of the cell wall targeting antibiotic oxacillin (Figure 5.2B).

Although more SpoIIIE foci were observed in the absence of Noc and upon treatment with Nalidixic acid, the expression levels of SpoIIIE-YFP (controlled by *spoIIIE* promoter) were not altered (Figure 5.2C). This observation indicates that, the formation of SpoIIIE pumps does not result from the production of more SpoIIIE in the cell but from the reorganization/recruitment of the already existing SpoIIIE molecules, that would otherwise be scattered along the membrane.

These results indicate that SpoIIIE has a transient function that is not always essential. Only when the activity of a DNA pump is required, does SpoIIIE assemble in a complex to rescue chromosomes trapped by the septum.

Deletion of *S. aureus* Slp causes a severe cell division phenotype

In an initial approach to study the function of Slp, we designed a strategy to delete *slp* gene from NCTC8325-4 genome without leaving any resistance marker, using the pMAD plasmid (Arnaud *et al.*, 2004). The procedure to obtain the knockout mutant, which involves integration and excision of pBCBHV012 plasmid (for more details see materials and methods) was performed several times and 10 independent PCR-confirmed Slp knockout mutants were obtained from 5 different integration colonies. When labelled with membrane and DNA dyes and observed by fluorescence microscopy, these 10 mutants presented different phenotypes. Six Slp knock-out mutants, named 8325-4ΔSlp*, were obtained from 4 independent colonies isolated during the plasmid integration step and showed a mild phenotype (here denominated phenotype A), while 4 mutants (8325-4ΔSlp), obtained from a fifth colony with integrated plasmid, all showed strong cell division defects (here dominated phenotype B) (Figure 5.3).

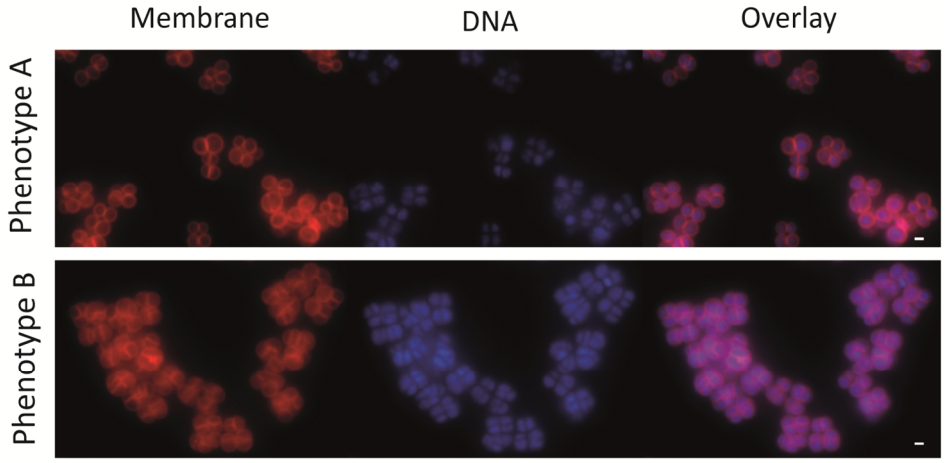
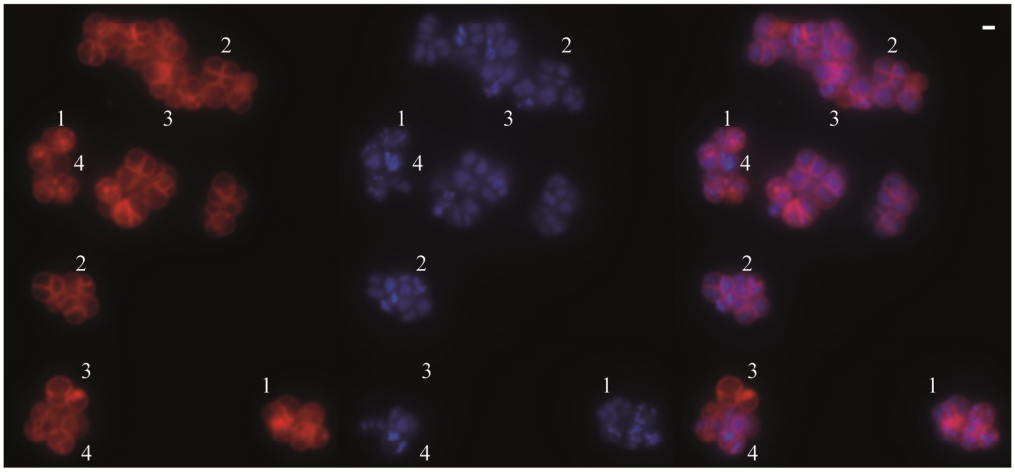


Figure 5.3. Two groups of Slp deletion mutants have different phenotypes. Top panel shows the mild phenotype (here denominated phenotype A) of six 8325-4ΔSlp* mutants. Bottom panel shows the severe cell division defects (phenotype B) of four 8325-4ΔSlp mutants. The cells were labelled with the membrane dye Nile Red and DNA dye Hoechst 33342. Images are false-coloured. Scale bars, 1µm.

Importantly, only the mutants with phenotype B could be complemented by ectopic expression of Slp from the *spa* locus, under the control of IPTG-inducible promoter P_{spac} (data not shown). These results strongly suggest that the colonies with phenotype A had accumulated suppressor mutations during the *slp* deletion process and that phenotype B is most likely the real consequence of the absence of Slp. To confirm this hypothesis and to obtain a Slp mutant without suppressor mutations, we constructed the Slp inducible mutant BCBHV027. This mutant was constructed by first inserting a complete copy of *slp* gene in the *spa* locus, under the control of P_{spac} promoter, and subsequently, while in the presence of IPTG, deleting *slp* from its native locus.

When grown in the absence of IPTG, the Slp inducible mutant showed the same phenotype B already observed in the cells that presumably had not accumulated suppressor mutations (Figure 5.4). The absence of Slp caused cell division and/or morphological chromosome defects in 79.4% of BCBHV027 cells (n=966). Abnormal phenotypes included cells with multiple incorrectly oriented septa (18.1%; phenotype 1 Figure 5.4) and cells that seemed to have already proceeded to a second round of division before finishing the first one (18.0%; phenotype 2 Figure 5.4). We also observed cells that had a normal morphology but were anucleate (3.5%; phenotype 3 Figure 5.4) or presented a disorganized and/or wrongly positioned chromosome (39.8%; phenotype 4 Figure 5.4). These phenotypes were partially complemented upon expression of Slp induced by the presence of IPTG (Figure 5.4B).The deletion of Slp also affected growth, as duplication time of BCBHV027 strain, at 37°C in rich medium not supplemented with IPTG, was 1.5 times higher than in the presence of the inducer (Figure 5.4B). It is possible that we are underestimating the effect on growth since we cannot exclude the possibility that residual quantities of the Slp protein were still present during BCBHV027 growth.

A



B

	NCTC8325-4 (n =1083)	BCBHV027 No IPTG (n =966)	BCBHV027 IPTG 0.5mM (n =1432)
1: Multiple incorrectly oriented septa Normal or abnormal DNA morphology	0%	18.1%	1.2%
2: Two division septa Normal or abnormal DNA morphology	0%	18.0%	0.8%
3: Anucleated cells	0%	3.5%	1.0%
4: Abnormal DNA morphology Normal cell morphology	0%	39.8%	6.1%
Duplication time (min)	27	44	29

Figure 5.4. The absence of Slp protein causes severe cell division and chromosome segregation phenotypes. (A) Images of BCBHV027 Slp inducible mutant, grown in the absence of IPTG and labelled with membrane dye Nile Red and DNA dye Hoechst 33342. In the population of *S. aureus* cells lacking the entire Slp protein it was possible to identify cells with multiple incorrectly oriented septa that could also have DNA morphological defects (labelled "1"), cells that assembled a second septum before finishing the first division cycle and could also present abnormal nucleoids (labelled "2"), anucleate cells (labelled "3") and cells that have a normal cell morphology but an irregular DNA morphology (labelled "4"). Images are false-coloured. For simplification, only some cells of each phenotype are labelled. Scale bar, 1µm. (B) Quantification of the phenotypes described above in the parental and in BCBHV027 strains grown with or without IPTG. n=total number of cells analysed. Bottom of the table: duplication times for NCTC8325-4 and BCBHV027 grown at 37°C in rich medium, in the absence or in the presence of the inducer IPTG.

Slp is a multifunctional protein

The *B. subtilis* homologue of Slp, SftA, is a monofunctional protein, involved only in DNA translocation (Kaimer *et al.*, 2009, Biller & Burkholder, 2009). In contrast, the *E. coli* homologue, FtsK, has different functions that are performed independently by its various domains (Bigot *et al.*, 2007). In order to establish if the strong phenotype observed in the Slp full-length depletion mutant was a consequence of the lack of the DNA translocase motor or if it resulted from a second function of Slp, possibly associated with its N-terminal and linker domains, we constructed NCTC8325-4 strains in which the C-terminal domain of Slp was removed or inactivated. The Slp C-terminal deletion mutant was constructed by removing from the genome the sequence encoding for this domain, namely after codon 816. To inactivate the C-terminal DNA motor, a conserved lysine of the Walker A motif was substituted for an alanine (K971A). This substitution was already shown, in *E. coli* and *B. subtilis* (Kaimer *et al.*, 2009, Bigot *et al.*, 2004), to inactivate the ATPase activity of FtsK and SftA DNA motor domains, respectively, which prevents DNA translocation.

The Slp C-terminal deletion mutant, BCBHV023, and the Slp Walker A mutant, BCBHV024, had the same duplication rate as the parental strain (27 min) and did not present any obvious phenotypic defects (Figure 5.5A). This shows that it is not the DNA translocase motor, but the N-terminal and linker domains (Slp_{NL}, between amino acids 1 and 815), that are necessary for normal cell morphology. In other words, Slp_{NL} has an activity, independent from the translocation motor, which seems to be related with cell division. These results were confirmed by the fact that expression of Slp_{NL} from the replicative plasmid pCNX, complemented the phenotype of 8325-4ΔSlp (Figure 5.5B). Interestingly, the expression of Slp N-terminal domain (Slp_N, between amino acids 1 and 174), without the linker region, did not restore the morphological defects of 8325-4ΔSlp (Figure 5.5B), showing that Slp linker (Slp_L, between amino acids 175 and 815) is necessary for the role of Slp_{NL}or for the correct folding of Slp_N.

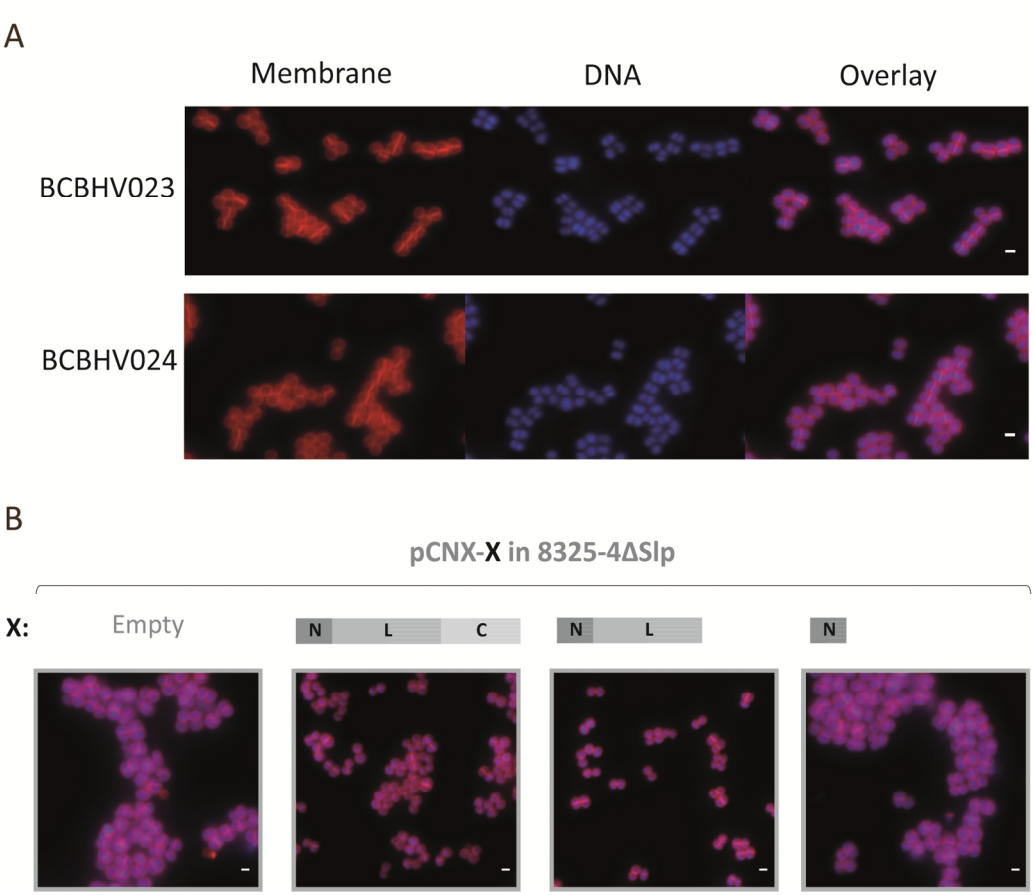


Figure 5.5. Slp C-terminal domain is not required to maintain normal cell and chromosome morphology. (A) Images of BCBHV023 Slp C-terminal deletion mutant (top panel) and BCBHV024 Slp Walker A mutant (bottom panel), showing that the absence or inactivation of Slp C-terminal domain did not cause observable phenotypic defects. Cells were labelled with membrane dye Nile Red and DNA dye Hoechst 33342. (B) Fluorescence images of the Slp deletion mutant 8325-4ΔSlp cells, expressing truncated forms of Slp from pCNX replicative plasmid. The expression of the Slp N-terminal and linker domains complemented the morphological defects of 8325-4ΔSlp (third panel), showing that these domains are sufficient for normal cell division and confirming that the absence of the Slp C-terminal domain does not cause observable defects. The expression of the Slp N-terminal domain alone could not complement *slp* deletion (fourth panel), showing that the role of Slp in cell division is dependent on both Slp_N and Slp_L. Cells containing an empty pCNX plasmid or expressing the entire Slp protein are shown, in first and second panels respectively, as controls. X-Fragment expressed from pCNX plasmid; N-Slp N-terminal domain; L-Slp linker domain; C-Slp C-terminal domain. Cells membranes labelled with Nile Red dye and DNA with Hoechst 33342 dye. Images are false-coloured. Scale bars, 1µm.

The lack of an obvious phenotype upon deletion/inactivation of the Slp C-terminal domain could indicate that this domain does not have a DNA translocase activity or that this activity is not necessary during normal growth. To determine which of these hypotheses was true, we tested the ability of BCBHV023 and BCBHV024 cells to avoid chromosome bisection by the septum, after treatment with Nalidixic acid. We observed that, 14.2% of BCBHV023 cells (n=1322) and 12.6% (n=1320) of BCBHV024 cells had the septum positioned on top of the DNA, while in the parental strain NCTC8325-4, this occurred in only 8.8% of the cells (n=1083). This indicates that the absence or inactivation of Slp C-terminal domain in BCBHV023 and BCBHV024, respectively, impairs the movement of DNA retained at the division site after Nalidixic acid treatment, indicating that the Slp C-terminal domain has a DNA translocase activity.

Slp localizes early to the division septum

To determine the localization of Slp in live cells, we constructed a NCTC8325-4 strain that expressed, as the only Slp copy in the cell, sfGFP-Slp, a super-fast folding GFP (Fisher & DeLisa, 2008) N-terminal fusion to Slp. This fusion was functional since its expression, controlled by *slp* native promoter at its native locus, did not cause observable phenotypic defects.

Like its *B. subtilis* homologue SftA (Biller & Burkholder, 2009, Kaimer *et al.*, 2009), Slp does not have a predicted N-terminal transmembrane domain but it was found to localize at the division septum throughout the constriction process (Figure 5.6A).

In order to estimate the time of Slp arrival to the septa, we constructed BCBHV036 and BCBHV037 strains that expressed both sfGFP-Slp and, respectively, the early divisome proteins EzrA-mCherry (Veiga *et al.*, 2011, Jorge *et al.*, 2011) or PBP4-mCherry, which arrives late at the septum to crosslink glycan chains of the peptidoglycan (Atilano *et al.*, 2010).

In 95.2% of BCBHV036 cells expressing sfGFP-Slp and EzrA-mCherry (n=418), the two proteins colocalized (Figure 5.6B), showing that Slp is present at the septum almost

since the beginning of its formation. The two proteins only displayed different localization patterns in 4.8% of the cells analysed. In these cases, EzrA-mCherry localized along a completely formed septa (line across the cell), while sfGFP-Slp was seen as a focus in the middle of the septum (arrowhead in Figure 5.6B).

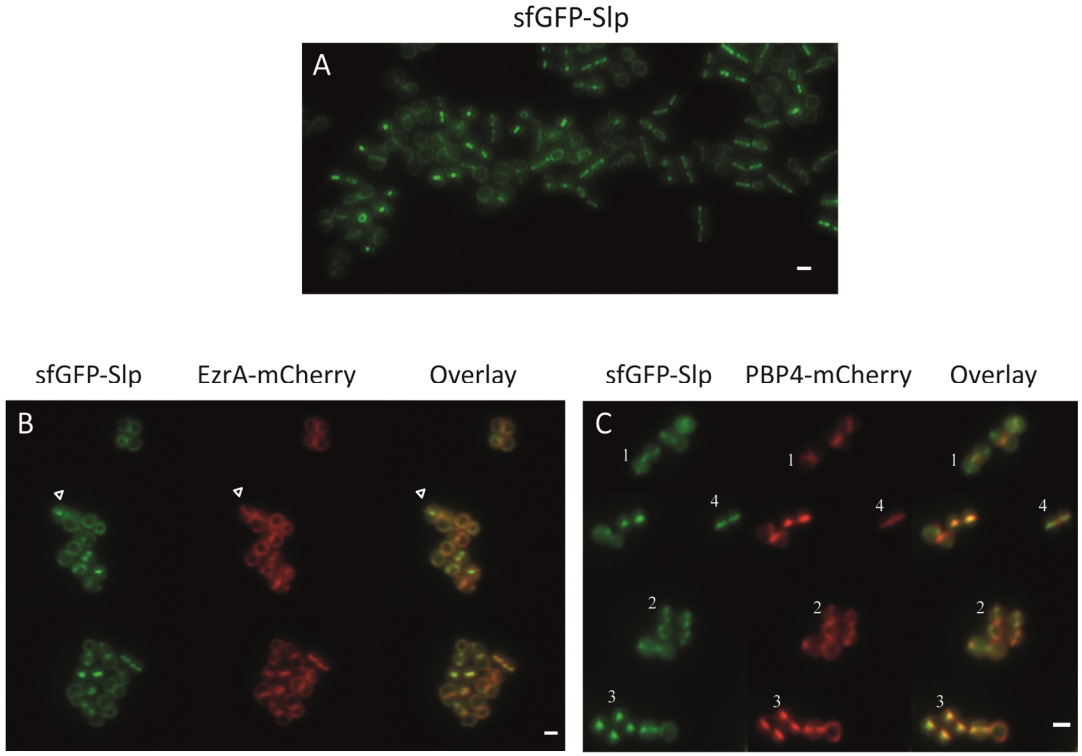


Figure 5.6. Slp localizes early to the division septa. (A) Localization of sfGFP-Slp in BCBHV033 cells, showing that Slp is a septal protein. (B) sfGFP-Slp co-localized with the early divisome protein EzrA-mCherry in the majority of BCBHV036 cells. Only in 4.8% of the cells the two proteins did not show the same localization pattern. In these cases, EzrA-mCherry was seen as a line across the cell, while sfGFP-Slp was seen as a focus in the middle of the septum (white arrowhead). (C) In BCBHV037 cells, sfGFP-Slp was always seen arriving at the septum before the late divisome protein PBP4-mCherry. At the beginning of septum formation, when PBP4 was not yet present, it was already possible to see sfGFP-Slp localizing at the septal ring (seen as two septal spots) (labelled "1"). As division proceeded, PBP4 was seen as two septal spots and Slp was already positioned across the entire septum (labelled "2"). Later in the constriction process, PBP4 was seen as a septal line while Slp appeared as a focus in the middle of the septum (leading edge of the septum) (labelled "3"). In 10.5% of BCBHV037 cells, PBP4 and Slp had the same localization (labelled "4"). For simplification, only some cells of each phenotype are labelled. Images are false-coloured. Scale bars, 1µm.

Fluorescence microscopy analysis of sfGFP-Slp and PBP4-mCherry localization in BCBHV037 cells confirmed that Slp is an early septal protein. In 89.5% of the BCBHV037 cells (n=784), it was clear that Slp arrived at the septum before PBP4. It was possible to observe sfGFP-Slp localizing to the septal ring (seen as two septal spots) when PBP4 was not yet present at the septum (cell 1 Figure 5.6C) and also Slp positioned across the entire septum while PBP4 was only seen as two septal spots (cell 2 Figure 5.6C). Additionally, later in the constriction process, PBP4 was seen as a septal line while Slp appeared as a focus in the middle of the septum (cell 3 Figure 5.6C). PBP4 and Slp had similar localization in 10.5% of BCBHV037 cells (e.g. cell 4 Figure 5.6C).

Slp localization depends on its N-terminal and linker domains

Slp does not have an obvious membrane targeting domain but, in strain BCBHV035, a sfGFP-Slp_{NL} fusion protein, expressed in substitution of Slp, from the *slp* locus and under the control of its native promoter, localized properly at the septum (Figure 5.7A), showing that, as expected, the DNA translocase C-terminal domain is not involved in Slp localization. It is not clear, however, if it is only the N-terminal domain that localizes the protein, like in the case of *E. coli* FtsK (Yu *et al.*, 1998a), or if the presence of the linker domain, that we showed to be important for Slp role in cell division, is essential for its proper localization. To address this question, we determined the ability of the different Slp domains, fused to sfGFP and expressed from pCNX plasmid, to properly localize at the septum in the background of 8325-4ΔSlp strain, the Slp mutant with no suppressor mutations. As shown in Figure 5.7B, sfGFP -Slp_{NL} was recruited to the septum similarly to sfGFP-Slp. Neither the C-terminal, the N-terminal nor the linker domains individually were able to localize to the septum. Although in some cells, sfGFP-Slp_N and sfGFP-Slp_{LC} were partially visible at the septa, the majority of the protein was in the cytoplasm. This shows that Slp localization is strictly dependent on both N-terminal and linker domains or on the N-terminal/linker alone, if the other domain is required for correct folding.

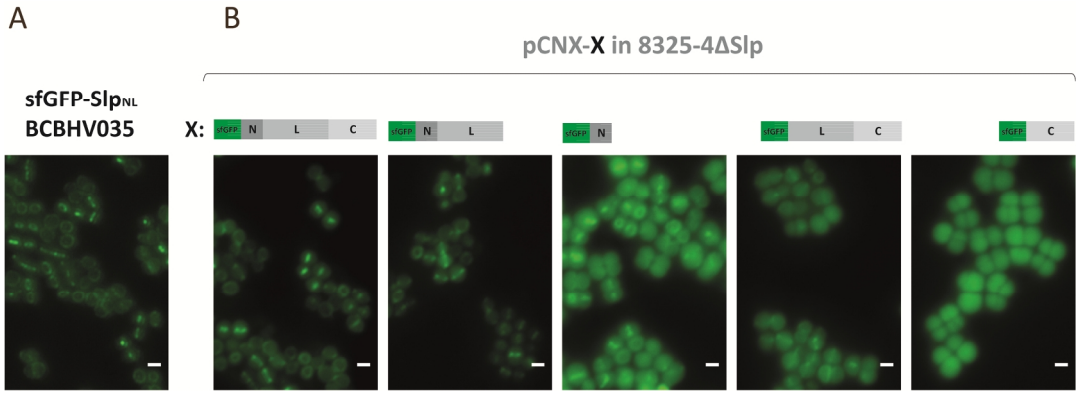


Figure 5.7. Slp N-terminal and linker domains are both necessary for Slp localization. (A) A sfGFP fusion to Slp_{NL} (truncated form of Slp without the C-terminal domain) localizes at the septum, showing that Slp C-terminal domain is not necessary for Slp localization. (B) Fluorescence images of 8325-4ΔSlp cells expressing truncated forms of sfGFP-Slp from pCNX replicative plasmid, under the control of P_{cad} promoter, in the absence of Cadmium. Cells expressing a complete Slp protein, fused to sfGFP, are shown as control (first panel).The expression of Slp N-terminal and linker domains (second panel) was sufficient for a proper Slp localization. The C-terminal domain is soluble (fifth panel) and, the N-terminal and linker domains separately (third and fourth panels, respectively) cannot completely localize to the septum. X- Fragment expressed from pCNX plasmid; N-Slp N-terminal domain; L-Slp linker domain; C-Slp C-terminal domain. Images are false-coloured. Scale bars, 1µm.

SpoIIIE and Slp have different localization patterns

As a first step towards understanding if the two *S. aureus* DNA translocases are functionally redundant, act through independent pathways or influence the activity of each other, we monitored the localization of both SpoIIIE and Slp in live cells.

For co-visualization of SpoIIIE and Slp we constructed BCBHV038 strain expressing the previously described SpoIIIE-YFP fusion and a Slp-CFP fusion, which was used instead of sfGFP-Slp to avoid overlap between the YFP and GFP signals using fluorescence microscopy. We were unable to obtain a fluorescent N-terminal CFP fusion to Slp. The Slp-CFP was not fully functional as its expression did not cause the severe phenotype of Slp knockout mutant, but it resulted in a small percentage of cells with more than one septum (data not show). Therefore, in BCBHV038 strain, in addition to the fusion protein controlled by the *slp* native promoter, a native copy of *slp* under the control of P_{spac}

promoter was also expressed. Importantly, the Slp-CFP protein showed the same location pattern as sfGFP-Slp.

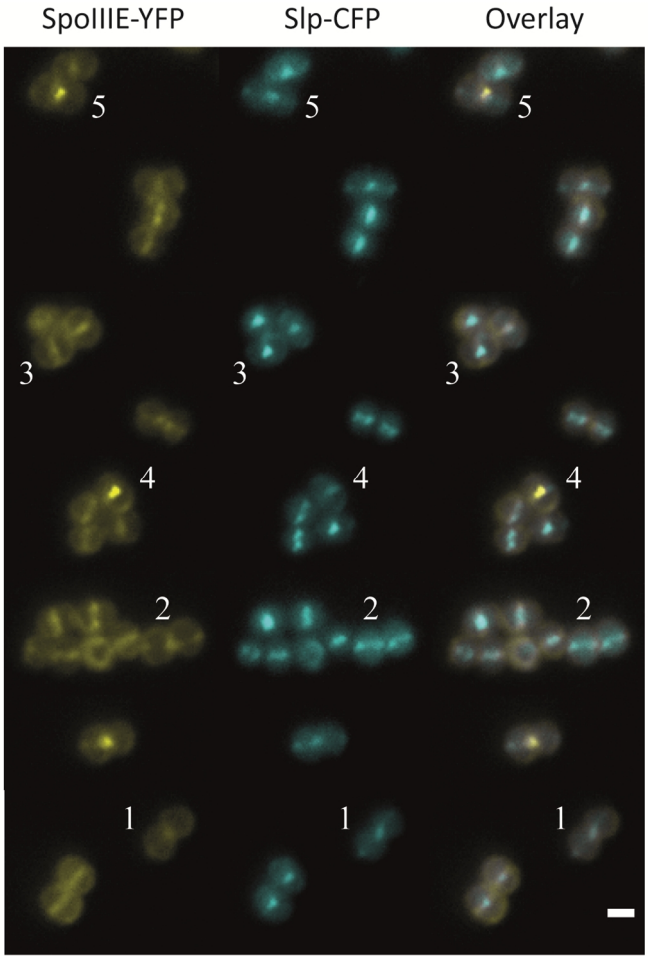


Figure 5.8. SpoIIIE and Slp do not colocalize in dividing *S. aureus* cells. Fluorescence images of SpoIIIE-YFP and Slp-CFP localization in strain BCBHV038. In the initial stage of septum synthesis, Slp was seen at the septum (two septal spots) and SpoIIIE at the cell membrane (labelled "1"). With the progression of division, when Slp was visible as a line across the cell (completely formed septum), SpoIIIE was seen as two septal spots (ring around the division site) (labelled "2"). When both proteins localized at a completely formed septa, Slp was seen as a focus in the middle of the septum (leading edge of the septum) and SpoIIIE as a line (membrane at the outer edge of the septum) (labelled "3"). When a SpoIIIE focus was assembled at the middle of the septa, Slp-CFP was distributed across the entire septum (line with the cell diameter) (labelled "4"). At end of the division cycle, when SpoIIIE-YFP foci were placed between two splitting daughter cells, Slp was located at the septal rings forming in the new daughter cells (labelled "5"). For simplification, only some cells of each phenotype are labelled. Images are false-coloured. Scale bar, 1µm.

As described above, Slp localizes early to the septum and follows septum constriction, while SpoIIIE is distributed throughout the cell membrane (including the septum) and forms bright foci in 8.9% of the cells. By analysing 763 BCBHV038 cells at different stages of septum formation we observed that these two proteins do not colocalize during the entire *S. aureus* cell division cycle (Figure 5.8). BCBHV038 cells in the initial stage of septum synthesis showed Slp as two septal foci (corresponding to a septal ring), while SpoIIIE was found at the cell membrane (cell 1, Figure 5.8). With the progression of division, Slp became visible as a line across the cell (completely formed septum), while SpoIIIE was seen as two septal spots (ring around the division site) (cell 2, Figure 5.8). In the final stages of division, the two proteins had a septal localization. However, Slp was located at the edge of the septum (spot in the middle of the septum), far from SpoIIIE (line along the septum) (cell 3, Figure 5.8).

In all the cases where a bright SpoIIIE-YFP focus was visible, it did not colocalize with Slp-CFP (examples of cells 4 and 5, Figure 5.8).

The data collected shows that SpoIIIE and Slp act at different cellular locations and time points. Therefore, and assuming that the activity of these proteins is strictly dependent on their localization, it is likely that SpoIIIE and Slp have independent roles.

The localization and expression of SpoIIIE and Slp is not interdependent

The two *S. aureus* DNA translocases do not share the same localization pattern, but we wondered if the absence of one motor could alter the expression, localization or activity of the other.

To test if SpoIIIE and Slp localization patterns changed in the absence of the other DNA motor we constructed strain BCBHV034, that expressed sfGFP-Slp in the absence of SpoIIIE, and strains BCBHV028, BCBHV029 and BCBHV030 that correspond, respectively, to the Slp C-terminal deletion mutant, the Slp Walker A mutant and the Slp inducible mutant, expressing SpoIIIE-YFP.

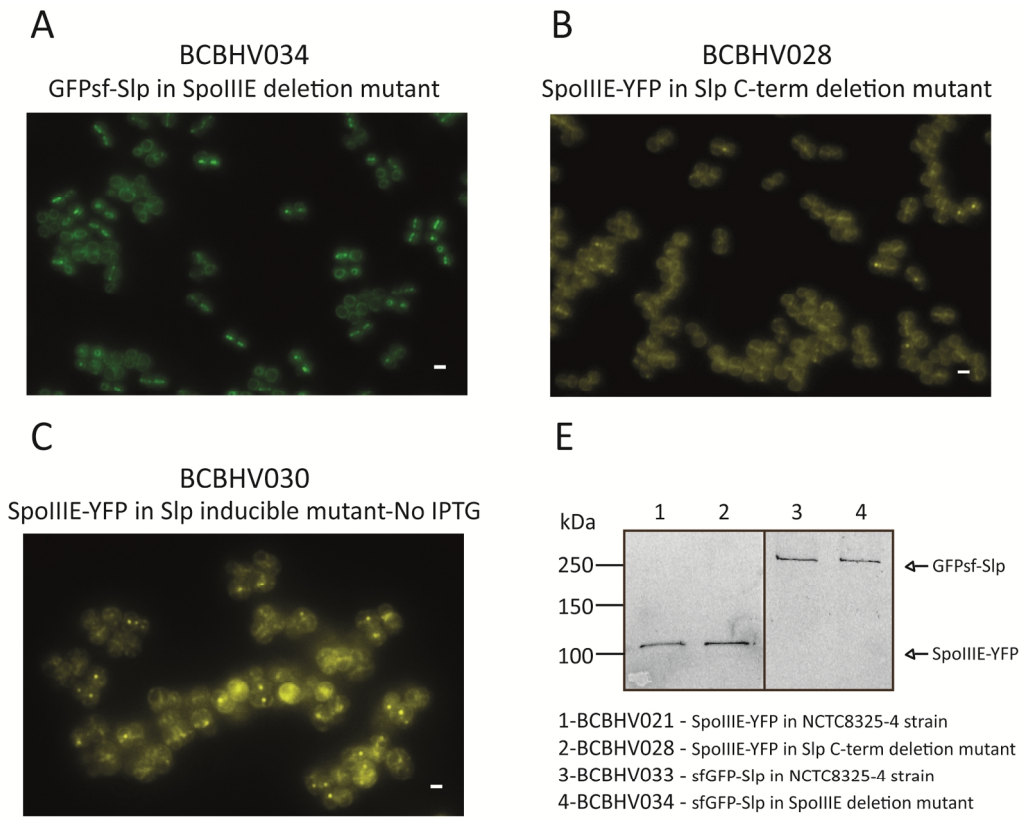
As shown in Figure 5.9A, sfGFP-Slp was still recruited to the division septum in the absence of SpoIIIE. Likewise, the absence of a functional Slp DNA motor, in strains

BCBHV028 and BCBHV029, did not change the normal localization pattern of SpoIIIE. The protein still bound throughout the cell membrane and, like in a wild-type situation, formed foci in approximately 8% of the population (Figure 5.9B,D).Similarly, in the Slp inducible strain BCBHV030 grown in the absence of IPTG, SpoIIIE-YFP still localized at the membrane (Figure 5.9C,D), showing that the localization of SpoIIIE is completely independent of Slp.

If SpoIIIE could compensate for the absence of the Slp DNA motor, we would expect that the number of cells assembling a SpoIIIE pump in Slp C-terminal deletion or Walker A mutants would be higher than in the presence of a functional Slp protein. However, the percentage of BCBHV028 and BCBHV029 cells with a SpoIIIE focus was the same as in the wild type (Figure 5.9D), suggesting that SpoIIIE does not substitute Slp DNA translocase activity. The number of cells with SpoIIIE-YFP foci increased upon depletion of Slp in BCBHV027 Slp inducible mutant but this was probably not a direct effect of the absence of the Slp DNA translocation activity but rather a response to the large chromosomal morphologic defects of BCBHV030.

Since in strains BCBHV021, BCBHV028 and BCBHV033, BCBHV034, SpoIIIE-YFP and sfGFP-Slp were expressed as sole copies of SpoIIIE and Slp in the cell and were under the control of their respective native promoters, respectively, we determined if the expression levels of SpoIIIE and Slp changed in response to the absence of the other DNA motor. Through fluorescence imaging of BCBHV028 and BCBHV029 protein extracts, it was possible to observe that the SpoIIIE-YFP expression level was not altered in the absence of Slp C-terminal domain. Similarly, sfGFP-Slp was expressed at the same level in the SpoIIIE knockout mutant BCBHV034 as in the parental strain BCBHV033 (figure 5.9E).

Together these results show that SpoIIIE and Slp DNA translocases localize and are expressed independently of each other, reinforcing the idea that the two proteins act through independent pathways.



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	BCBHV021 (n=1135)	BCBHV028 (n=1115)	BCBHV029 (n=1112)	BCBHV030 No IPTG (n=874)	BCBHV030 0.5mM IPTG (n=974)
Percentage of SpoIIIE-YFP foci	8.9%	7.3%	7.9%	40.0%	14.9%

Figure 5.9. SpoIIIE and the DNA translocase domain of Slp do not depend on each other for expression and localization. (A) sfGFP-Slp localized at the septum in SpoIIIE deletion mutant, similarly to in the BCBHV021 parental strain (see Figure 5.6A). (B) Likewise, the SpoIIIE-YFP pattern of localization is not altered in the absence of Slp C-terminal domain or (C) the entire Slp protein. Images are false-coloured. Scale bars, 1µm. (D) Quantification of the frequency of SpoIIIE-YFP foci in BCBHV021, BCBHV028 Slp C-terminal deletion mutant, BCBHV029 Slp Walker A mutant, BCHV030 Slp inducible mutant, showing that, even though SpoIIIE was more frequently recruited in the absence of the entire Slp protein, it did not assemble more foci in the absence of a functional Slp C-terminal domain. n=total number of cells analysed. (E) Analysis of SpoIIIE-YFP (111kDa) and sfGFP-Slp expression in the wild-type strains (1 and 3 respectively) and in the absence of each other (2 and 4 respectively), showing that SpoIIIE and Slp expression was not interdependent. Notice that, although sfGFP-Slp protein has a predicted molecular weight of 172 kDa, it runs above the 250 kDa marker.

Deletion of both DNA translocases exacerbates the single mutants' phenotypes

All evidences points towards the idea that SpoIIIE and Slp have independent roles and if this is true, depriving *S. aureus* cells of both DNA motors would cause a cumulative or exacerbated phenotype. To study the effect of a double deletion of the DNA translocases, we generated strains BCBHV031 and BCBHV032 by knocking-out *spoIIIE* gene in the backgrounds of Slp C-terminal and Slp Walker A mutants.

As expected, BCBHV031 and BCBHV032 double mutants presented much more severe chromosome morphological and partioning defects than the single mutants (Table 5.4). 31.4% of BCBHV031 cells, lacking SpoIIIE and the C-terminal domain of Slp (n=977), had a condensed nucleoid and 3.9% had a condensed nucleoid trapped by the division septa (Table 5.4). Accordingly, 23.9% of BCBHV032 SpoIIIE/Slp Walker A mutant cells (n=1033) had condensed chromosomes and 2.2% of the population presented a condensed nucleoid bisected by the septum.

Table 5.4. Phenotypes of SpoIIIE and Slp C-terminal single and double mutants

	Single mutants				Double mutants	
	WT	BCBHV018	BCBHV023	BCBHV024	BCBHV031	BCBHV032
Percent of cells with condensed nucleoid	0.3%	6.7%	0.2%	0.1%	31.4%	23.9%
Percent of cells with condensed nucleoid bisected by the septa	0%	0.9%	0%	0%	3.9%	2.2%
MIC Nalidixic acid (µg/mL)	160	80	160	160	40	40
MIC HPUra (µg/mL)	4	4	2	2	1	1
MIC Oxacillin (µg/mL)	0.16	0.16	0.16	0.16	0.16	0.16
Duplication time (min)	27	27	27	27	36	34

BCBHV031 and BCBHV032 double mutants also had longer duplication times, at 37°C in rich medium, and were less resistant to the DNA-targeting antibiotics Nalidixic acid and HPUra, than each individual mutant or the wild-type strain (Table 5.4). The double mutants' minimal inhibitory concentration (MIC) for Nalidixic acid was 4 times lower than that of the parental strain. Likewise, the HPUra MIC decreased from 4 µg/ml in the wild type and SpoIIIE deletion strains, to 1 µg/ml in the double mutants (Table 5.4).

Together these results confirm that the two *S. aureus* DNA translocases have independent activities in the cell. Since the double mutants' phenotypes were stronger than just the addition of the defects caused by the absence of each DNA translocase, it also seems likely that SpoIIIE and Slp act synergistically, substituting each other whenever needed.

Discussion

FtsK/SpoIIIE DNA translocases have a vital role in synchronizing segregation of the chromosomes with cell division during sporulation and vegetative growth of several bacteria (Kaimer & Graumann, 2011, Barre, 2007, Wang *et al.*, 2007, Wang *et al.*, 2006). Nevertheless, it remains unclear at which level these proteins are required for the division cycle of cocci, like *Staphylococcus aureus,* that segregate the chromosomes along three consecutive perpendicular axes. In this study, we investigated the two putative FtsK/SpoIIIE DNA translocases of *S. aureus*, SpoIIIE and Slp.

Here we showed that, the absence of SpoIIIE caused a nucleoid condensation phenotype in approximately 8% of *S. aureus* cells and, in a few cases, the condensed DNA was bisected by the septa. This phenotype could result from changes in the organization of the chromosome but could also be indicative of deficient chromosome segregation. *S. aureus* XerC mutants, for example, had a similar, though more severe, nucleoid condensation phenotype (Veiga H., unpublished data). The XerC protein of *S. aureus*, like its orthologues in other bacteria (Lesterlin *et al.*, 2004, Sciochetti *et al.*, 1999, Recchia &

Sherratt, 1999), was suggested to be a chromosome resolvase, necessary for the conversion of chromosome dimers into monomers and so for proper completion of the segregation process (Chalker *et al.*, 2000). Therefore, it is very likely that the condensation phenotype of *xerC* deletion mutant results from deficient chromosome segregation. Similarly, nucleoid condensation observed in the absence of SpoIIIE may also be indicative of partitioning defects.

As shown in figure 5.2, *S. aureus* SpoIIIE molecules are usually scattered along the cell membranes and are recruited to the centre of the septum to form a SpoIIIE focus, only in a subpopulation of 8% of the cells. *B. subtilis* SpoIIIE forms similar foci when the nucleoid is bisected by the septum and these foci are known to be DNA conducting channels (Burton *et al.*, 2007). Therefore, we can assume that the bright spots observed in *S. aureus* represent the same type of structures, which assemble only when DNA translocase activity is needed. In agreement with this, the number of *S. aureus* wild-type cells with septal localized SpoIIIE complex/bright focus (approximately 9% of the population) was almost the same as the number of defective cells in the SpoIIIE deletion mutant. Moreover, the frequency of foci in the population increased under conditions that require a DNA translocase, like when the normal mechanisms of septum placement and chromosome replication/segregation are perturbed.

Importantly, the fact that approximately 9% of a *S. aureus* population require the formation of SpoIIIE foci to avoid nucleoid condensation/segregation defects clearly shows that *S. aureus* SpoIIIE activity is required even during normal growth. This is in contrast to the function of its *B. subtilis* homologue, which is not necessary for normal vegetative growth of *B. subtilis* cells (Sharpe & Errington, 1995).

The second *S. aureus* FtsK/SpoIIIE protein, Slp, is critical for proper cell division. The absence of the entire coding sequence for this protein caused severe cell division and chromosome morphological defects in the majority of a *S. aureus* population. However, these defects do not result from the lack of an active DNA translocase but rather from a second, still unknown, function of the Slp N-terminal and linker domains. Under normal conditions, the absence or inactivation of Slp C-terminal region did not cause any

observable alterations in *S. aureus* growth rate or in the morphology and partioning of the nucleoid. Nevertheless, this domain was required to translocate unsegregated DNA, retained at the division site after Nalidixic acid treatment, showing that indeed Slp C-terminal domain (Slp_C) has a DNA translocase activity.

Contrarily to what we have observed for Slp_C, deletion of SftA or of the DNA translocation domains of FtsK, in *B. subtilis* and *E. coli* cells respectively, causes cell division defects. In the absence of SftA, a small percentage of *B. subtilis* cells have their DNA bisected by the septum (Biller & Burkholder, 2009, Kaimer *et al.*, 2009) and approximately 20% of *E. coli* cells lacking $FtsK_C$ have abnormal positioned chromosomes (Yu *et al.*, 1998b, Liu *et al.*, 1998, Steiner *et al.*, 1999). Importantly, deletion of the RecA protein, necessary for the recombination events that originate chromosome dimers, can rescue these phenotypes, thus showing that they result from a failure to resolve chromosome dimers (Steiner *et al.*, 1999, Yu *et al.*, 1998b, Liu *et al.*, 1998, Biller & Burkholder, 2009). Taking this in consideration, we hypothesize that *S. aureus* Slp_C is not involved in the resolution of chromosome dimers but only in the translocation of DNA in critical situations.

We also showed that Slp is a septal protein that arrives early to the septum and stays at that location throughout the constriction process. Slp N-terminal and linker domains are both necessary for proper localization of the protein, and since these domains are predicted to be soluble, Slp positioning should be dependent on the interaction with one, or even more, early divisome proteins.

Although the activity of both *S. aureus* DNA translocases is dependent on their localization at the septum, the two proteins never colocalized. Even when SpoIIIE was located at the septum, dispersed or as a focus, it was positioned at the septal membrane and therefore distant from Slp that was located at the leading edge of the septum. Moreover, the localization and expression of SpoIIIE and Slp was independent from each other and lack of both DNA motors caused much more severe defects in *S. aureus* cells than the lack of only one, indicating that the two *S. aureus* DNA translocases operate in independent pathways. We therefore propose the following roles for the *S. aureus* DNA

translocases: Slp always localizes at the septum and, when chromosome segregation is compromised, promotes DNA movement to prevent bisection of the non-separated chromosomes; SpoIIIE forms DNA translocation channels to resolve specific DNA entrapment situations that, under normal growth, occur in approximately 9% of *S. aureus* population.

Since defective phenotypes in a SpoIIIE/Slp_C double mutant are much more severe than the sum of the defects caused by the absence of each DNA translocase, it seems likely that the two *S. aureus* DNA translocases act synergistically, to complement, when required, the absence of each other. This complementation implies that at least one of the DNA translocases can substitute for the activity of the other. However, it is not yet clear which protein could have this ability. If *S. aureus* SpoIIIE acts post-septationally like its *B. subtilis* homologue, and Slp, like SftA (Biller & Burkholder, 2009), cannot form channels across closed septum thus it seems unlikely that Slp can substitute for SpoIIIE. Theoretically, it is more likely that SpoIIIE substitutes for Slp. However, we observed that the number of cells with a SpoIIIE focus in Slp C-terminal and Walker A mutants is almost the same as in the parental strain, contrary to what would be expected if SpoIIIE could replace Slp.

As described above and contrarily to its *B. subtilis* homologue SftA (Biller & Burkholder, 2009, Kaimer *et al.*, 2009), *S. aureus* Slp is a bifunctional protein. Its N-terminal and linker domains have a second, independent function from the already established C-terminal DNA translocase activity. However, the exact nature of this second role is not yet known. Like Slp, *E. coli* FtsK, has a second function. In this case, the protein is essential to recruit divisome proteins to the septum in formation (Draper *et al.*, 1998, Chen & Beckwith, 2001). To investigate if Slp_{NL}, like $FtsK_N$, is involved in the recruitment of late septal proteins, we observed the localization pattern of FtsW, PBP2, PBP4 and TagO in the Slp inducible mutant. The septal localization of these proteins was not altered in the absence of Slp protein (preliminary data, not shown), showing that Slp_{NL} is not

responsible for their recruitment. However, at this point, we cannot discard the possibility that other septal proteins require Slp for their localization.

As we have described, the majority of *S. aureus* cells lacking the entire Slp protein had severe defects (Figure 5.4), including multiple irregularly oriented septa and/or abnormal nucleoid morphology. It is particularly interesting that a significant percentage of the mutated cells seemed to have assembled a second septum at a new division plane, before the end of the previous round of division. In an alternative approach to determine the role played by Slp_{NL}, we investigated the origin of these cells. For that, we followed the growth of Slp inducible mutant in the absence of IPTG, by time-lapse-microscopy (data not shown), and we found that these cells were still capable of finishing the first round of division and separated, even though the second division was already in an advanced state. This suggests that the absence of Slp_{NL} does not impair cell separation. Therefore, an attractive hypothesis is that, in the absence of Slp, *S. aureus* loses an important cell division check-point that regulates the timing of septum placement.

References

Arnaud, M., A. Chastanet & M. Debarbouille, (2004) New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, Gram-positive bacteria. *Appl Environ Microbiol* **70**: 6887-6891.

Atilano, M. L., P. M. Pereira, J. Yates, P. Reed, H. Veiga, M. G. Pinho & S. R. Filipe, (2010) Teichoic acids are temporal and spatial regulators of peptidoglycan cross-linking in *Staphylococcus aureus*. *Proc Natl Acad Sci U S A* **107**: 18991-18996.

Aussel, L., F. X. Barre, M. Aroyo, A. Stasiak, A. Z. Stasiak & D. Sherratt, (2002) FtsK Is a DNA motor protein that activates chromosome dimer resolution by switching the catalytic state of the XerC and XerD recombinases. *Cell* **108**: 195-205.

Barre, F. X., (2007) FtsK and SpoIIIE: the tale of the conserved tails. *Mol Microbiol* **66**: 1051-1055.

Bath, J., L. J. Wu, J. Errington & J. C. Wang, (2000) Role of *Bacillus subtilis* SpoIIIE in DNA transport across the mother cell-prespore division septum. *Science* **290**: 995-997.

Bigot, S., J. Corre, J. M. Louarn, F. Cornet & F. X. Barre, (2004) FtsK activities in Xer recombination, DNA mobilization and cell division involve overlapping and separate domains of the protein. *Mol Microbiol* **54**: 876-886.

Bigot, S., V. Sivanathan, C. Possoz, F. X. Barre & F. Cornet, (2007) FtsK, a literate chromosome segregation machine. *Mol Microbiol* **64**: 1434-1441.

Biller, S. J. & W. F. Burkholder, (2009) The *Bacillus subtilis* SftA (YtpS) and SpoIIIE DNA translocases play distinct roles in growing cells to ensure faithful chromosome partitioning. *Mol Microbiol* **74**: 790-809.

Boucher, H. W. & G. R. Corey, (2008) Epidemiology of methicillin-resistant *Staphylococcus aureus*. *Clin Infect Dis* **46 Suppl 5**: S344-349.

Burton, B. M., K. A. Marquis, N. L. Sullivan, T. A. Rapoport & D. Z. Rudner, (2007) The ATPase SpoIIIE transports DNA across fused septal membranes during sporulation in *Bacillus subtilis*. *Cell* **131**: 1301-1312.

Chalker, A. F., A. Lupas, K. Ingraham, C. Y. So, R. D. Lunsford, T. Li, A. Bryant, D. J. Holmes, A. Marra, S. C. Pearson, J. Ray, M. K. Burnham, L. M. Palmer, S. Biswas & M. Zalacain, (2000) Genetic characterization of Gram-positive homologs of the XerCD site-specific recombinases. *J Mol Microbiol Biotechnol* **2**: 225-233.

Charpentier, E., A. I. Anton, P. Barry, B. Alfonso, Y. Fang & R. P. Novick, (2004) Novel cassette-based shuttle vector system for Gram-positive bacteria. *Appl Environ Microbiol* **70**: 6076-6085.

Chen, J. C. & J. Beckwith, (2001) FtsQ, FtsL and FtsI require FtsK, but not FtsN, for co-localization with FtsZ during *Escherichia coli* cell division. *Mol Microbiol* **42**: 395-413.

Dorazi, R. & S. J. Dewar, (2000) Membrane topology of the N-terminus of the *Escherichia coli* FtsK division protein. *FEBS Lett* **478**: 13-18.

Draper, G. C., N. McLennan, K. Begg, M. Masters & W. D. Donachie, (1998) Only the N-terminal domain of FtsK functions in cell division. *J Bacteriol* **180**: 4621-4627.

Dubarry, N., C. Possoz & F. X. Barre, (2010) Multiple regions along the *Escherichia coli* FtsK protein are implicated in cell division. *Mol Microbiol* **78**: 1088-1100.

Fisher, A. C. & M. P. DeLisa, (2008) Laboratory evolution of fast-folding green fluorescent protein using secretory pathway quality control. *PLoS ONE* **3**: e2351.

Jorge, A. M., E. Hoiczyk, J. P. Gomes & M. G. Pinho, (2011) EzrA contributes to the regulation of cell size in *Staphylococcus aureus*. *PLoS ONE* **6**: e27542.

Kaimer, C., J. E. Gonzalez-Pastor & P. L. Graumann, (2009) SpoIIIE and a novel type of DNA translocase, SftA, couple chromosome segregation with cell division in *Bacillus subtilis*. *Mol Microbiol* **74**: 810-825.

Kaimer, C. & P. L. Graumann, (2011) Players between the worlds: multifunctional DNA translocases. *Curr Opin Microbiol* **14**: 719-725.

Kaltwasser, M., T. Wiegert & W. Schumann, (2002) Construction and application of epitope- and green fluorescent protein-tagging integration vectors for *Bacillus subtilis*. *Appl Environ Microbiol* **68**: 2624-2628.

Klevens, R. M., M. A. Morrison, J. Nadle, S. Petit, K. Gershman, S. Ray, L. H. Harrison, R. Lynfield, G. Dumyati, J. M. Townes, A. S. Craig, E. R. Zell, G. E. Fosheim, L. K. McDougal, R. B. Carey & S. K. Fridkin, (2007) Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *J Amer Med Assoc* **298**: 1763-1771.

Koyama, T., M. Yamada & M. Matsuhashi, (1977) Formation of regular packets of *Staphylococcus aureus* cells. *J Bacteriol* **129**: 1518-1523.

Lesterlin, C., F. X. Barre & F. Cornet, (2004) Genetic recombination and the cell cycle: what we have learned from chromosome dimers. *Mol Microbiol* **54**: 1151-1160.

Liu, G., G. C. Draper & W. D. Donachie, (1998) FtsK is a bifunctional protein involved in cell division and chromosome localization in *Escherichia coli*. *Mol Microbiol* **29**: 893-903.

Liu, N. J., R. J. Dutton & K. Pogliano, (2006) Evidence that the SpoIIIE DNA translocase participates in membrane fusion during cytokinesis and engulfment. *Mol Microbiol* **59**: 1097-1113.

Massey, T. H., C. P. Mercogliano, J. Yates, D. J. Sherratt & J. Lowe, (2006) Double-stranded DNA translocation: structure and mechanism of hexameric FtsK. *Mol Cell* **23**: 457-469.

Oshida, T. & A. Tomasz, (1992) Isolation and characterization of a Tn*551*-autolysis mutant of *Staphylococcus aureus*. *J Bacteriol* **174**: 4952-4959.

Pereira, P. M., H. Veiga, A. M. Jorge & M. G. Pinho, (2010) Fluorescent reporters for studies of cellular localization of proteins in *Staphylococcus aureus*. *Appl Environ Microbiol* **76**: 4346-4353.

Pinho, M. G., H. de Lencastre & A. Tomasz, (2001) An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant *Staphylococci*. *Proc Natl Acad Sci U S A* **98**: 10886-10891.

Recchia, G. D. & D. J. Sherratt, (1999) Conservation of *xer* site-specific recombination genes in bacteria. *Mol Microbiol* **34**: 1146-1148.

Sciochetti, S. A., P. J. Piggot, D. J. Sherratt & G. Blakely, (1999) The *ripX* locus of *Bacillus subtilis* encodes a site-specific recombinase involved in proper chromosome partitioning. *J Bacteriol* **181**: 6053-6062.

Sharp, M. D. & K. Pogliano, (1999) An *in vivo* membrane fusion assay implicates SpoIIIE in the final stages of engulfment during *Bacillus subtilis* sporulation. *Proc Natl Acad Sci U S A* **96**: 14553-14558.

Sharp, M. D. & K. Pogliano, (2003) The membrane domain of SpoIIIE is required for membrane fusion during *Bacillus subtilis* sporulation. *J Bacteriol* **185**: 2005-2008.

Sharpe, M. E. & J. Errington, (1995) Postseptational chromosome partitioning in bacteria. *Proc Natl Acad Sci U S A* **92**: 8630-8634.

Steiner, W., G. Liu, W. D. Donachie & P. Kuempel, (1999) The cytoplasmic domain of FtsK protein is required for resolution of chromosome dimers. *Mol Microbiol* **31**: 579-583.

Tzagoloff, H. & R. Novick, (1977) Geometry of cell division in *Staphylococcus aureus*. *J Bacteriol* **129**: 343-350.

Veiga, H., A. M. Jorge & M. G. Pinho, (2011) Absence of nucleoid occlusion effector Noc impairs formation of orthogonal FtsZ rings during *Staphylococcus aureus* cell division. *Mol Microbiol* **80**: 1366-1380.

Wang, L. & J. Lutkenhaus, (1998) FtsK is an essential cell division protein that is localized to the septum and induced as part of the SOS response. *Mol Microbiol* **29**: 731-740.

Wang, L., Y. Yu, X. He, X. Zhou, Z. Deng, K. F. Chater & M. Tao, (2007) Role of an FtsK-like protein in genetic stability in *Streptomyces coelicolor* A3(2). *J Bacteriol* **189**: 2310-2318.

Wang, S. C., L. West & L. Shapiro, (2006) The bifunctional FtsK protein mediates chromosome partitioning and cell division in *Caulobacter*. *J Bacteriol* **188**: 1497-1508.

Wu, L. J. & J. Errington, (1994) *Bacillus subtilis* SpoIIIE protein required for DNA segregation during asymmetric cell division. *Science* **264**: 572-575.

Wu, L. J. & J. Errington, (1997) Septal localization of the SpoIIIE chromosome partitioning protein in *Bacillus subtilis*. *EMBO J* **16**: 2161-2169.

Wu, L. J., P. J. Lewis, R. Allmansberger, P. M. Hauser & J. Errington, (1995) A conjugation-like mechanism for prespore chromosome partitioning during sporulation in *Bacillus subtilis*. *Genes Dev* **9**: 1316-1326.

Yu, X. C., A. H. Tran, Q. Sun & W. Margolin, (1998a) Localization of cell division protein FtsK to the *Escherichia coli* septum and identification of a potential N-terminal targeting domain. *J Bacteriol* **180**: 1296-1304.

Yu, X. C., E. K. Weihe & W. Margolin, (1998b) Role of the C-terminus of FtsK in *Escherichia coli* chromosome segregation. *J Bacteriol* **180**: 6424-6428.

Zapun, A., T. Vernet & M. G. Pinho, (2008) The different shapes of cocci. *FEMS Microbiol Rev* **32**: 345-360.

Chapter 6

Concluding remarks and future perspectives

Concluding remarks and future perspectives

The characteristic mode of division of *Staphylococcus aureus*, as well as of other cocci, has been largely unexplored. This is because most of the work in the bacterial cell division field has been concentrated in rod-shaped organisms, such as *Escherichia coli* and *Bacillus subtilis*, which always place the septum at the same medial plane. Differently, *S. aureus* spherical cells divide in three orthogonal planes over successive division cycles (Tzagoloff & Novick, 1977, Koyama *et al.*, 1977).

In this thesis, the mechanisms behind *S. aureus* cell division and chromosome segregation were investigated. This study revealed that division plane selection in *S. aureus* is intimately dependent on nucleoid segregation. Before the initiation of chromosome partioning, all potential division planes would result in bisection of the nucleoid. Only after segregation of the two newly formed chromosomes, a nucleoid-free area, competent for the assembling of the division apparatus, is opened. Therefore, the orientation of chromosome segregation restricts the number of possible division planes to a single one. This effect is mediated by the nucleoid occlusion effector Noc that impedes the formation of Z-rings on top of the nucleoid.

In addition to Noc, two other regulators of the coordination between chromosome segregation and cell division were revealed. These are SpoIIIE and Slp DNA translocases that act synergistically to ensure proper clearance of the nucleoid from the division site before septum placement, particularly when normal chromosome segregation is impaired.

In order to perform these cell division studies, molecular genetic tools were developed to better manipulate *S. aureus*: (i) the restriction-modification gene *hsdR* was deleted from the genome of non-transformable *S. aureus* strains, in an attempt to make them able to accept foreign DNA; (ii) a series of plasmids for expression of fluorescent fusions to *S. aureus* proteins were constructed; (iii) an additional vector, pBCB13, was designed to permit ectopic expression of any fragment of interest, under the control of the IPTG-inducible P_{spac} promoter, in the ectopic *spa* locus of *S. aureus* chromosome.

Development of genetic tools to better manipulate *S. aureus*

The availability of *S. aureus* laboratory and clinical strains capable of accepting foreigner DNA, would be remarkably useful for genetic studies in this bacteria. However, at the time this work was initiated, the only transformable *S. aureus* strain was the highly mutagenized RN4220. To introduce DNA isolated from *E. coli* into other *S. aureus* strains, it was first necessary to introduce, by electroporation, the DNA into this transformable strain, before transferring it, to the *S. aureus* strain of interest.

The ability of RN4220 to receive foreign DNA was presumably the result of a stop mutation in the *hsdR* gene that encodes the conserved restriction endonuclease of the Sau1 Type I restriction-modification system, responsible for the degradation of foreign unmodified DNA (Waldron & Lindsay, 2006). We therefore deleted the *hsdR* gene from the genome of widely used *S. aureus* laboratory strains NCTC8325-4, SH1000 (methicillin-susceptible) and COL (methicillin-resistant), which have their genomes sequenced. However, inactivation of *hsdR* in the genome of these untransformable *S. aureus* strains was not sufficient to make them able to uptake foreign DNA, which suggested the existence of additional restriction barriers. In fact, after the publication of our work, Corvaglia *et al.* (Corvaglia *et al.*, 2010) reported the identification of the second factor that, when deleted in combination with *hsdR*, was sufficient to make two *S. aureus* clinical isolates (UAMS-1 and SA564) transformable. Initially identified as a type III-like endonuclease (Corvaglia *et al.*, 2010), this second factor is now known to be a type IV modification-dependent restriction endonuclease, named SauUSI (Xu *et al.*, 2011). This endonuclease specifically recognizes cytosine methylated DNA sequences and thus destroys any DNA isolated from *E. coli* K-12 derivatives, such as DH5α and XL1-Blue, that express the DNA cytosine methyltransferase protein, Dcm (Xu *et al.*, 2011, Monk *et al.*, 2012). Importantly, inactivation of both HsdR and SauUSI in *S. aureus* strains Newman (Monk *et al.*, 2012), NCTC8325-4 and COL (Veiga H. unpublished data) also renders them transformable, confirming that these two pathways are the major *S. aureus* barriers to transformation.

Using a different approach to facilitate transformation of *E. coli* DNA into *S. aureus* strains other than RN4220, Monk *et al.* (Monk *et al.*, 2012) constructed an *E. coli* Dcm mutant in the background of the high-efficiency cloning strain DH10B. Plasmids cloned in this strain are not cytosine methylated by Dcm and so are not recognized by SauUSI, which means that they can be directly transformed into *S. aureus* without being degraded.

To facilitate visualization of *S. aureus* protein localization, we constructed a toolkit of vectors designed to allow the expression of fluorescent derivatives of *S. aureus* proteins. We also constructed plasmid pBCB13 to insert fragments, under the control of the IPTG-inducible P_{spac} promoter, in the ectopic *spa* locus of *S. aureus* chromosome. The vector pBCB13 was used for complementation studies that required a low dosage of the mutated gene and for the generation of inducible mutants. It also allowed the construction of strains expressing fluorescent protein derivatives at a distant place from their native locus, which was particularly important when polar effects on the downstream genes of the same operon had to be avoided.

The direction of chromosome segregation defines *S. aureus* planes of division

In this thesis we show that, similarly to its *B. subtilis* and *E. coli* homologues (Wu & Errington, 2004, Bernhardt & de Boer, 2005), *S. aureus* Noc is a division inhibitor directly involved in preventing Z-ring assembly on top of the nucleoid. In *B. subtilis,* and most likely also in *S. aureus*, this protein is associated with chromosomal regions absent from the *ter* region (Wu & Errington, 2012) and so, only when the bulk of the chromosomes are completely segregated, a Noc-free area, competent for Z-ring assembly, is opened. This implies that the axis of chromosome segregation determines which plane will be used for Z-ring placement and consequently for division. Therefore, to understand how *S. aureus* divides, it is necessary to identify the key factors determining the orientation of chromosome segregation.

In theory, the axis of chromosome segregation can be established solely by determining the directionality of *oriC* segregation, as the other chromosomal loci that are replicated/segregated after *oriC*, will orderly move in the same direction. Through observation of *oriC* localization in *S. aureus*, it seems that after being completely segregated, the origins of replication localize near the intersection points between the two previous division planes (represented in Figure 6.1). Therefore, an attractive hypothesis for the movement of the chromosomes in a specific axis, is the existence of an origin binding factor at these cross junctions, that would direct the movement of the *oriC* region, and so of the chromosome, to these points. In fact, "scars" of previous division planes can be seen by electron microscopy and atomic force microscopy (Yamada *et al.*, 1996, Turner *et al.*, 2010) and it is tempting to speculate that a putative origin binding factor would be positioned at these locations, generating a protein gradient with higher concentration at the cross junctions. However, currently, there are no potential candidates for the role of origin binding factors in *S. aureus*. DivIVA of *S. aureus* was envisioned to have a role in this process since its *B. subtilis* homologue mediates the RacA-dependent anchoring of the chromosomes to the cell poles, at the onset of sporulation (Ben-Yehuda *et al.*, 2003, Thomaides *et al.*, 2001). However, *S. aureus* cells lacking DivIVA have no chromosome segregation defects and this protein does not colocalize with *oriC* regions, which indicates that it is most certainly not involved in chromosome segregation (Pinho & Errington, 2004) and moreover, *S. aureus* do not possess a RacA homologue.

Hypothetically, a chromosomal ParABS system could mediate *oriC* positioning in *S. aureus*, as in *C. crescentus* and *V. cholerae* (Toro *et al.*, 2008, Fogel & Waldor, 2006). In fact, it is the ParABS system of *B. subtilis* that drives the movement of *oriC* towards RacA/DivIVA, during sporulation (Ben-Yehuda *et al.*, 2003, Wu & Errington, 2003). However, a bioinformatics search revealed that *parA* homologues are not encoded in the genomes of bacteria from the Staphylococcus genus (Livny *et al.*, 2007) and deletion of the *S. aureus* ParB homologue (named Spo0J) does not cause obvious chromosome segregation defects (Veiga H. unpublished data). A possible strategy to identify the

mechanisms behind the establishment of the axis of chromosome segregation would be to screen a mutagenesis library for mutants that present mislocalized origins of replication and so are most likely affected in the orientation of chromosome segregation.

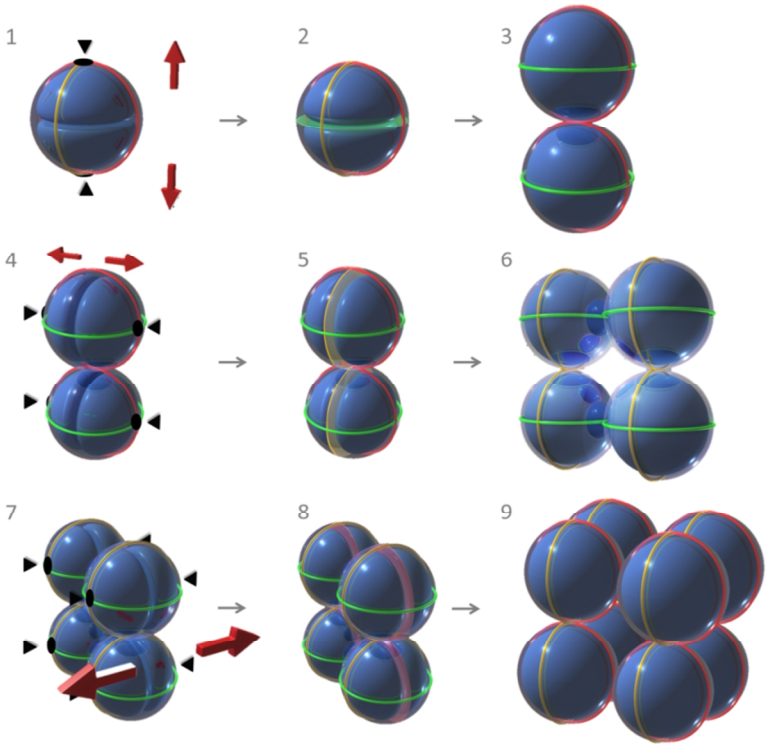

Figure 6.1. *S. aureus oriC* regions seem to always segregate towards to cross junctions of the two previous division planes. (1) A cell that in the previous two divisions cycles placed the septum in the circular planes marked with the orange and red lines, segregates its origins of replication towards the regions near the cross junctions of these two planes (black dots indicated by the black arrowheads), then (2) places the septum in the green plane and (3) divides. (4) The two daughter cells that used the green plane in the last division and the red plane in the second to last division segregate the *oriC* regions toward the intersection points between the green and red planes (black dots indicated by the black arrowheads), then (5) place the septum in the orange plane and (6) divide giving rise to 4 cells. (7) Like in previous division cycles, these four daughter cells position their newly replicated *oriC* regions near the cross junctions of the two previous division planes (black dots indicated by the black arrowheads), in this case the orange and green. (8) These cells then place the septa in the red plane and (9) divide. The red arrows are indicative of the direction of chromosome segregation.

S. aureus SpoIIIE and Slp DNA translocases act in synergy to promote chromosome segregation

Bacterial cells have evolved several mechanisms to closely synchronize the processes of chromosome segregation and cytokinesis, so that genetic information is faithfully passed onto the next generation. Among regulators required for this synchronization are the Noc protein (see above) and the DNA translocases of the FtsK/SpoIIIE family, whose role in *S. aureus* cell division was also explored in this thesis. We showed that the two *S. aureus* DNA translocases of FtsK/SpoIIIE family, SpoIIIE and Slp, act in two pathways to promote the movement of the chromosomes away from the division site. SpoIIIE is necessary, during normal growth, for proper chromosome segregation in approximately 9% of the population and, when required, it forms foci in the middle of the septum. Slp is a multifunctional septal protein with a C-terminal DNA translocase domain that seems to be required only in situations of impaired chromosome segregation.

We observed that the absence of both *S. aureus* SpoIIIE and Slp DNA translocase motors caused a more severe phenotype than the combined defects observed in the single mutants. This indicates that the two proteins, even if acting independently, cooperate to ensure the clearance of the division site. This data also suggests that at least one of the DNA translocases has the ability to substitute the activity of the other. However, for now, it is not clear how this could occur. In *B. subtilis,* which also expresses two DNA translocases with a synergistic activity, it was shown that in the absence of SftA (Slp homologue), more cells assemble SpoIIIE foci at the centre of the septum (Kaimer *et al.*, 2009). Also, SftA was shown to be unable to transport DNA through a closed septum (Biller & Burkholder, 2009) and so it cannot substitute for *B. subtilis* SpoIIIE post-septational activity (Burton *et al.*, 2007). Therefore, in *B. subtilis*, it is SpoIIIE that compensates for SftA absence (Kaimer *et al.*, 2009, Biller & Burkholder, 2009). However, in *S. aureus,* the situation could be different. The number of *S. aureus* SpoIIIE foci in a population, indicative of the level of requirement for SpoIIIE, did not rise in the absence of the Slp DNA translocase motor. Therefore, it is not likely that SpoIIIE can compensate

for the absence of Slp DNA translocase activity. The reverse situation, however, cannot be discarded. Assuming that, like their *B. subtilis* homologues, *S. aureus* SpoIIIE acts after septum closure and Slp cannot form translocation channels across two membranes and a peptidoglycan layer, it seems unlikely that Slp could compensate for the absence of SpoIIIE. However, if these conditions are not verified for the *S. aureus* homologues, it is possible that Slp could rescue DNA which was not properly segregated in the absence of SpoIIIE, explaining why the SpoIIIE knock-out mutant has 8% of defective cells, but in the absence of both DNA motors, 35% of the cells have chromosome segregation defects. The clarification of this situation requires further studies in which it is particularly important to determine if *S. aureus* SpoIIIE and Slp proteins transport DNA before or after septum closure.

An additional subject that still requires investigation is the putative role of SpoIIIE and Slp in chromosome dimer resolution in *S. aureus*. The lack of obvious chromosome segregation defects in the Slp C-terminal deletion mutant suggests that Slp, contrarily to its *E. coli* FtsK and *B. subtilis* SftA homologues (Biller & Burkholder, 2009, Liu *et al.*, 1998, Steiner *et al.*, 1999, Yu *et al.*, 1998), does not promote chromosome dimer resolution. It is possible, though, that *S. aureus* SpoIIIE could participate in this process, which would explain why approximately 8% of *S. aureus* population cannot segregate their DNA properly in the absence of SpoIIIE. These cases could represent the situations in which chromosome dimers were formed, due to recombination events during replication, but could not be resolved because SpoIIIE was absent and Slp could not promote chromosome dimer resolution. In fact, if Slp was able to rescue unsegregated DNA in the absence of SpoIIIE, but not to resolve chromosome dimers, we could have an explanation for the fact that in the presence of Slp, it is possible to avoid most miss segregation events observed in the double mutant, but not to avoid the presence of chromosome dimers which would be responsible for the defects observed in 8% of the cells. The first approach to test this hypothesis would be to investigate if the chromosome segregation defects of the SpoIIIE knockout mutant could be relieved in the absence of RecA, as RecA-mediated recombination events are the main routes for the generation of chromosome

dimers. The hypothesis that *S. aureus* SpoIIIE is involved in chromosome dimer resolution gains more strength with the observation that its *B. subtilis* homologue is also necessary to facilitate this process (Kaimer *et al.*, 2011).

We have determined that Slp is a multifunctional protein since the elimination of its entire coding sequence causes a much more severe phenotype than just the deletion of its C-terminal DNA motor. However, at the moment, the second role of Slp, performed by its N-terminal and linker domains, remains unknown. By establishing the parallelism with the function of FtsK N-terminal domain in recruiting divisome proteins (Chen & Beckwith, 2001, Draper *et al.*, 1998), we tested the ability of FtsW, TagO, PBP2 and PBP4 (late septal proteins) to localize in the absence of Slp. We observed that these four proteins still localize at the septum in the absence of Slp (data not shown). However, many other proteins have still to be tested before determining with certainty if Slp functions in the recruitment of septal proteins. A second hypothesis for the role of Slp was prompted by the observation that, in the absence of Slp, several mutated cells have more than one septum and, in some cases, it is clear that a second septum was assembled at a new division plane, before the end of the previous round of division. A preliminary study indicated that these cells are able to complete the first round of division, even if the second septum is already in an advanced state of assembly, suggesting that the Slp mutant cells are not deficient in the autolysis/splitting process. Alternatively, Slp could function as a cell division check-point that regulates the timing of septum placement so that a second division would not start before the end of the previous one.

Overall, this thesis work led to the identification of a crucial role for the nucleoid in the establishment of *S. aureus* planes of division and revealed the important role of Noc, SpoIIIE and Slp regulators in the synchronization of chromosome segregation and cell division in *S. aureus*. This work also highlighted the importance of studying organisms with different morphologies and modes of division from the more traditional model

organisms, to gain a better understanding of the mechanisms behind bacteria cell division.

References

Ben-Yehuda, S., D. Z. Rudner & R. Losick, (2003) RacA, a bacterial protein that anchors chromosomes to the cell poles. *Science* **299**: 532-536.

Bernhardt, T. G. & P. A. de Boer, (2005) SlmA, a nucleoid-associated, FtsZ binding protein required for blocking septal ring assembly over chromosomes in *E. coli*. *Mol Cell* **18**: 555-564.

Biller, S. J. & W. F. Burkholder, (2009) The *Bacillus subtilis* SftA (YtpS) and SpoIIIE DNA translocases play distinct roles in growing cells to ensure faithful chromosome partitioning. *Mol Microbiol* **74**: 790-809.

Burton, B. M., K. A. Marquis, N. L. Sullivan, T. A. Rapoport & D. Z. Rudner, (2007) The ATPase SpoIIIE transports DNA across fused septal membranes during sporulation in *Bacillus subtilis*. *Cell* **131**: 1301-1312.

Chen, J. C. & J. Beckwith, (2001) FtsQ, FtsL and FtsI require FtsK, but not FtsN, for co-localization with FtsZ during *Escherichia coli* cell division. *Mol Microbiol* **42**: 395-413.

Corvaglia, A. R., P. Francois, D. Hernandez, K. Perron, P. Linder & J. Schrenzel, (2010) A type III-like restriction endonuclease functions as a major barrier to horizontal gene transfer in clinical *Staphylococcus aureus* strains. *Proc Natl Acad Sci U S A* **107**: 11954-11958.

Draper, G. C., N. McLennan, K. Begg, M. Masters & W. D. Donachie, (1998) Only the N-terminal domain of FtsK functions in cell division. *J Bacteriol* **180**: 4621-4627.

Fogel, M. A. & M. K. Waldor, (2006) A dynamic, mitotic-like mechanism for bacterial chromosome segregation. *Genes Dev* **20**: 3269-3282.

Kaimer, C., J. E. Gonzalez-Pastor & P. L. Graumann, (2009) SpoIIIE and a novel type of DNA translocase, SftA, couple chromosome segregation with cell division in *Bacillus subtilis*. *Mol Microbiol* **74**: 810-825.

Kaimer, C., K. Schenk & P. L. Graumann, (2011) Two DNA translocases synergistically affect chromosome dimer resolution in *Bacillus subtilis*. *J Bacteriol* **193**: 1334-1340.

Koyama, T., M. Yamada & M. Matsuhashi, (1977) Formation of regular packets of *Staphylococcus aureus* cells. *J Bacteriol* **129**: 1518-1523.

Liu, G., G. C. Draper & W. D. Donachie, (1998) FtsK is a bifunctional protein involved in cell division and chromosome localization in *Escherichia coli*. *Mol Microbiol* **29**: 893-903.

Livny, J., Y. Yamaichi & M. K. Waldor, (2007) Distribution of centromere-like *parS* sites in bacteria: insights from comparative genomics. *J Bacteriol* **189**: 8693-8703.

Monk, I. R., I. M. Shah, M. Xu, M. W. Tan & T. J. Foster, (2012) Transforming the untransformable: application of direct transformation to manipulate genetically *Staphylococcus aureus* and *Staphylococcus epidermidis*. *mBio* **3**: e00277-11.

Pinho, M. G. & J. Errington, (2004) A *divIVA* null mutant of *Staphylococcus aureus* undergoes normal cell division. *FEMS Microbiol Lett* **240**: 145-149.

Steiner, W., G. Liu, W. D. Donachie & P. Kuempel, (1999) The cytoplasmic domain of FtsK protein is required for resolution of chromosome dimers. *Mol Microbiol* **31**: 579-583.

Thomaides, H. B., M. Freeman, M. El Karoui & J. Errington, (2001) Division-site-selection protein DivIVA of *Bacillus subtilis* has a second distinct function in chromosome segregation during sporulation. *Genes Dev* **15**: 1662-1673.

Toro, E., S. H. Hong, H. H. McAdams & L. Shapiro, (2008) *Caulobacter* requires a dedicated mechanism to initiate chromosome segregation. *Proc Natl Acad Sci U S A* **105**: 15435-15440.

Turner, R. D., E. C. Ratcliffe, R. Wheeler, R. Golestanian, J. K. Hobbs & S. J. Foster, (2010) Peptidoglycan architecture can specify division planes in *Staphylococcus aureus*. *Nat Commun* **1**: 26.

Tzagoloff, H. & R. Novick, (1977) Geometry of cell division in *Staphylococcus aureus*. *J Bacteriol* **129**: 343-350.

Waldron, D. E. & J. A. Lindsay, (2006) Sau1: a novel lineage-specific type I restriction-modification system that blocks horizontal gene transfer into *Staphylococcus aureus* and between *S. aureus* isolates of different lineages. *J Bacteriol* **188**: 5578-5585.

Wu, L. J. & J. Errington, (2003) RacA and the Soj-Spo0J system combine to effect polar chromosome segregation in sporulating *Bacillus subtilis*. *Mol Microbiol* **49**: 1463-1475.

Wu, L. J. & J. Errington, (2004) Coordination of cell division and chromosome segregation by a nucleoid occlusion protein in *Bacillus subtilis*. *Cell* **117**: 915-925.

Wu, L. J. & J. Errington, (2012) Nucleoid occlusion and bacterial cell division. *Nat Rev Microbiol* **10**: 8-12.

Xu, S. Y., A. R. Corvaglia, S. H. Chan, Y. Zheng & P. Linder, (2011) A type IV modification-dependent restriction enzyme SauUSI from *Staphylococcus aureus* subsp. *aureus* USA300. *Nucleic Acids Res* **39**: 5597-5610.

Yamada, S., M. Sugai, H. Komatsuzawa, S. Nakashima, T. Oshida, A. Matsumoto & H. Suginaka, (1996) An autolysin ring associated with cell separation of *Staphylococcus aureus*. *J Bacteriol* **178**: 1565-1571.

Yu, X. C., E. K. Weihe & W. Margolin, (1998) Role of the C-terminus of FtsK in *Escherichia coli* chromosome segregation. *J Bacteriol* **180**: 6424-6428.

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