Chromosome dynamics in *Bacillus subtilis* – Characterization of the Structural Maintenance of Chromosomes (SMC) complex

Dissertation
for the doctor's degree of natural sciences
(Dr. rer. nat. corresponding to Ph.D.)
submitted to the Fachbereich Biologie
Philipps Universität Marburg

by
Judita Mascarenhas
from Bhadravathi, India

Marburg an der Lahn
2004

Von Fachbereich Bioloogie der Philipps-Universität Marburg als Dissertation am 27 April 2004 angenommen.

Tag der mündlichen Prüfung: 28.04.2004

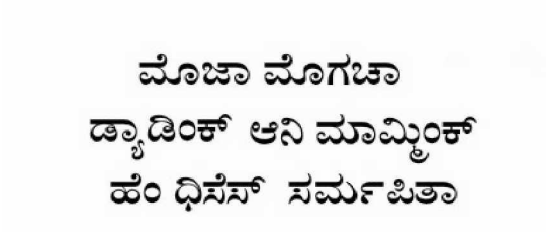
Erstgutachter: Prof. Dr. E. Bremer

Zweitgutachter: Dr. P. Graumann

Drittgutachter: Prof. Dr. M. Bölker

Viertgutachter: Prof. Dr. A. Batschauer

To my dear parents



and in the memory of Babuji, my dear uncle

Zusammenfassung

Alle Zellen müssen ihr Erbmaterial verdoppeln und dafür Sorge tragen, daß jede Tochterzelle einen kompletten Satz des Erbguts vor der Zellteilung erhält. In Bakterien müssen die Chromosomen organisiert und kompaktiert werden, während sie gleichzeitig dynamisch sein müssen, um laufende zelluläre Prozesse wie DNA Reparatur, Rekombination, Transktiption und Replikation zu ermöglichen. SMC (Structural Maintenance of Chromosome) Proteine bilden eine ubiquitäre Proteinfamilie, die eine zentrale Rolle in verschiedenen Chromosomendynamiken spielt. Das Hauptaugenmerk in dieser Arbeit ruht auf der Charakterisierung der SMC Proteine und ihrer Partner aus *Bacillus subtilis*.

Genbanksuchen haben zu der Identifizierung zweier Interaktionspartner des SMC Proteins geführt. Diese Proteine, ScpA und ScpB, sind in Bakterien und Archaen konserviert. Die Deletion des *scpA* oder des *scpB* Gens führte zu einem der *smc* Deletionsmutante ähnlichen Phänotyp, d.h. temperatursensitivem langsamen Wachstum (unterhalb 23°C), dekondensierten Nukleoiden (zelluläre Struktur der Chromosomen) und einem ausgeprägten Segregationsdefekt. Die gleichzeitige Deletion der Gene erzeugte keinen veränderten Phänotyp, was zeigt, dass alle drei Proteine im gleichen Aspekt der Chromosomen-Kondensation und Segregation fungieren. Um ihre Funktion *in vivo* zu untersuchen, wurden die Proteine in Zellen mit Hilfe von voll funktionellen GFP Fusionen lokalisiert. Alle drei Proteine bildeten diskrete Foci in den Zellen, einem bis dato unbekannten Lokalisationsmuster, das sich dynamisch während des Zellzyklus veränderte: zu Beginn des Zellzyklus befanden sich die Foci in der Zellmitte, und nach der Verdopplung des Focus wanderten die beiden Foci rasch entgegengesetzt in Richtung der Zellpole. In diesen bipolaren Foci verblieben die drei Proteine für den Rest des Zellzyklus. Die Bildung des Proteinkomplexes konnte durch Fluoreszenz Resonanz Energie Transfer (FRET) und durch Depletionsstudien belegt werden. So konnte die Bildung der Foci nur in Anwesenheit aller Proteine beobachtet werden, nicht jedoch in Abwesenheit eines der drei Proteine. Die spezifische Lokalisierung des SMC Komplex hing auch von fortlaufender DNA Replikation ab, von zellulärer Gyrase Aktivität (d.h. von der Struktur der DNA), sowie von der ATPase-Aktivität von SMC. Die Überproduktion von SMC führte zu einer Über-Kondensation der Nukleoide, wobei die Lokalisation

des SMC Komplexes erhalten blieb, was darauf hin deutet, daß die beobachteten Foci aktive Kondensationszentren darstellen.

Weiterhin zeigten die Proteine des SMC Komplex wachstumsabhängige Expression. SMC und ScpB waren nur in wachsenden Zellen vorhanden, und wurden rasch beim Übergang in die Statonärphase abgebaut. Die Analyse der RNA Mengen in verschiedenen Wachstumsphasen durch Primer Extensionsanalyse zeigte, daß das *smc* Transkript im Übergang zur Stationärphase nicht abnimmt. Diese Experimente zeigten einen bisher nicht identifizierten *smc* Promotor auf, und erbrachten den Nachweis, daß SMC posttranskriptionell reguliert wird. Die *smc*, *scpA*, und *scpB* Deletionsmutanten wiesen ebenfalls eine ausgeprägte Sensitivität gegenüber Mitomycin C (MMC) auf, welches Doppelstrangbrüche (DSBs) in die DNA einführt. Demnach wird der SMC Komplex ebenfalls für die Reparatur von DSBs benötigt.

Weiterhin wurde die Funktion des SMC Proteins YirY untersucht, welches homolog zum DNA Reparatur Protein SbcC aus *Escherichia coli* ist. Die *yirY* Deletion führte ebenfalls zu einer deutlichen Sensitivität zu MMC, was eine Rolle in der DSB Reparatur belegt. In MMC behandelten Zellen bildete YirY Foci auf der DNA, welche aktive DSB Reparaturzentren darstellen könnten. In Gegensatz dazu waren die anderen Proteine aus dem gleichen Operon, AddA, AddB, and SbcD überall in den Zellen vorhanden und bildeten keine speziellen Strukturen, was darauf hindeutet, daß SbcC und AddAB in verschiedenen Reparaturwegen fungieren.

Die subzelluläre Lokalisation der Topoisomerase IV Untereinheiten ParC und ParE wurde ebenfalls in dieser Arbeit beleuchtet. ParC lokalisierte auf dem gesamten Nukleoid, ganz im Gegenteil zu einer früheren Studie, in der ParC ausschließlich in der Nähe der Zellpole vorhanden war, wonach ParC eine spezialisierte Rolle bei der Dekatenierung von Chromosomen zugesprochen wurde. Durch Überproduktion von ParC und ParE wurden die Nukeloide noch stärker kompaktiert, was zusammen mit der Lokalisierung eine generelle Rolle in der Chromosomenkompaktierung belegt.

Ein weiterer Aspekt in dieser Arbeit war die Lokalisierung von Ribosomen. Das L1 Protein aus der großen Untereinheit lokalisierte in wachsenden Zellen in den zytoplasmatischen Stellen, die das Nukeloid umgeben, wohingegen es in stationären Zellen und nach Inhibition der Transkription überall in der Zelle vorlag. Demnach hängt die spezifische Lokalisierung von Ribosomen von aktiver RNA Synthese in den Zellen ab.

Insgesamt läßt sich schlußfolgern, daß die Lokalisation von Proteinen, die an der Chromosomensegregation, DNA Reparatur und Translation beteiligt sind, ein wesendlich definierteres Bild der räumlichen Funktion der Proteine in lebenden Bakterien erbrachte.

Summary

All cells need to duplicate and separate their genetic material faithfully into the future daughter cells before cell division takes place. In bacteria, the chromosome has to be organized and compacted whilst, at the same time, it needs to be dynamic to allow other ongoing cellular processes like repair, recombination, transcription, replication and segregation to take place. SMC (Structural Maintenance of Chromosome) protein belongs to a ubiquitous protein family that play crucial roles in chromosome dynamics. The main interest of this work is to characterize the function of the SMC protein in *Bacillus subtilis*.

Data base searches have led to the identification of two interaction partners of SMC. These proteins, ScpA and ScpB are conserved among bacterial and archaeal species possessing SMC. The *scpA* or *scpB* deletions showed a similar phenotype to that of a *smc* disruption, namely temperature sensitive slow growth (below 23°C), decondensed nucleoids and a strong segregation defect. Their simultaneous deletion did not exacerbate the phenotype, suggesting that all the three proteins function in the same pathway in chromosome condensation. To investigate their *in vivo* function, the proteins where localized in the cells using functional GFP fusions. The subcellular localization showed bipolar foci, a unique pattern of localization that was dynamic and cell cycle dependent. The foci were present at mid-cell position in smaller cells and separated towards opposite cell poles within a few minutes. The formation of a complex between SMC, ScpA, and ScpB *in vivo* was confirmed using fluorescence resonance energy transfer (FRET) and depletion studies. Formation of foci was only seen in the presence of all three proteins, but not in the absence of any one of them. The specific localization pattern of these proteins also depended on ongoing DNA replication, on active gyrase and thus on DNA topology, as well as on SMC's ATPase activity. Overproduction of SMC led to increased compaction of nucleoids but the localization was retained in the form of foci suggesting that the foci represent-active chromosome condensation centers.

The proteins of the SMC complex showed growth dependent protein expression. SMC and ScpB proteins were present in actively replicating exponential phase cells, but were rapidly depleted as the cells entered stationary phase. Analysis with total RNA extracts from various growth phases by primer extension studies

showed a strong transcript for SMC that was present even in stationary phase. This experiment led to the identification of a new promoter for *smc*, and suggests that SMC is regulated at the protein level by a protease that is induced at the onset of stationary phase. *Smc*, *scpA*, and *scpB* deletion mutant cells were also sensitive to Mitomycin C (MMC) treatment, which induces double strand breaks (DSB) into DNA. This finding revealed a role of the SMC complex in DSB repair.

I also investigated the role of YirY, a homolog of the DSB repair protein SbcC which is a proposed member of SMC family. Upon disruption of *yirY/sbcC*, the cells did not show any visible phenotype but the cells were sensitive to MMC, suggesting its role in repair. SbcC formed foci only in MMC treated cells, so the foci in the cell might represent a DNA repair centers. Other proteins located in the same operon as SbcC, AddA, AddB, and SbcD, did not show any specific pattern of localization, but were present throughout the cell and showed slight increase in their fluorescence intensity after MMC treatment, suggesting that SbcC and AddAB function in different in repair pathways.

The localization of topoisomerase IV subunits ParC and ParE has also been investigated in this work. The fluorescent protein fusion of ParC localized throughout the nucleoid, contrarily to the previously published bipolar localization as foci, which had suggested a specialized function of topoisomerase IV in chromosome decatenation. Upon over expression of ParC and ParE, the cells contained more condensed nucleoids, revealing a general role of topoisomerase IV in global chromosome compaction.

A further aspect of this work was the study of dynamic localization of ribosomes. The large subunit ribosome protein L1 showed specific localization in the cytoplasmic space surrounding the nucleoid in growing cells, and was seen diffused throughout the cell in the stationary phase. The same effect was observed upon inhibition of transcription, suggesting the dependence of specific ribosome localization on active transcription.

In toto, localization of DNA segregation, DNA repair and the ribosomal proteins has provided a more defined view of the spatial organization of these cellular processes in live bacterial cells.

Abbreviations

ATP	adenisine-5'- triphosphate
amyE	gene coding for α-amylase
bp	base pair
cDNA	complementary DNA
Cm^r	chloramphenicol resistant
DAPI	4',6-diamidino-2-phenylindole
DSBR	double strand break repair
dsDNA	double stranded DNA
EDTA	ethylene diamine tetra acetic acid
EM	electron microscopy
EtBr	ethidium bromide
Fig	figure
FP	fluorescent protein
FRET	fluorescent resonance energy transfer
GFP/YFP/CFP	green/ yellow/cyan fluorescent protein
h	hour
IPTG	isopropanol-b-D-thiogalactopyranoside
kb	kilo base(s)
LB	Luria-Bertani medium
MCS	multiple cloning sites
min	minute(s)
mls	macrolide lincosamine streptogramidine B
MMC	mitomycin C
nm	nanometer
$O.D_{xxx}$	optical density at xxx nm
Ori	origin of replication
PCR	polymerase chain reaction
RNase	ribonuclease
RT	room temperature
rpm	revolutions per minute
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SMC	structural maintenance of chromosome protein
T_m	melting temperature of dsDNA

TB	tris boric acid buffer
TE	tris EDTA buffer
tet^r	tetracyclin resistance
Tris	tris-(hydroxymethyl) aminomethane
U	unit of enzyme activity
UV	ultraviolet light
wt	wild type strain
Σ	sum of
Δ	deletion
::	gene replacement at chromosome by double crossover

1 Introduction

Life on earth persists because of its propagation through cell division - a central cellular process that is shared by all living organisms. Before a cell divides it has to duplicate a number of sub cellular components, most importantly the DNA molecule(s) carrying the genetic information and depending on the organism, organelles and then segregate them into the appropriate daughter cell compartments. This process is maintained by well-coordinated action of many dedicated proteins that make up a functional network whose complexity depends on the nature of the respective organism. Although compared to eukaryotes, prokaryotic cell division seems much simpler with most of the time only one major DNA molecule and lack of membrane-dependent organelles, many basic principles are functionally conserved. The DNA of bacterial chromosome is several thousand micrometers long and therefore are condensed into a compact structure called 'nucleoids' that has the diameter of only 0.5 µm (Rouviere-Yaniv *et al.*, 1979). A typical bacterial cell contains >250 different species of DNA binding proteins (Robinson and Kadonaga, 1998), which include DNA polymerases, topoisomerases, helicases, histone-like proteins, etc. These proteins are associated with the nucleoid and take part in chromosome organization during various cellular processes like replication, recombination, repair, modification and transcription of DNA. One among these players is the SMC protein, which belongs to a ubiquitous protein family and plays a key role in maintaining chromosome organization. This work is focused on the *in vivo* characterization of SMC and proteins interacting with SMC by making use of genetic and microscopic approaches.

From the important model organisms *Escherichia coli* a Gram-negative enterobacterium, *Bacillus subtilis* a Gram-positive soil bacterium, and *Caulobacter cresentus* a dimorphic Gram-negative aquatic bacterium all of which have been under thorough investigation for several years now, our laboratory decided to focus on *Bacillus subtilis* which is a broadly distributed, rod-shaped micro-organism that resides in the upper layers of soil. *B. subtilis* is a facultative aerobe and capable of converting to anaerobic nitrate respiration under oxygen limiting conditions (Hoffmann *et al.*, 1995). It has the ability to form extremely resistant endospores in response to nutrient deprivation or slow dehydration (Stragier and Losick, 1996) and

because it is genetically easily accessible, it has been accepted as one of the best-studied bacteria even before its genome was entirely sequenced a few years ago (Kunst *et al.*, 1997; Weber and Marahiel, 2003).

1.1 Basic mechanisms of bacterial replication and cell division

A cell divides only after molecular sensors have detected that its genetic material, DNA, providing the molecular blueprint for daughter cells to survive, has been faithfully duplicated in a damage-free manner. In eukaryotes, this sensor is a cell division cycle molecule Cdc25, that turns on the proteins required for the actual cell division event (Jinno *et al.*, 1994). Precise DNA replication has to be followed by the segregation process which involves a complex sequence of structural events termed mitosis in eukaryotes. This is a marked difference compared to prokaryotes where replication is not followed by but coupled to the segregation process and is therefore coordinated with the cell growth and division (Helmstetter, C, 1996).

In bacteria, chromosome replication is initiated when a critical size of a growing cell is reached (Messer, W., and Weigel, C, 1996). This parameter is called the initiation mass. In *Vibrio harveyi* the protein CgtA was shown to be involved in coupling of chromosome replication to cell growth and division. In *B. subtilis,* its homologue, Obg, has been proposed to control DNA replication and regulate initiation of sporulation by sensing the intracellular GTP level and stimulating the activity of a phosphorelay system which in turn activates several proteins involved in replication processes (Sikora-Borgula *et al.*, 2002).

Initiation of replication commences when an ATP-bound replication initiation protein DnaA binds to the AT rich DnaA boxes in the replication origin, *OriC,* regions and causes local strand melting (Moriya *et al.*, 1988). The DnaB helicase is then recruited to the unwound region. Together with other proteins of the primosome complex the strands are loaded on the DNA polymerase replication machinery, which is located at the mid cell. In *B. subtilis,* the DNA now moves through a stationary replisome complex (Lemon and Grossman, 1998) whereas in case of *C. cresentus* the replisome is mobile (Jensen *et al.,* 2002). During the replication process, replicated chromosomes move outward towards each cell halves. The termination of replication takes place through arresting of the replication forks by complex formation of the

replication termination protein (RTP) with the *ter* sites located at approximately 172° on the chromosome (Bussiere and Bastia, 1999). The replicated chromosomes are thereafter separated by decatenation process involving topoisomerase IV and the site-specific recombinases, CodV and RipX (Sciochetti and Piggot, 2000). Once the mid cell region is cleared from the replicated chromosomes by FtsK/SpoIIIE, bacterial cells assemble a ring like cytoskeletal structure at the division site, which is composed of tubulin-like FtsZ protein that constricts the cellular membrane and forms the septum. This FtsZ ring structure or the divisome is localized to the division site by the Min proteins (Raskin and De Boer, 1997). The Min system plays an important role in division site placement by inhibiting FtsZ ring formation at polar regions. It comprises the MinC and MinD complex and the inhibitor protein which is called DivIVA in *B. subtilis* and MinE in *E. coli*, that ensures the inhibition only at the polar regions (Cha and Stewart, 1997; Edwards and Errington, 1997; Marston *et al.*, 1998). Under normal conditions, bacterial cell division is symmetric. However, *B. subtilis* undergoes asymmetric division when conditions of nutrient limitation and high population density result in the initiation of a sporulation pathway that culminates in the formation of a heat- and desiccation-resistant spore. During sporulation, FtsZ forms a septum close to one of the cell poles - a process regulated by the master sporulation regulator, Spo0A (Levin and Losick, 1996; Stragier and Losick, 1996). In case of *Caulobacter*, the cell cycle is inherently asymmetric, a sessile-stalked cell undergoes asymmetric cytokinesis releasing a flagellated motile swarmer cell. This motile cell has to re-differentiate into a sessile-stalker cell before becoming able to undergo a further round of cell division (Wheeler *et al.*, 1998). This is achieved by repression of the replication process by a response regulator (CtrA) that is later proteolyzed when the swarmer cell differentiates into a sessile cell. (Shapiro and Losick, 2000).

The mechanism of bacterial chromosome partitioning was explained by an ‘extrusion-capture’ model proposed by (Lemon and Grossman, 2001) (fig. 1). This model assumes that the energy from the replication factory is used to power partitioning of the replicated chromosomes. The replicated chromosomes are captured at the cell quarter position and are organized through compaction and supercoiling which further assists the segregation process. This model was then further refined by a ‘push, direct, condense, hold and clear’ model by (Sawitzke and Austin, 2001) and

assumes that daughter DNA strands are actively transported to the cell halves, possibly by the Par proteins.

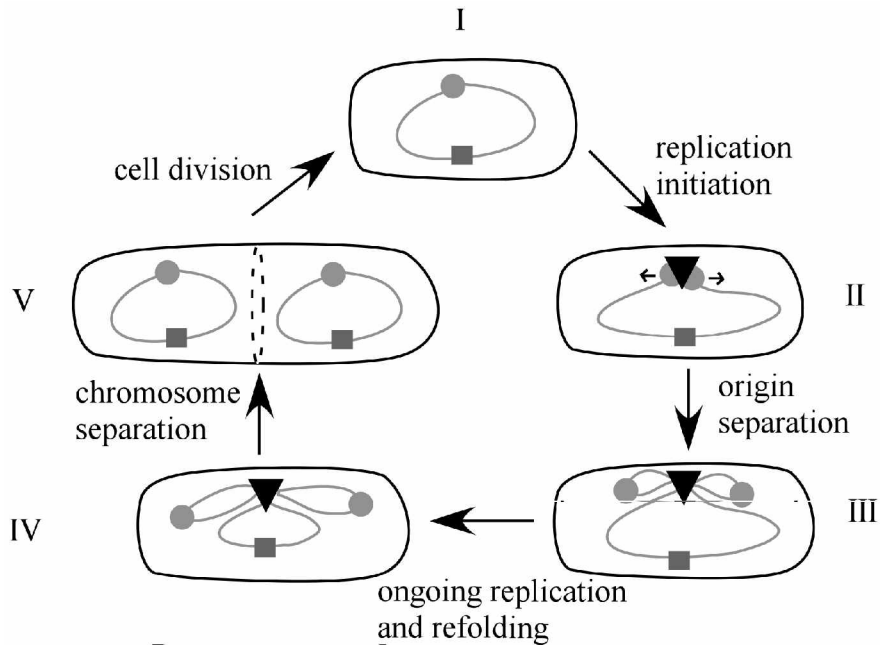


Fig. 1: Simplified model of bacterial cell cycle. DNA (grey lines), *oriC* (grey circles), terminus, *terC* (dark grey square), DNA polymerase (triangles), and cytokinetic ring FtsZ (dashed line). DNA replication initiates at the mid cell. The sister origins separate out bidirectionally. The replication continues followed by compaction of a newly replicated DNA until there are two complete and separate chromosomes. Finally the cell divides medially by the FtsZ ring formation. Figure adapted from (Lemon and Grossman, 2001).

1.2 Organization of bacterial chromosome

With some exceptions such as *Streptomycetes coelicolor* that possesses linear DNA and the *Borrelia* genus whose genome is made from linear DNA with hairpin ends, most bacterial cells possess a closed circular genomic DNA molecule. As stated earlier, the bacterial chromosome is about 1000-fold longer than the cell size (Drlica, K., 1986) and is condensed into a compact structure called ‘nucleoid’. The bacterial nucleoid is functionally analogous to the eukaryotic nucleus, e.g. the packing density of the DNA in the nucleoid is like that of eukaryotic interphase nuclei and would thus allow diffusion in and out of even large macromolecules (Kellenberger, 1991). Early attempts to elucidate the nucleoid structure using techniques of fixation led to a number of artefacts caused by the fixation technique itself. Nucleoids prepared from the cryo-freeze substituted cells showed a central dense regions and long, thinner

cytoplasmic protrusion-like clefts, see fig. 2 (Bohrmann *et al.*, 1991; Hobot *et al.*, 1985):

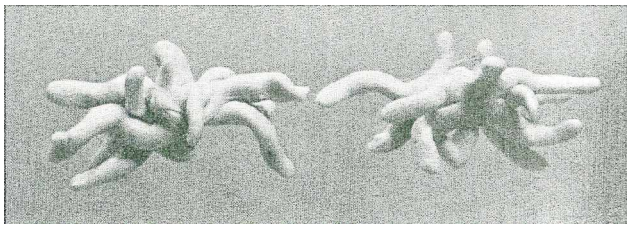


Fig. 2: Schematic model of bacterial nucleoid from the sections of cryofixed, freeze substituted *E. coli* cells. Figure adapted from Bohrmann et al., 1991.

The nascent RNA was shown to localize at the nucleoid periphery (Ryter and Chang, 1975), hence these edges of chromosomal protrusions from the nucleoids were interpreted as areas of a metabolically active nucleoid undergoing active transcription. Furthermore, DNA from isolated nucleoids was shown to possess a negatively supercoiled topology and these supercoils could not be relaxed by a single nick. This observation was interpreted in terms of topologically independent chromosomal domains that were calculated as 50 per genome for *E. coli* (Sinden and Pettijohn, 1981); (Drlica, 1986).

1.2.1 Membrane attachment of nucleoids

The compact nucleoid structure is maintained by membrane-DNA, protein-DNA and RNA-DNA interactions (Guillen and Bohin, 1986). In *B. subtilis* or *E. coli*, it was not possible to obtain membrane-free nucleoids (Harmon and Taber, 1977) (fig. 3), which led to the hypothesis that nucleoids are anchored to the membrane. Specifically, in *B. subtilis,* the chromosome origin region isolates were enriched in membrane fractions and these attachments are thought to facilitate chromosome replication and segregation processes. DnaB was one among the proteins involved in DNA attachment process playing an essential role in DNA replication and membrane attachment of the Ori of replication of chromosomes (Laurent and Vannier, 1973). It was shown that DnaB forms specific foci and localizes at the OriC region (Imai *et al.*, 2000). Recent investigations identified a novel protein RacA that localized near the poles and on the nucleoid and acts as an adhesion component bridging the origin region to the cell poles (Ben-Yehuda *et al.*, 2003).

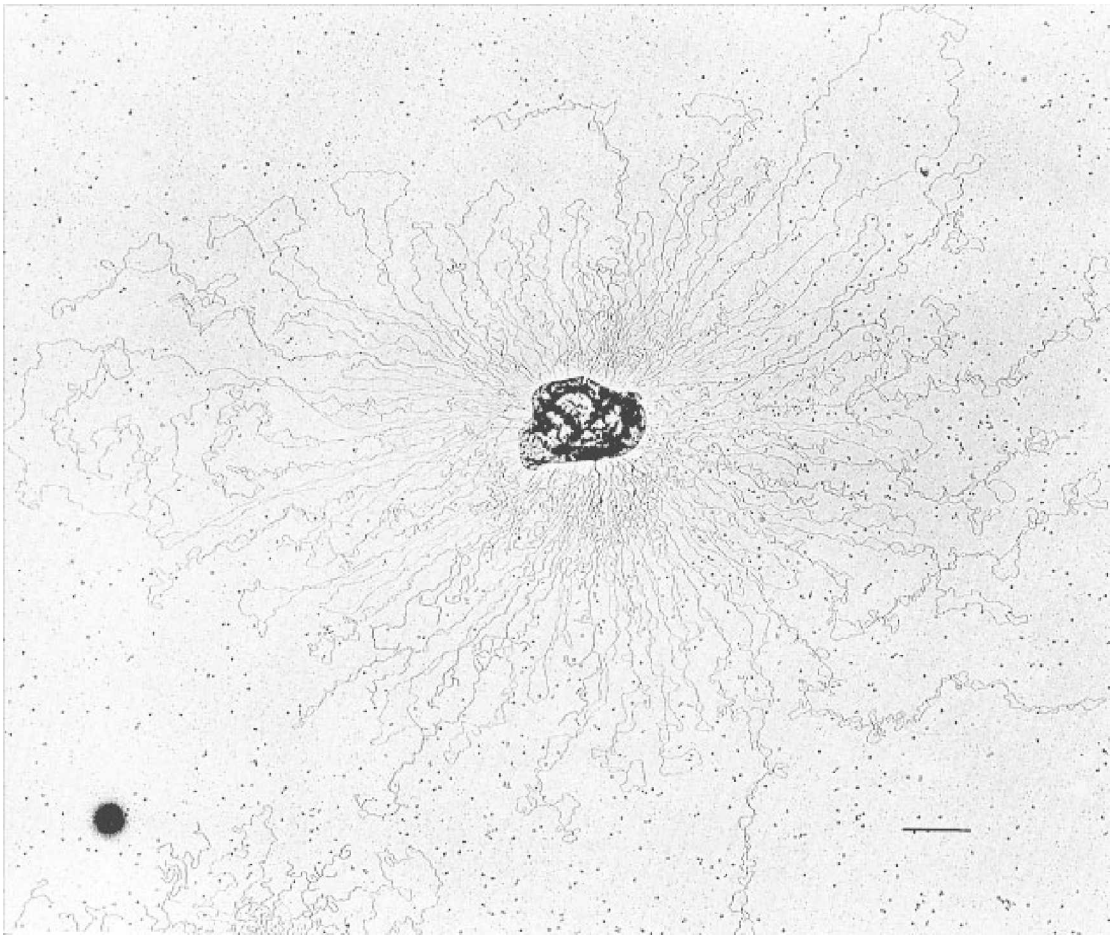


Fig. 3: Membrane attachment of nucleoid- EM of isolated *E. coli* nucleoid spread in the presence of spermidine. (Scale bar 1µm). Fig. adapted from '*Escherichia coli* and *Salmonella typhimurium*', Cellular and Molecular Biology, Vol 1, 1987 (ASM press).

1.2.2 The nucleoid structure is dynamic

Nucleoids in rapidly growing cells appear in complex shapes (Zimmerman, 2003). This is due to the occurrence of cellular processes like transcription and replication that require a very dynamic state of chromosomes. Moreover, chromosomes appear to have a defined orientation within the cell which has been determined using various origin region markers (Levin and Grossman, 1998; Losick and Shapiro, 1999; Webb *et al.*, 1997). Soon after replication, origin regions separate from each other and move to opposite sides of the cell, while the terminus regions is found in the mid cell (Teleman *et al.*, 1998; Webb *et al.*, 1997). The newly replicated origin regions then position near the cell quarter regions for the next round of replication. In vegetative cells of *B. subtilis*, the nucleoid appears as a discrete mass

centered close to mid cell, with prominent gaps at each cell pole. Soon after the onset of sporulation, the nucleoid undergoes a conformational change, in which it approximately doubles in length so that it reaches from pole to pole in this state, it is termed as axial filament (Errington, 2001). In spores, the nucleoid is packed into donut-like ring of approximately 1 micrometer in diameter (Pogliano *et al.*, 1995). During germination, the ring-shaped nucleoid disappears and the nucleoid becomes more dense while later in spore outgrowth the shape of the nucleoid is reverted to the diffuse lobular shape seen in growing cells (Ragkousi *et al.*, 2000).

Changes in the nucleoid structures have been observed during cell growth phase and in various environmental stress conditions, depicting the altered transcription. Upon cold shock, the nucleoid appears more condensed (Weber *et al.*, 2001). Elevated hydrostatic pressure perturbs cell division and nucleoid structure (Welch *et al.*, 1993) and the addition of transcription inhibitor rifamycin leads to decondensed nucleoid in *B. subtilis* (Guillen and Bohin, 1986). Addition of chloramphenicol in exponentially growing cells showed changes in appearance from irregular spheres and dumbbells to large, brightly stained spheres and ovals, while the late exponential phase cells showed elongated axial filament structures (Bylund *et al.*, 1993).

1.3 Nucleoid-associated proteins

DNA topology plays a critical role during dynamic chromosomal processes and is affected by changes in growth phase, environmental stress situations, and by several DNA-interacting proteins. Proteins that bind and organize DNA structure are vital components of the cell. By interacting with their DNA substrate, they affect gene expression, growth efficiency, and cell viability through change in the state of chromosome condensation and relaxation. Some of the relevent nucleoid-associated proteins are briefly discussed below.

HBsu - The histone-like protein in *B. subtilis*, belongs to a highly conserved HU protein family. HBsu is coded by *hbs* gene, which is regulated by two promoters. Throughout the cell cycle, HBsu is the most abundant protein associated with

nucleoid, is essential for growth and differentiation and has been shown to modulate DNA topology (Klein and Marahiel, 2002; Micka and Marahiel, 1992). HBsu was extracted from isolated nucleoids and characterized by its ability to introduce negative supercoils into DNA in the presence of topoisomerase I (Le Hegarat *et al.*, 1993). HBsu binds to DNA as homodimer in a sequence-independent manner with a preference for curved DNA (Kohler and Marahiel, 1997). HBsu localizes to the nucleoids in growing cell and colocalizes with SASPs (see below) on the ring-shaped nucleoid of the germinating spores (Ross and Setlow, 2000). HBsu has also been demonstrated to play a role as part of the bacterial signal recognition particle involved in presecretory protein translocation (Nakamura *et al.*, 1999) and in DNA repair and recombination (Alonso *et al.*, 1995).

SASPs - Small acid-soluble spore proteins are found in two forms, the alpha- and beta-type, encoded by six *sspA-F* genes in *B. subtilis* that are expressed during sporulation and are implicated in packaging of DNA in spores. The SASPs bind with greater affinity to GC rich DNA regions and increase DNA persistence length tremendously by changing the DNA conformation B to A (Mohr *et al.*, 1991). SASPs protect the spore chromosome against damages induced by heat, oxidizing agents, desiccation, and UV irradiation (Mason and Setlow, 1987; Setlow and Setlow, 1995). SASPs also act as an amino acid reservoir for protein synthesis during spore germination (Setlow, 1988) and colocalize with the nucleoid until early germination (Ross and Setlow, 2000). During the germination process, the donut shaped nucleoid transforms into a more compact mass due to the degradation of most of the spore's pool of major alpha/beta-type SASPs (Ragkousi *et al.*, 2000).

1.3.1 Partition proteins

Plasmid segregation system in E. coli. Plasmids are autonomously replicating genetic entities that are ubiquitous in bacteria. Plasmids control their own replication (Hiraga, 1992), utilizing the standard cellular replication machinery and are actively segregated between daughter cells (Gordon and Wright, 2000; Hiraga, 2000). The presence of a partition cassette (*par*) allows for the inheritance of the plasmid copy in each daughter cell. This partition cassette encodes two structural genes and a *cis*-acting *parS* site (a centromere analog). Par^+ plasmids form clusters and localize to the

cell poles, while *Par*$^{-}$ plasmids localize randomly in a cell (Weitao *et al.*, 2000). Plasmid partitioning either uses ParA, an ATPase with a Walker-type ATP-binding motif or ParM, an actin-type-ATPase (Bignell and Thomas, 2001; van den Ent *et al.*, 2002). In both cases, ParB, a second partition protein, binds to the *cis*-acting DNA partitioning *parS* site and recruits the ATPase into the nucleoprotein partition complex (Bouet and Funnell, 1999). In the ParA system, plasmid pairs translocate to the mid-cell position shortly before septation and are then propelled bidirectionally by the partition apparatus into the daughter cell halves (Li and Austin, 2002). For plasmid partition involving an actin-type ParM protein, extensive polymerization of this protein is likely to direct plasmid movement during segregation (van den Ent *et al.*, 2002).

Spo0J and Soj constitute the Par protein system in *B. subtilis* and are homologs of ParB and ParA proteins involved in plasmid and chromosome segregation in *E. coli*. Spo0J and Soj were originally identified as proteins required for an early stage of the sporulation pathway. The ParB homolog Spo0J controls the expression of early acting sporulation genes which require expression of the Spo0A transcription factor. Soj is related to the ParA ATPase family and is a transcriptional regulator that functions antagonistically to Spo0J. The Spo0J and Soj have been demonstrated to function as partition proteins in *E. coli* and were required for the specific localization of plasmids at cell quarters when heterologously expressed in *E. coli* (Yamaichi and Niki, 2000). The Soj-Spo0J system operates a checkpoint that couples chromosome partitioning to developmental gene expression. When chromosome partitioning is incomplete, Soj represses the activity of Spo0A. The completion of partitioning results in Spo0J inactivating the Soj repression. Spo0J binds to specifically conserved 16-bp *parS* sequences clustered around the *soj-spo0J* operon. These sequences occur approximately ten times within the *Ori* region of *B. subtilis* genome (Lin and Grossman, 1998). Immunofluorescence and GFP tagging of Spo0J show that they localize as foci near the origin region (Lewis and Errington, 1997). Deletion of Spo0J affects nucleoid organization and segregation and leads to a 100-fold increase in anucleate cells suggesting its active role in chromosome partitioning (Ireton *et al.*, 1994); (Draper and Gober, 2002). Spo0J was also thought to be involved in the spatial organization of *Ori* regions of the chromosome, since its mutations led to defects in the orientation of the prespore chromosome. Soj oscillates

from one pole to the other within the period of 20 seconds in a Spo0J-dependent manner and in its absence, Soj localizes to the nucleoid (Marston and Errington, 1999a). Deletion of *soj* (*parA* homolog) does not result in a DNA segregation defect, but it is required for the stability of *parS*-containing plasmids. In contrast to *B. subtilis*, inactivation of either *parA* or *parB* in *C. crescentus* is lethal to the cell (Marczynski and Shapiro, 2002).

1.3.2 Proteins involved in chromosome dynamics

Topoisomerases participate in maintaining chromosome function by adjusting DNA topology appropriately to meet the requirements of changing conditions such as temperature, growth phase, nutrient availability, etc. and facilitate fundamental cellular processes such as chromosome segregation, transcription, and DNA replication (Brill *et al.*, 1987). Topoisomerases possess the unique ability to create a transient break in a DNA molecule that allows the passage of one strand through another and then religate the cut molecule (Hsieh and Brutlag, 1980). Type I topoisomerases cleave one strand of the DNA duplex in an ATP-independent manner, while type II topoisomerases cleave both strands and utilize ATP. Topoisomerase II activity facilitates DNA replication and transcription by removing superhelical twists that result from the progression of the DNA and RNA polymerases along the chromosome (Koshland and Strunnikov, 1996). While the DNA molecules of mesophilic bacteria are negatively supercoiled, which facilitates the DNA processes of replication, transcription and recombination (Declais *et al.*, 2001), those of hyperthermophilic archaea possess positively supercoiled DNA that are maintained by the activity of a unique enzyme termed reverse gyrase that protects their chromosomes from denaturation (Lopez-Garcia and Forterre, 1999).

Bacillus subtilis harbours four topoisomerases: *topA*, coding for topoisomerase I, unwinds DNA by removing negative supercoils and has been shown to play a role in illegitimate plasmid recombination that allows recombination between non-homologous sequences and recognizes a consensus sequence 5'-A/(T)CAT(A)/(T)TA(A)/(A)(T)/(T)A-3' (Meima *et al.*, 1998). *TopB* codes for topoisomerase III, which has been characterized in *E. coli*, where it acts as a cellular decatenase during the process of chromosome segregation. *topB* is a multicopy suppressor of a *topA* null mutation (Broccoli *et al.*, 2000). Both *topA* and *topB* are

type I topoisomerases and required for proper chromosomal segregation in *E. coli* (Zhu *et al.*, 2001). Gyrase and topoisomerase IV finally constitute the type II topoisomerases. Gyrase is formed by two subunits coded by *gyrA* and the ATP-binding subunit *gyrB*. Similarly, *parE* and *parC* code for topoisomerase IV. Gyrases are involved in the initial stages of replication easing the positive supercoils and the topoisomerase IV acts at the final stage of replication as a decatenase (Huang *et al.*, 1998) and are essential for cell cycle progression and developmental regulation in *Caulobacter crescentus* (Ward and Newton, 1997).

SpoIIIE is a DNA tracking protein with ATPase activity and a member of a large family of bacterial proteins involved in DNA translocation (Dworkin, 2003; Errington *et al.*, 2001). It is required for complete segregation of chromosomal DNA into the pre-spore during asymmetric division in sporulating *B. subtilis*. Chromosome partitioning during sporulation differs from vegetative chromosome partitioning in that it occurs after formation of the septum. SpoIIIE localizes to the prespore septum where it is proposed to pump the remaining chromosome from the mother cell compartment into the prespore (Wu and Errington, 1997). Mutations in the *spoIIIE* gene prevent proper partitioning of one chromosome into the developing prespore during sporulation but has no effect on partitioning in vegetatively dividing cells (Pedersen and Setlow, 2000). The gene encoding SpoIIIE is expressed constitutively and plays a role in chromosome segregation during vegetative growth by translocating trapped DNA from enclosing septum during cell division (Pedersen and Setlow, 2000).

PrfA, the penicillin-binding protein-related factor A, also designated as RecU, is located downstream in an operon with *ponA*, a penicillin-binding protein (PBP1) involved in peptidoglycan crosslinking. PrfA/RecU is implicated in several cellular processes such as cell wall synthesis, chromosome segregation, and DNA recombination and repair (Pedersen and Setlow, 2000). A *prfA* deletion rendered cells more sensitive to DNA-damaging agents, decreased the transformation efficiency (Fernandez *et al.*, 1998), and led to 0.9-3% anucleate cells and cells with abnormal nucleoid staining patterns. Inactivation of *prfA* also exacerbated *smc* and *spo0J* chromosome segregation phenotype and its overexpression in *E. coli* caused nucleoid condensation (Pedersen and Setlow, 2000). PrfA has been shown to possess

endonuclease activity and is structurally related to the restriction enzyme *Pvu*II (Rigden *et al.*, 2002).

1.4 SMC - Structural/stable maintenance of chromosomes protein

SMC proteins are ubiquitous and are an essential part of a high order complex which is involved in chromosome dynamics. In 1985, as pioneers of SMC research Larinov and Strinnikov, observed an increase in the copy number of artificial mini chromosomes in a *Saccharomyces cerevisiae* mutant with an impaired segregation process. These mutants were mapped in four genes, AMC1, AMC2, AMC3, and AMC4 (AMC = Arificial Mini Chromosome) which control the segregation of natural chromosomes in yeast. AMCs were later rediscovered as SMC for stability of mini chromosomes (Larionov and Strunnikov, 1987; Strunnikov *et al.*, 1993). While eukaryotes code for 6 different types of SMC protein represented by SMC1-6, to date, prokaryotes contain only a single allele for an SMC homolog (Hirano, 2002).

1.4.1 Structure of SMC

The proteins belonging to the SMC family are large proteins in the range between 110 and 170 kDa (Harvey *et al.*, 2002) and share common principles in domain organization: A globular N-terminus contains a conserved sequence resembling a Walker A ATP-binding motif (G-NGSGKSN) and a C-terminal domain harbors both the highly conserved LSGG motif signature, called C motif, and P-P-DE-DAALD which corresponds to a Walker B motif (Walker *et al.*, 1982). The N- and C-terminal domains are connected via two long coiled coil domains (of variable length) separated by a globular hinge domain of approx. 150 amino acids in length:

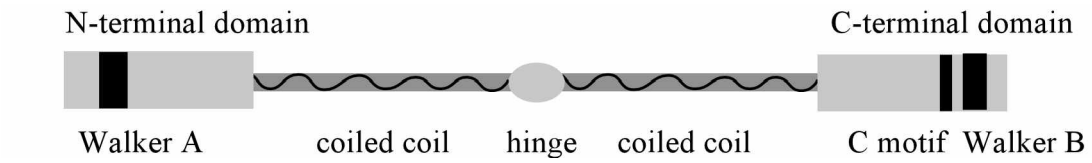


Fig. 4: Schematic diagram of domain organization in a typical SMC protein.

Based on the primary sequence it was predicted that SMC proteins might form antiparallel dimers, and that dimerization is probably mediated by inter- or intramolecular interactions (Saitoh *et al.*, 1994). Electron microscopic analysis of *B. subtilis* SMC by (Melby et al., 1998), showed various conformations of SMC and the most prominent 'V' shaped conformation, see fig. 5. In their model, they suggested that SMC proteins form antiparallel dimers connected through the coiled coil segments with the N- and C-termini of each monomer forming a head domain located at the ends of a 'V'-like structure (Melby *et al.*, 1998):

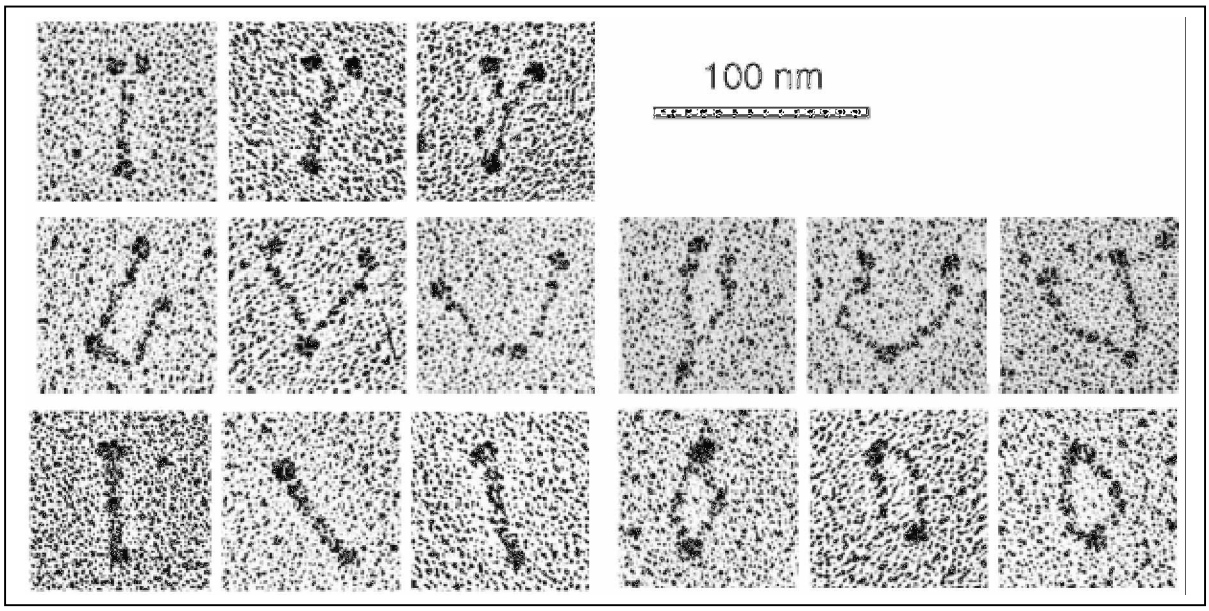


Fig. 5: Electron micrographs from *B. subtilis* SMC representing the most common conformations. Images were adapted from (Melby *et al.*, 1998).

So far, no crystallization of the whole SMC molecule has been reported, which might be due to its large and flexible nature, but several workgroups have come up with the crystal structures of different domains. Analysis of the crystal structure of N-terminal domain of MukB which is a member of SMC family and a functional analog of SMC protein in *E. coli*, showed that the N- and C-terminal domains of SMC molecules have to come together to create an ATPase activity pocket (van den Ent *et al.*, 1999). Rad50 is a member of eukaryotic SMC-like proteins and differs from other true SMC in having shorter coiled coil arms and a conserved CxxC motif within the hinge domain. The crystal structure of the Rad50 catalytic domain showed two ATP molecules being sandwiched between the P loop of Walker A and the signature C motif (Hopfner *et al.*, 2000). Furthermore, the crystal structure of the head domain

comprising the N- and the C-terminal domain of SMC from *Thermotoga maritima*, showed close similarity to the ABC ATPases (Lowe *et al.*, 2001).

Based on electron microscopic analysis of SMC and MukB, it was earlier proposed that SMC forms antiparallel dimers mediated by the coiled coil interaction between two different subunits, i.e. the catalytic ATP cassette or the head domain is formed by the intermolecular interaction between the N- and the C-terminal domains of the dimer (Melby *et al.*, 1998). But the recent evidence from the crystal structure of the SMC hinge domain and the hinge domain with a part of the coiled coils from *Thermotoga maritima* showed that the hinge forms a donut-like dimer (Haering *et al.*, 2002), proving that the dimer formation mediated by the hinge and the head domain is formed by the intramolecular interaction of N- and C-terminal domains of the same SMC molecule. This view was supported by biochemical studies with various point mutations at the hinge region and site-directed protein-protein cross linking experiments (Hirano *et al.*, 2001; Hirano and Hirano, 2002).

1.4.2 SMC in Eukaryotes

Eukaryotic SMC proteins have been well investigated in the model organisms *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, Drosophila, and *Xenopus*. So far, six different SMC family members have been identified which take part in various chromosomal events (Hagstrom and Meyer, 2003). SMC1 and SMC3 form a part of cohesin, that acts like glue between sister chromatids, which is laid down during DNA replication at S phase until the sister chromatids are subsequently segregated away from each other in metaphase stage. The cohesin complex comprises the SMC1 and SMC3 heterodimer and two non-SMC proteins, termed as Scc1 and Scc3, in S. *cerevisiae* that helps the SMC's fix to the sister chromatids (Koshland and Guacci, 2000). Once chromosomes are properly aligned in the mitotic spindle, cohesin is cleaved by proteolysis of Scc1 to allow sister chromatids to segregate into the two daughter cells. Condensin is required for the substantial reorganization of chromosome structure as chromosomes compact during mitosis and is also crucial for resolving connections between sister chromatids. The SMC2 and SMC4 form a part of the 13S condensin complex, together with three non-SMC subunits, namely Ycs4, Ycs5, and Brn1 in *S. cerevisiae* (Hirano *et al.*, 1997). The condensing complex

introduces positive writhe into the DNA by bending the DNA into the coils that remodel the chromosome into a more compact structure (Kimura and Hirano, 1997).

Apart from these most canonical roles of SMC in cohesin and condensin of chromosomes, they also play a role in gene regulation in part by influencing enhancers, silencers and insulators (Cobbe and Heck, 2000). In *C. elegans* dosage-compensation factors resemble condensin subunits. This condensin-like complex assembles on hermaphrodite X chromosomes to downregulate X-linked gene expression. The switch in the mating type of *S. cerevisiae* from *a*- to α-mating type by intrachromosomal gene conversion is brought about by cohesin. Other gene regulatory functions carried out by SMC's are nerve-cell formation and wing patterning in *Drosophila* (Cobbe and Heck, 2000).

Yet another role of SMC proteins is in DNA repair. The first indication that cohesin subunits are involved in DNA repair was the discovery of mammalian SMC1 and SMC3 as a part of biochemically purified recombinational repair complex (Cobbe and Heck, 2000). Two new additional SMC proteins, SMC5 and SMC6 were identified as being involved in repair (Fujioka *et al.*, 2002). SMC6 was identified as a gene product of Rad18 in *S. pombe* whose mutants were hypersensitive to UV and γ-radiation (Taylor *et al.*, 2001). SMC5 and SMC6 are essential to maintain checkpoint arrest after DNA damage. In *Arabidopsis*, SMC6 mutants were defective in intrachromosomal homologous recombination in somatic cells (Hirano, 2002; Mengiste *et al.*, 1999). Yet another member of specialized subfamily of SMC proteins, Rad50 in complex with Mre11 and Nbs1, take part in double stranded break repair pathways, homologous recombination and non-homologous end joining (Smith, 2002). Thus SMC proteins in eukaryotes play a central role in almost all chromosome related processes.

1.4.3 SMC in prokaryotes

In prokaryotes, *E. coli* was the first identified to possess MukB (a member of SMC protein sub family). MukB was originally isolated in a genetic screen to detect mutants with chromosome segregation defects (Hiraga *et al.*, 1991; Niki *et al.*, 1991). Two genes, the non-SMC like subunits, MukE and MukF located immediately upstream of MukB, were also shown to be involved in chromosome partitioning.

Later it was demonstrated that MukE and MukF interact and form a complex with MukB (Yamanaka *et al.*, 1996; Yamazoe *et al.*, 1999). Homologs of MukB, MukE, and MukF were found in the other *E. coli*-related gamma subdivision of proteobacteria (*Klebsiella, Salmonella, Yersinia, Vibrio, Actinobacillus, Haemophilus, and Pasteurella*). Mutations in MukB, MukE, and MukF resulted in (i) slow growth, (ii) 5% anucleate cell production at the permissive temperature 22°C apparently caused by chromosome segregation defects, and (iii) restricted growth at 42°C in rich media. The nucleoids showed aberrant chromosomal condensation (Niki *et al.*, 1991) and the mutants were hypersensitive to novobiocin (Weitao *et al.*, 1999).

Several suppressors of MukB mutants have been isolated that rescue the temperature sensitive and chromosome segregation defect. Some of these are SmbA (Yamanaka *et al.*, 1992), CspC and CspE (Yamanaka *et al.*, 1994), the N-terminus of RNase E (Kido *et al.*, 1996), and *topA* (Sawitzke and Austin, 2000). Temperature sensitivity, anucleate cell production and poor nucleoid folding phenotype from the *mukB* strain were suppressed by a *seqA* null mutation, whereas filamentation, asymmetric septation and compact folding of the nucleoids observed in the *seqA* strain were suppressed by inactivation of the *mukB* gene function (Weitao *et al.*, 1999). Mutants suppressing the hypersensitivity of *mukB* mutant to novobiocin has been recently mapped near *gyrB* (Adachi and Hiraga, 2003).

In *B. subtilis*, the *smc* gene was detected while characterizing the *rnc* operon. It shared 26.6% amino acid identity with SMC1 (Oguro *et al.*, 1995). Null mutations in *B. subtilis* SMC resulted in inability to form colonies in rich medium at elevated temperatures as well as in 10-15% anucleate cell formation and aberrant nucleoids at the permissive temperature (Britton *et al.*, 1998; Graumann *et al.*, 1998; Moriya *et al.*, 1998). A similar phenotype was observed in a *Caulobacter* SMC mutant, but the formation of anucleate cells was not significant (Jensen and Shapiro, 1999).

B. subtilis SMC is required for proper placement of the origins which mislocalize in its absence but are still able to separate (Britton *et al.*, 1998; Graumann, 2000). Depletion of SMC in a *spoIIIE* mutant resulted in cessation of growth and cells with bisected nucleoid by invaginating septa (Britton and Grossman, 1999). *smc* mutants were also synthetically lethal if combined with mutations in *spo0J* (Ireton *et al.*, 1994) and *recU* (Pedersen and Setlow, 2000), both protein products being involved in chromosome partitioning. *B. subtilis* SMC affected DNA supercoiling *in*

vivo and a *smc* null mutant proved to be hypersensitive to gyrase inhibitors in a manner similar to a *mukB* mutant in *E. coli*, whereas the depletion of topoisomerase I suppressed the partitioning effect of the *smc* null mutation (Lindow *et al.*, 2002a).

B. subtilis SMC were shown to localize as bipolar foci on the nucleoids and it was demonstrated that its C-terminal region was required for viability but dispensable for polar localization (Britton *et al.*, 1998; Graumann *et al.*, 1998). Similar bipolar localization was also shown for MukB of *E. coli* (den Blaauwen *et al.*, 2001).

Based on similarity in sequences and function, SMC protein family has been grouped into eight subfamilies, as shown in the tree form in fig. 6. The first six sub families comprise SMC1-SMC6 of the eukaryotic SMC proteins. Sub family 7 and 8 are constituted by the bacterial and archaeal SMC proteins (Cobbe and Heck, 2000, 2003). The phylogenetic analysis of SMC related proteins have shown that the closest relatives to the SMC proteins are the archaeal Rad50 proteins, followed by eukaryotic Rad50 and eubacterial SbcC proteins (Cobbe and Heck, 2000, 2003).

The existence of six SMC variants in eukaryotes has been attributed to a symmetric duplication of genes encoding the larger and smaller eukaryotic SMC proteins. The relatively close proximity of the SMC1/SMC4 or the SMC2/SMC3 lineages to the prokaryotic SMC root also suggests that the first duplication event, giving rise to the primordial eukaryotic SMC heterodimer, occurred very early in the evolution (Cobbe and Heck, 2003).

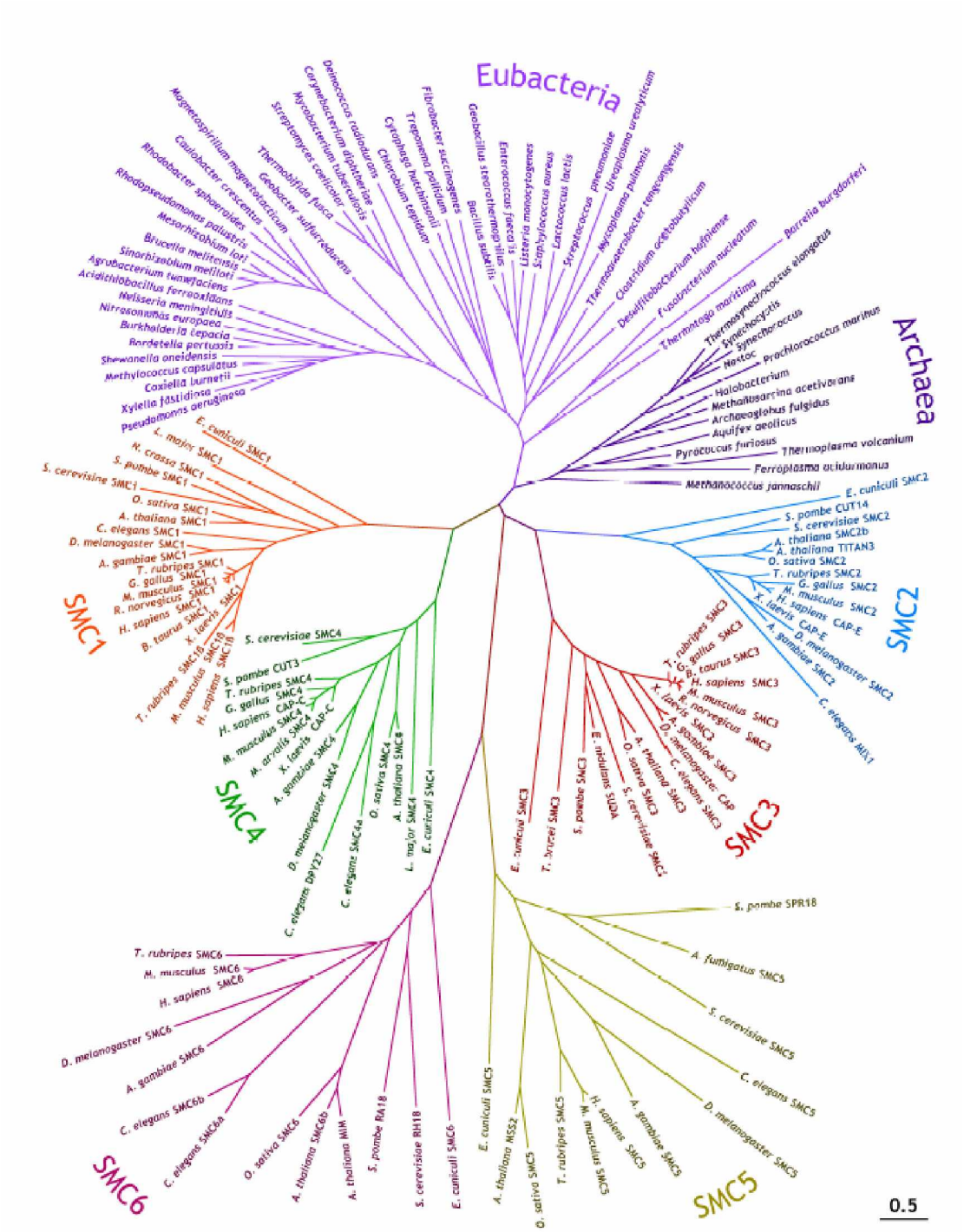


Fig. 6: Phylogenetic tree showing the divergence of known SMC proteins. Constructed by Cobbe and Heck (Cobbe and Heck, 2003).

1.5 Basis and aim of this work

At the beginning of this work, the knowledge of prokaryotic SMC, and especially SMC from *Bacillus subtilis* was limited to genetic and physiological studies of its deletion mutant, which revealed its importance in chromosome condensation and segregation. This work aimed at further characterization of the SMC protein and identification of proteins interacting with it using a combination of genetic and fluorescence microscopic approaches.

2 Materials and Methods

2.1 Materials

2.1.1 Equipment used in this study

Table 1

Equipment	Manufacturer
Automated DNA sequence analyzer	ABI PRISM 301 Genetic analyzer, Perkin Elmer
Western blotting chamber	Semi dry blotting chamber Trans-Blot SD, Sigma-Aldrich
Centrifuge	Heraeus Microfuge pico, Eppendorf 5415 D
Digital pH meter	CG8400 Schott
Documentation of agarose gel	Video camera Cybertech CS1
DNA thermocycler	Eppendorf Mastercycler personal
Digital camera for microscope	MircoMax CCD
Fluorescence microscope	AX70, Olympus
Electroporation system	Biorad Gene pulser II
Gel electrophoresis apparatus	Philipps-Universität Marburg workshop
Sonicator	Bandelin sonopuls HD2070
Photometer	Pharmacia Ultraspec 3000 UV/Visible spectrophotometer
Water bath shaker	C76, New Brunswick scientific
Speed-Vac	Uniequip Univapo 150H

2.1.2 Materials and reagents:

Most of the chemicals were of analytical grade and were purchased from Fluka (Deisenhofen), Gibco BRL (Karlsruhe), Merck (Darmstadt), Roth (Karlsruhe), Serva (Heidelberg), and Sigma (München).

Table 2

Materials	manufacturer
Sterile filters - 0.45 µm and 0.2 µm	Roth (Karlsruhe)
Electroporation cuvettes	Eurogenetec (Belgium)
Spectrophotometer cuvettes	Roth (Karlsruhe)
Quartz cuvettes	Hellma (Müllheim)
For western blotting:	
Whatman 3MM filter paper	Schleicher and Schuell (Dassel)
Nitrocellulose membrane type BA85	Schleicher and Schuell (Dassel)
Conjugated secondary antibody	Amersham Biosciences (Freiburg)
X-ray film Biomax MR	Kodak (Rochester, USA)
Strep-tactin Sepharose column	IBA (Göttingen)
Enzymes for molecular biology:	
Restriction endonuclease, DNA modifying enzymes, DNA and protein markers	New England Biolabs (Schwalbach)
Expand Long template PCR system	Boehringer (Mannheim)
Turbo *pfu*	Stratagene (Heidelberg)
Protease inhibitors:	
PMSF (phenylmethylsulfonylfluoride)	Sigma (München)
RNase inhibitors:	
DEPC (diethylpyrocarbonate)	Sigma (München)
ribonuclease-inhibitor (RNAsin)	Promega (Mannheim)
Vital stains for microscopy:	
DAPI, FM646, Syto59	Molecular Probes TM (Netherlands)
Radionuceotides	
α-^{32}P-dATP, α-^{35}S-ATP	Amersham Pharmacia Biotech (Freiburg)

Computer softwares:	Chromas 1.45, DNAstar 5.0, Clone manager, Metamorph 4.6 (Universal Imaging)

Only deionized and/or distilled water was used for the preparation of buffer solutions and growth media, and was sterilized prior to use in all the enzymatic reactions.

2.1.3 Kits

Table 3

Kit designation (manufacturer)	**Usage description**
ABI Prism dRhodamine terminator cycle sequencing ready reaction kit (ABI, Foster City, USA)	Reaction ingredients for automated DNA sequencing
DyeExSpin kit (Qiagen)	Purification of reactions for automated sequencing
Nucleospin Extract (Macheary Nagel AG)	Purified plasmid extraction
QIAquick gel extraction kit (Qiagen)	Purification of DNA fragments from agarose gels
QIAquick PCR purification kit (Qiagen)	Purification of DNA fragments from PCR reactions
RNeasy mini kit (Qiagen)	Isolation of total RNA from cells.
Sequenase Version 2.0 DNA Sequencing Kit (USB)	Manual sequencing reactions
Strep-tag (IBA)	Detection and purification of proteins tagged with strep tags

2.1.4 Antibodies

Table 4

Primary antibodies	**Proteins purified, antibody source**
Rabbit anti SMC	A. Strunnikov (NIH, USA)
Rabbit anti ScpB	A. Volkov, Eurogenetec
Rabbit anti GFP	D. Rudner, Eurogenetec
Mouse anti RGSHis	Qiagen
Strep	IBA
Secondary antibodies	
Goat anti Rabbit IgG, peroxidase conjugated	Amersham Life Sciences
Goat anti Mouse IgG, peroxidase conjugated	Amersham Life Sciences

2.1.5 Oligonucleotides

Synthetic oligonucleotides for PCR were supplied by MWG-Biotech AG and Qiagen-Operon. The annealing temperature was calculated using an empirical formula provided by MWG-Biotech AG:

$$Tm = 69.3 + 0.41 \cdot \left(\frac{100 \cdot \sum G + C}{L} \right) - \frac{650}{L}$$

With *Tm* = annealing temperature of the primer, *L* = length of the primer, and Σ*G*+*C* = sum of G and C residues within the primer sequence. For a more convenient use, a table was constructed using the 'Excel spread sheet' (see table 16 in the appendix, p. 118).

2.1.6 Bioinformatic tools and computer programs

All sequence comparisons, restriction analysis and *in silico* cloning procedures were performed using Clone Manager version 5.0 from Scientific and educational software, DNA sequencing data analysis was carried out using chromas 1.45 software. Most other bioinformatic analyses were undertaken using public internet resources:

Table 5

Task:	**Reference:**
collection of bioinformatic tools	http://us.expasy.org/tools/ http://www.ncbi.nlm.nih.gov/
BLASTP protein similarity searches	http://www.ncbi.nlm.nih.gov/blast/
multiple protein sequence alignments using ClustalW	http://www.ebi.ac.uk/clustalw/
retrieval of *E. coli* genome data	http://genolist.pasteur.fr/Colibri/
retrieval of *B. subtilis* genome data	http://genolist.pasteur.fr/SubtiList/ http://locus.jouy.inra.fr/cgibin/genmic/madbase/progs/madbase.operl

2.1.7 Bacterial host strains

Table 6

Escherichia coli	**Genotype**	**reference**
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* lac[F' proAB lacI^{q}ZΔM15 Tn10 (Tet^{r})]	Stratagene
Top10F’	F- *mcr*A (*mrr-hsd*RMS-*mcr*BC) 80*lac*Z M15 *lac*X74 *rec*A1 *ara*D139 *gal*U *gal*K (*ara-leu*)7697 *rps*L (StrR) *end*A1 *nup*G	Invitrogen
GM48	*ara dam dcm galK galT leu supE44 thi-1 ton tsx*	(Yanisch-Perron *et al.*, 1985)
Bacillus subtilis		
PY79	prototrophic, *Bacillus subtilis* subsp. *subtilis*	P.Youngman (Webb *et al.*, 1997)

2.1.8 Plasmids used in this study

pBluescript®SKII(+) is a derivative of pUC19 [Yanish-Perron, 1985 #210] from Stratagene. It possesses a filamentous phage origin of replication. The plasmid harbours the β-lactamase gene conferring ampicillin resistance to the bacteria and thus helps in the selection of transformants. The multiple cloning cassette is inserted in frame at the 5' of β-galactosidase which is under the control of IPTG-inducible lac promoter. β-galactosidase hydrolyses Xgal (an analogous substrate of β-galactosidase) present in the bacterial growth medium resulting in blue coloured colonies. Insertion of a DNA fragment in the cloning cassette disrupts the β-galactosidase gene and consequently the expression of the protein. In addition the plasmid vector has promoter sequences of RNA polymerases of T3 and T7 phages flanking the multiple cloning cassette. In this study this plasmid was used to subclone various resistance genes selectable in *Bacillus subtilis*.

pDG vector series were obtained from Bacillus genetic stock center (BGSC), Ohio StateUniversity, originally constructed at Patric Stragier's lab (Guerout-Fleury *et al.*, 1995). These plasmids contain a *bla* (β-lactamase gene) for amipicillin resistance and one of the resistance genes (tetracyclin, kanamycin, erythromycin, or spectinomycin) selectable in single copy number in *Bacillus subtilis*. These plasmids are useful in constructing insertion mutants for *Bacillus subtilis* genes.

Table 7

pDG vectors used :	Resistance cassette:
pDG646	erythromycin
pDG780	kanamycin
pDG1515	tetracyclin
pDG1726	spectinomycin

pSG vectors were obtained from Peter Lewis at the University of Newcastle, Australia (Feucht and Lewis, 2001; Lewis and Marston, 1999). These plasmids are designed for constructing fluorescent protein fusions at C-terminus of protein and can integrate via single cross over into the *B. subtilis* genome at the homologous gene locus. pSG1192 is another fusion vector to the N-terminal of cfp and contains *amyE* front and back fragments flanking a spectinomycin resistance (*spec*^{r}) gene that allows for the stable integration of the cloned gene at the *amyE* locus. The gene fusion from *amyE* locus is transcribed from a P*xyl* promoter.

Table 8

pSG vectors used:	genotype
pSG1151	*bla, cat, -'gfpmut1*
pSG 1164	*bla, cat,* P*xyl -'gfp*
pSG1170	*bla, cat,* P*spac -'gfpuv*
pSG1186	*bla, cat, -'cfp*
pSG1187	*bla, cat, -'yfp*
pSG1192	*bla, amyE3' spec* P*xyl -'cfp amyE5'*

pMUTIN-YFP/CFP vectors were originally constructed at the laboratory of Wolfgang Schumann at the University of Bayreuth, Germany (Kaltwasser *et al.*, 2002), were obtained from BGSC. pMUTIN-YFP/CFP are integration vectors for tagging C-terminus of gene products with yellow/cyan fluorescent protein (FP). Upon transformation into *B. subtilis*, the plasmid can integrate into the chromosome by a single recombination event. The integrants are erythromycin resistant in *B. subtilis*, the transcription of the downstream genes in the operon of the integrants is controlled by IPTG inducible P*spac* promoter.

pMUTIN-YFP	(*bla, erm,* P*spac-'yfp*)
pMUTIN-CFP	(*bla, erm,* P*spac-'cfp*).

pJQ43 and pDr111 are integration vector for controlling gene expression *B. subtilis* obtained from D. Rudner (Harvard University, Cambridge, Massachusetts). pJQ43 has an IPTG-inducible *hyperspac* promoter that allows for the conditional expression of the gene cloned downstream to it, the vector carries resistance cassette for chloramphenicol and can integrate into the chromosome by homologous recombination event. pDr111 is a cloning vector for integration into the ectopic, *amyE* site that carries an advanced version of IPTG-inducible *hyperspank* promoter and has spectinomycin resistance. The gene cloned downstream of the promoter can be tightly regulated or overexpressed using IPTG.

pCm::tet is a plasmid (Steinmetz and Richter, 1994) obtained from BGSC. This plasmid was used to exchange the chloramphenicol (*cat*) resistance gene with tetracyclin (*tet*) resistance by double crossover event. Upon transformation into *B. subtilis* strains carrying a *cat* gene, the transformants become cm^s and tet^r.

List of plasmids and strains constructed and used in this work are listed in tables 13 and 14 in appendix (5.2 and 5.2.1)

2.2 Molecular biology methods

2.2.1 Growth medium

LB / LB agar medium, (Sambrook et al., 1989):

Bactotryptone	10g
Yeast extract	10g
NaCl	5g
dH_2O to 1L	

Ingredients were dissolved in water, the resulting solution adjusted to pH 7.4 using 1M NaOH and sterilized by autoclaving at 121°C, 1.5bar for 30min. LB agar, 1.5% agar was added to LB medium before autoclaving. After autoclaving, the medium was cooled down to approx. 50°C and the antibiotics (table 9) were added, swirled to mix and poured into petridishes.

2.2.2 Antibiotic Solutions

Table 9

Antibiotic	**stock solution**	**working solution**
Ampicillin	50 mg/ml in H_2O	50µg/ml (*E. coli*)
Kanamycin	10 mg/ml in H_2O	50µg/ml (*E. coli*)
Kanamycin	10mg/ml in H_2O	10µg/ml (*B. subtilis*)
Chloramphenicol	25 mg/ml in EtOH	7.5µg/ml (*B. subtilis*)
Spectinomycin	25mg/ml in 50%EtOH	25µg/ml (*B. subtilis*)
Tetracycline	20mg/ml in 50%EtOH	20µg/ml (*B. subtilis*)
Erythromycin [1]	4 mg/ml in EtOH	1 µg/ml (*B. subtilis*)
Lincomycin [1]	25 mg/ml in 50% EtOH	25 µg/ml (*B. subtilis*)

[1]MLS - collective term addressing the macrolide lincosamine streptogramidine B antibiotic family which is applied as a combination of lincomycin and erythromycin

All stock solutions listed above were sterile filtered and the antibiotic stocks were stored at -20°C.

2.2.3 Techniques related to DNA

2.2.4 Agarose gel electrophoresis of DNA

DNA molecules can be separated according to their sizes by electrophoretic migration. Depending on the sizes of the DNA fragments to be resolved, for preparation of agarose gels 0.8-2 % (w/v) agarose was suspended in TB buffer, 0.5 µg/ml of ethidium bromide were added and the mixture was heated until the agarose had completed dissolved. After cooling to approx. 50°C, the gel was poured and allowed to solidify. The gel was submerged in a chamber with TB buffer. DNA samples suspended in DNA loading buffer and electrophoresis was carried out at 50-75 mA. After the run, DNA was visualized by ultraviolet (UV) irradiation.

DNA loading buffer:	50% (v/v) glycerol
	0.1 M EDTA
	0.1% (w/v) SDS
	0.05% (w/v) bromo-phenol blue
	0.05% (w/v) xylene cyanol FF
TB buffer:	90 mM Tris-HCl pH 8.0
	90 mM boric acid

2.2.5 Digestion of DNA by restriction enzymes

The digestion of plasmids/DNA by restriction enzymes was carried out according to the manufacturer's instructions (NEB). Typically, a restriction digest reaction contained 1-2 µg of DNA, 1/10 volume of an appropriate 10x restriction buffer, 1-2 units of the restriction enzyme. Preparative digestions were carried out in 50 µl volumes and qualitative digestions in 10 µl final volumes. The reactions were carried out by incubation for 2 h at 37°C and analyzed by agarose gel electrophoresis. For preparative digestions, the DNA fragment of interest was excised from the gel and purified through QIAquick gel extraction kit following the manufactures protocol.

2.2.6 Ligation of vector and insert DNA

Vector and insert DNA were digested with appropriate restriction enzymes to generate compatible ends for cloning. A typical ligation reaction was carried out in a total volume of 10 µl containing vector and insert DNA (molar ratio vector: insert was approx. 1:5), 1/10 volume of 10x T4 ligase buffer and 3 U of T4 Ligase. The ligation reaction was carried out at room temperature or at 16°C overnight.

2.2.7 E. coli transformation

Electrocompetent bacteria were prepared by repeatedly washing bacteria harvested in the exponential growth phase (OD_{600}=0.6-0.75) with sterile ice cold water to remove salt and were then stored at -80°C in 10% glycerol. 1 µl of a typical ligation reaction (or the whole ligation mix after dialysing against water for 15 minutes on 0.025 µm membrane) was mixed with 40 µl of electrocompetent bacterial cells and transfered into a 0.2 cm electoporation cuvette and placed into the Biorad Gene Pulser electroporator. Settings were 25 µFD capacitance, 12.5 kV/cm field strength, 200 Ω resistance. The electric pulse creates transitory pores in the bacterial cell wall which allows the entry of the DNA. The transformed bacteria were diluted in 1 ml of pre-warmed LB medium and incubated at 37°C for 45 minutes. This incubation permits the bacteria to reconstitute their cell walls and start to express the antibiotic resistance gene present on the plasmid. For selection of transformants, bacteria were plated on LB-Agar plates containing the appropriate antibiotic and incubated overnight at 37°C.

2.2.8 Preparation of plasmid DNA

In order to check transformants for the presence of the expected plasmid, small scale DNA plasmid preparation (mini-prep) was carried out. Individual transformant colonies were grown under vigorous shaking by overnight incubation at 37°C in 3 ml LB medium supplemented with appropriate antibiotics. Cells were harvested by centrifugation and the cell pellet was resuspended in 300 µl of solution I and then lysed by alkali treatment in 300 µl of solution II, which also denatures the chromosomal DNA and proteins. The lysate was neutralized with 300 µl of solution III and plasmid DNA was then precipitated by adding 600 µl of isopropanol. The precipitated pellet was washed with 70% ethanol, dried and resuspended in 40 µl of dH_2O.

solution I : 25 mM Tris/HCl pH 8.0
10 mM EDTA pH 8.0

solution II : 0.2 N NaOH
1% w/v SDS

solution III : 60 ml of 5 M potassium acetate
11.5 ml glacial acetic acid
28.5 ml d.H_2O

For large scale isolation of plasmids (midi-prep) the cultures were grown in 50 ml and treated similarly as above with volumes of solution I, II, and III adjusted to 5 ml each.

2.2.9 Polymerase chain reaction - PCR

PCR allows for the exponential amplification of DNA by utilizing repeated cycles of DNA denaturation, primer annealing and DNA synthesis. The reaction essentially requires a thermostable DNA polymerase, primers, dNTPs, and a DNA template. A typical 50 µl PCR reaction mix contained:

5 µl of 10x DNA polymerase buffer
20 pmol of each primer
200 µM of dNTPs
10-100 ng (approx. 1 µl) of template DNA (1:100 from chromosomal DNA and 1:1000 from plasmid from standard preparations)
1-2 U (1 µl) of DNA polymerase preparation (Turbo pfu or pol mix-Expand Long template PCR system polymerase)

The reaction was carried out in a PCR thermocycler, using the following program listed below. The resulting PCR products were analyzed on an agarose gel. For cloning purposes the PCR product was purified over the column using the QIAquick PCR purification kit before subjecting to endonuclease digestion.

	Temperature	Time (min)	Cycles
Initial denaturation:	95°C	2 :00	
Denaturation:	95°C	0 :30	10
Primer annealing:	T_m - 2°C	0 :30	
Extension :	72°C (for pfu)	1:00 / kb	
	68°C (for pol mix)	1:20 / kb	
Exponential amplification:	95°C	0 :30	25
	T_m+ 5°C	0 :30	
	72°C / 68°C	1:00 / kb	
Final extension :	72°C / 68°C	4:00	
	4°C		

2.2.10 DNA sequencing

In order to verify clones for the presence of any point mutations, appropriate DNA preparations were sequenced utilizing a fluorescent dye technique. Clean plamids were prepared using the Nucleospin plasmid prep kit or QIA plasmid prep kit. The purity and concentrations were analyzed spectroscopically using DNS method mode. For a sequencing PCR reaction, plasmid concentrations of 100 ng/kb were used in a reaction mix of 10 µl which contained 1 µl of 10 pmol primer and 3 µl of termination mix (dNTP's, ddNTP's, buffer, Ampilitaq DNA polymerase FS). A standardized PCR reaction program was used with an initial denaturation at 95°C for 60 sec, 30 cycles of denaturation at 95°C for 10 sec, primer annealing at T_m-2°C for 5 sec, extension at 60°C for 4 minutes, and terminated with 60°C for 5 min to facilitate the completion of extension reaction. After PCR completion, the products were purified either using a column from the Dye Ex kit (Qiagen) or were precipitated with 1µl 3M sodium acetate and 25µl absolute ethanol, the pellet was washed with 70% ethanol dried and resuspended in 40 µl HPLC-grade H_2O. The sample was denatured at 95°C for 2 min before subjecting to analysis by the ABI 310 sequence analyser.

Sequencing reactions for primer extension studies were carried out using the 'Sequenase Version 2.0 DNA Sequencing Kit' from USB.

2.2.11 Primer annealing cloning

This technique was used to introduce short sequences of 15-40bp, e.g. a strep tag or multiple cloning site extensions into plasmid DNA. Two complementary primers were used, which upon annealing generated sticky ends corresponding to the sites where the desired fragment would be introduced into the plasmid. The primers were phosphorylated separately with ATP and the T4 PNK enzyme as follows:

12 µl primer (100 pmol)
2 µl of 100 mM ATP
5 µl of 10x PNK buffer
1.5 µl of T4 PNK
dH_2O to 50 µl

After two hours of incubation at 37°C the PNK enzyme was denatured at 70°C for 15 min. Equal volumes of the reaction mixtures each containing one of the phosphorylated primers were mixed and they were denatured at 95°C for 2 min followed by cooling on ice, which enabled the two primers to anneal. The resulting annealed product was ligated with the previously cut plasmid. The clones were analyzed either by sequencing and or by digestion utilizing a newly introduced cutting site that belonged to the insert.

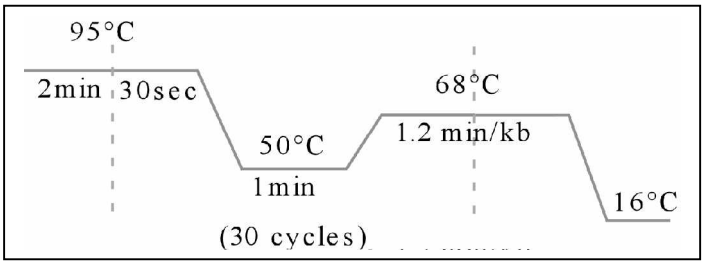
2.2.12 Site-directed mutagenesis

In vitro site-directed mutagenesis is a valuable technique for studying protein structure-function relationships. This procedure utilizes a vector carrying the gene to be modified and two complementary primers carrying the desired point mutation. These two complementary primers were designed such that the mutation region is located at the center:

(15-18 bp)-(mutation region)-(15-18 bp)

The primers each complementary to opposite strands of the vector, are extended in the PCR reaction with a *turbo pfu* polymerase. The PCR reaction was carried out in a reaction volume of 50 µl containing 1 µl of the plasmid (from standard plasmid prep).

Fig 7: PCR program for site directed mutagenesis:



The PCR reaction allows for the incorporation of the primers which results in a mutated plasmid with staggered nicks. In order to remove the parental vector the reaction was digested with *Dpn*I for 2-3 hours. *Dpn*I endonuclease is specific for methylated and hemimethylated DNA which is the case only for the parental vectors. The product was then purified through the column and used for transformation in *E. coli*. The presence of mutations was confirmed by sequencing the plasmids after their isolation from the transformants.

2.3 Techniques related to RNA

2.3.1 RNA extraction

Total RNA from cells was isolated by following the manufacturer's protocol describing the application of the Qiagen RNeasy mini kit. The concentration and purity of RNA was determined by measuring the absorbance at 260 nm (A_{260}) in an UV/VIS spectrophotometer and by visual inspection of the preparation on an RNase-free agarose gel to exclude degradation. An absorbance A_{260} of 1 corresponds to a RNA concentration of 40µg/ml such that the RNA concentration is given by:

$$[RNA] = a \cdot 40 \frac{\mu g}{ml}$$

Where [*RNA*] is the RNA concentration in µg/ml and *a*, the absorbance measured at 260 nm.

2.3.2 Primer extension

The primer extension technique is used to determine the relative abundance and the transcriptional start site of the mRNA of interest from a total cellular RNA preparation. A labelled oligonucleotide that is specifically complementary to the mRNA of interest is hybridized to the RNA and serves as a primer for cDNA synthesis carried out by a reverse transcriptase. This enzyme extends the primers 3' end until the 5' end of the template RNA is reached. The size of cDNA product therefore corresponds to the distance between the primers 5' end and the 5' end of the RNA. The start position of a transcript is then mapped by comparing the cDNA fragment size to a corresponding dideoxy sequencing reaction.

1. Primer labelling

Primer labelling was carried out in a total volume of 20 µl containing 5 µl of 100 pmol primer, 5µl ^{32}P-αATP, 2 µl of 10x PNK buffer, and 1.5 µl PNK. The reaction was carried out by incubation at 37°C for one hour followed by heat inactivation at 65°C for 5 minutes. The reaction product mixture was then passed through a G-25 column, to remove excess of ^{32}P-αATP.

2. Primer extension reaction

Reverse transcriptase was used to synthesize a DNA strand complementary to the RNA template (cDNA). In a 20 µl reaction, 3 µg of RNA, 1 µl of 10 mM dNTPs, 2 µl of labelled primer, 2 µl of 0.1 M DTT, and 4 µl of 5x first strand buffer were mixed and the primer allowed to anneal for 2 minutes at 42°C. 1 µl (200U) reverse transcriptase enzyme (Super ScriptII) was added and the reaction was incubated for one hour at 42°C. The reaction was terminated by adding an equal volume of stop solution (80% formamide; 10 mM EDTA; 1 mg/ml Xylene cyanol FF; 1 mg/ml bromophenol blue). The reaction was incubated at 70°C for 5 min before loading on a gel.

3. Extension product analysis

To determine the transcriptional start site of the RNA transcript, the reaction products were visualized on a 6% urea-polyacrylamide gel next to a sequencing reaction carried out using the same primer and a plasmid as template that carried the DNA region coding for the transcript.

6% sequencing gel mix:	210 g urea
	74 ml Rotiphorex 40 (acrylamide)
	50 ml 5x TBE
	Dissolved in 500 ml in H_2O

The solution was filtered and stored in dark at RT

To 50 ml of gel mix, 30 µl of TEMED and 400 µl of 10% APS was added and the mix was carefully poured into the space between the two plates avoiding bubbles and leakage. After polymerization, a pre-run was conducted with 0.5% TBE buffer for 10 min at 3000 V before loading the probes. 3-4 µl of the probes were loaded and electrophoresed for 1.5 hours at 2500 V / 300 mA / 300 W until the bromophenol blue front reached the lower buffer tank.

The gel was separated from the plates on a sequencing filter paper (Bio-Rad). After drying on a gel dryer the gel was exposed to a phospor imager screen overnight and scanned on phospor imager.

2.4 Techniques related to protein

2.4.1 Preparation of protein extracts

For the preparation of protein extracts from cell lysates, cell density was monitored spectroscopically at 600 nm (OD_{600nm}), culture volumes corresponding to OD_{600nm} values of 5 or 10 were harvested, and cells were isolated by centrifuging at 4°C. This procedure ensured that comparable amounts of cells were withdrawn for protein extract preparation even when cells of different growth stages had to be compared. The isolated cell pellets were lysed either by sonicating or by lysozyme treatment. For sonication, cells were resuspended in 400 µl of ice cold water supplemented with 1 mM of EDTA and PMSF as protease inhibitors. Cells were repeatedly (6 times) sonicated for 30 sec on ice using a sonicator. Between each repetition cycle, a 1 min pause was applied. The sonifier was set to deliver 80 % power, with a 25 % cycle. For cell lysis by lysozyme treatment, the cell pellet was resuspended in a lysis buffer (50 mM EDTA, 0.1 M NaCl, pH 7.5) containing 50

µg/ml of lysozyme and incubated for 10-15 min at 37°C until the dense solution started clearing.

Independent of the cell lysis method applied, the cell lysates were centrifuged to remove the cell debris. An aliquot of each lysate was stored at -20°C for future purpose and 100 µl of the lysates were boiled with the denaturing protein loading buffer and equal volumes of these samples were loaded on a SDS gel for inspection of whether the protein contents were of comparable concentration.

2.4.1 Separation of proteins by SDS-polyacrylamide gel electrophoresis

The protein sample to be resolved was denatured by heating at 95°C for 2 min in the presence of SDS and β-mercaptoethanol (see protein loading buffer composition below). While β-mercaptoethanol reduces disulfide bonds, SDS denatures and anneals to the amino acid chains of the proteins giving each protein a negative net charge that is proportional to the polypeptide chain length. As a consequence, the proteins are separated essentially based on their molecular mass (Laemmli, 1970). The sieving effect of the gel matrix is achieved by adjusting an appropriate ratio of acrylamide to N, N' methylene bisacrylamide (37.5/1). The polymerization of acrylamide is catalyzed by 0.1% APS (w/v) and 0.05% TEMED. The migration of the proteins was carried out in running buffer under a constant current of 25 mA for 2 h.

Loading buffer:	100 mM Tris/HCl, pH 6.8
	10 % (v/v) glycerol
	2 % (w/v) SDS
	3 % (v/v) β-mercaptoethanol
	0.1 % (w/v) bromophenol blue
Running buffer (Laemmli):	25 mM Tris/HCl, pH 8.3
	250 mM Glycine
	0.1% (w/v) SDS

Table 10: Composition of gel for SDS-PAGE

compounds	**separating gel (10ml)**		**stacking gel (5ml)**
	10%	7.5%	4%
acrylamide/bisacrylamide	3.33 ml	2.5 ml	0.66 ml
separating buffer (1.5 M Tris/HCl, pH 8.8)	2.5 ml	2.5 ml	-
stacking buffer (0.5 M Tris/HCl, pH 6.8)	-	-	1.2 ml
distilled water	4.0 ml	4.8 ml	3.01 ml
1% SDS	100 µl	100 µl	50 µl
10% ammonium persulfate	50 µl	50 µl	25 µl
TEMED	5 µl	5 µl	5 µl

*The volume corresponds to 2 gels, each of size: 8 cm x 10 cm x 0.1 cm

2.4.2 Protein staining with Coomassie blue

After electrophoresis, the proteins in the gel were fixed and stained in staining solution with gentle agitation for 1-2 hours. In order to remove non-specific dye from the protein gels, the gel was destained in the destaining solution.

staining solution: 0.125% (w/v) Coomassie blue
10% (v/v) acetic acid
25% (v/v) ethanol

destaining solution: 10% (v/v) acetic acid
20% (v/v) ethanol

2.4.3 Western blotting

For the detection of specific proteins on protein gels, a technique termed western blot was applied in which the protein bands were first transfered to a polyvinylidene fluoride (PVDF) 0.45 µm microporous membrane (Immobilon-P, Millipore). An air bubble-free sandwich was formed from Whatman 3MM filter papers embedding the membrane and the gel. All components were presoaked in transfer buffer and the electro transfer was carried out in a semi dry transfer system (Sigma-Aldrich) for 90 minutes under a constant current calculated by the area of the gel (in cm) multiplied by 0.8 mA. After transfer, the proteins were visualized by staining the membrane with amido black solution for 1-2 minutes and destaining with dH_2O.

transfer buffer: 48 mM Tris base
39mM glycine
1.3mM SDS
20% methanol, pH 9.2

amido black solution: 0.1% amido black
25% isopropanol
10% acetic acid

2.4.3.1 Immunodetection

After transferring the proteins to a PVDF membrane, the non-specific sites were blocked by incubating the membrane in blocking buffer for 30 min at RT. The membrane was then incubated in blocking buffer with a defined dilution of the primary antibodies (see below) overnight at 4°C or for 1 h at 37°C. The membrane was washed to eliminate the unbound antibodies, once for 10 minutes and twice for 5 min with PBS-T at RT. The membrane was then incubated in blocking buffer with the secondary antibody coupled to horse-radish peroxidase (1:10000 dilutions of anti-rabbit or 1:5000 of anti-mouse) for 1 h at 37°C. The membrane was washed again as described earlier to eliminate the unbound secondary antibodies. The proteins recognized by the primary antibodies were detected using ECL (enhanced chemiluminescence).

PBS-T:	80 mM Na_2HPO_4
	20 mM NaH_2PO_4
	100 mM NaCl, pH 7.5
	0.2% (v/v) Tween-20

primary antibody dilution:	
Rabbit anti SMC	1:1000
Rabbit anti ScpB	1:1000
Rabbit anti GFP	1:1000
Mouse anti His	1:1000
secondary antibody dilution:	
Goat anti Rabbit IgG, peroxidase-conjugated	1:10000
Goat anti Mouse IgG, peroxidase-conjugated	1:5000

2.4.3.2 Chemiluminescence-detection of proteins on nitrocellulose membrane

Immunolabeling was visualized by adding the luminol and H_2O_2 to the peroxidase-conjugated antibodies. The reaction was carried out in the dark by mixing two solutions:

solution1:	100 µl of 250 mM luminol
	44 µl of 90 mM coumaric acid
	1 ml of 1M Tris-HCl pH 8.5
	add H_2O to give 10 ml

solution 2:	6 µl of 30% H_2O_2
	1 ml of 1M Tris- HCl pH 8.5
	add H_2O to give 10 ml

The membrane was soaked for 1 minute in solution mix and luminescence was recorded by exposing the blots to an X-ray film for 5-30 min.

2.4.4 Purification by strep-tactin column

This purification procedure is based on the affinity of the so-called Strep-Tag II towards strep-tactin. The Strep-Tag II is a short peptide 'WSHPQFEK' which binds specifically to an engineered strep-tactin. The column material was provided by IBA. The column was equilibrated with buffer W (2.5 ml, rinsed twice) and thereafter 3-5 ml of the cell lysate were loaded on the column and allowed to flow through the column. Once the tagged protein has bound specifically to the column, the unspecific proteins were rapidly washed away with 5 times 1 ml of buffer W. The proteins were eluted with 3 times 5 ml of buffer E containing the specific competitor desthiobiotin and fractions of 0.5 ml were collected. The column was regenerated with 3 times 5 ml of buffer R, containing HABA (hydroxyazophenyl benzoic acid) that displaces desthiobiotin and regenerate the column.

buffer W: 100 mM Tris-Cl, pH 8.0
1 mM EDTA

buffer E: 100 mM Tris-Cl, pH 8.0
1 mM EDTA
2.5mM Desthiobiotin

buffer R: 100 mM Tris-Cl, pH 8.0
1 mM EDTA
1 mM HABA

2.5 Bacillus genetics

2.5.1 Preparation of chromosomal DNA from Bacillus subtilis cells

Cells from 2 ml overnight culture was harvested and resuspended in 0.5 ml of lysis buffer (50 mM EDTA, 0.1 M NaCl, pH 7.5) and incubated with 1 mg/ml lysozyme for cell lysis. The lysate was then extracted once with phenol and then with phenol:chloroform (1:1). Chromosomal DNA was precipitated by adding 40 µl of 3 M sodium acetate and ethanol. The precipitated DNA was spooled with a Pasteur pipette, and washed by dipping in 70% EtOH, it was then air dried before dissolving it in TE buffer, pH 8.

2.5.2 Preparation of competent Bacillus subtilis cells

B. subtilis develops a competent state at the onset of stationary growth phase during which exogenous DNA is trapped and processed to yield single-stranded DNA in cytoplasm. Through recombinational and replication processes such DNA is either established as a plasmid or integrated into the genome where homologous sequences are present (Dubnau, 1991). Preparation of competent *B. subtilis* cells is based on a modified two step procedure from Dubnau and Davidoffabelson (1971) and requires cells growing in SpC medium until the cells enter the stationary growth phase where they become naturally competent.

Overnight cultures were used to inoculate in 20 ml of freshly prepared SpC medium. Growth was maintained until the cells reached the stationary phase. When the OD_{600} remained unchanged for 20-30 min, the culture was diluted 1:10 into pre-warmed SpII medium and allowed to grow for 90 min. The cells were then pelleted by centrifugation at RT and resuspended in a mixture of 20 ml of supernatant containing 10% glycerol and aliquoted for storage at -80°C.

T-Base:	per litre	
	2 g	$(NH_4)_2SO_4$
	18.3 g	$K_2HPO_4 \cdot 3H_2O$
	6 g	KH_2PO_4
	1 g	trisodium citrate$\cdot 2H_2O$

Sterilized by autoclaving at 121°C at 1.5 bar for 30 min.

SpC Medium:

T-Base	20 ml
50 % (w/v) glucose	0.2 ml
1.2 % (w/v) $MgSO_4 \cdot 7H_2O$	0.3 ml
1 % (w/v) casamino acids	0.5 ml
10 % (w/v) bacto yeast extract	0.4 ml

SpII medium:

T-Base	200 ml
50 % (w/v) glucose	2 ml
1.2 % (w/v) $MgSO_4 \cdot 7H_2O$	14 ml
1 % (w/v) casamino acids	2 ml
10 % (w/v) bacto yeast extract	2 ml
0.1 M $CaCl_2$	1 ml

SpC and SpII media were prepared fresh from the sterile stock solutions and sterile filtered.

2.5.3 Transformation of Bacillus subtilis

100-200 µl of competent *Bacillus subtilis* cells were used for each transformation. Two different dilutions of the plasmid DNA (5 µl and 15 µl from normal plasmid mini-prep) or the chromosomal DNA (0.05 µl and 0.5 µl) were mixed with the competent cells in a culture tube and incubated at 37°C for 20-30 min in a roller drum. The cells were then plated on selective plates and incubated overnight. In case of temperature sensitive mutants the transformation was carried out at room temperature or 25°C for 30-40 min incubation.

2.5.4 Screening for gene integration at the amylase (amyE) locus

In order to screen the *Bacillus subtilis* transformants for the gene integration at the *amyE* locus, the clones were tested for their inability to utilize starch from the medium. A fraction of each of the *Bacillus* transformant colonies were picked from the master plate and were streaked in small patches on a LB-starch plate (with 1% (w/v) soluble starch) and LB plate supplemented with the appropriate antibiotic which served as a replica plate. After incubation overnight, the LB starch plate was tested by spreading 1-2 ml of Lugol's iodine solution and after approx. 1 min. Iodine present in the solution intercalates in the starch molecules and turns into blue. The test plate was examined for the non blue-halo zone around each colony. Those clones that showed 'halo' possessed the intact *amylase* gene and the ones without had the *amylase* gene disrupted by the integration of the transformed gene.

2.5.5 Promoter induction in Bacillus subtilis

0.5-1 mM of IPTG was used to induce all P*spac* derived promoters and 0.5% (w/v) of xylose for P*xyl* promoters.

2.5.6 PCR knockout technique for Bacillus subtilis

This technique involves gene disruption/deletion, using a PCR fragment carrying the resistance gene with the flanking sequences of the gene of interest. The procedure involved three rounds of PCR as illustrated in fig. 8a. In the first round, approx. 1 kb of the upstream and the downstream region of the gene of interest were amplified using the primers $P_{1,}$ P2 for upstream fragment and P3, P4 for downstream fragment. The primers P_2 and P_3 had approx. 19 bp homology to the resistance gene (e.g. the *tet* cassette). In the second round, the products form the first step PCR reaction were used as primers to PCR-amplify the resistance marker (*tet* gene was obtained from pSG1515 digestion). The resulting product was boosted in a third round using the primers $P_1$and P_4. The final product was visually inspected on an

agarose gel for the expected product and then transformed into *Bacillus subtilis*, selecting on the appropriate antibiotic (tetracycline).

Fig. 8a: Schematic representation of PCR knockout method:

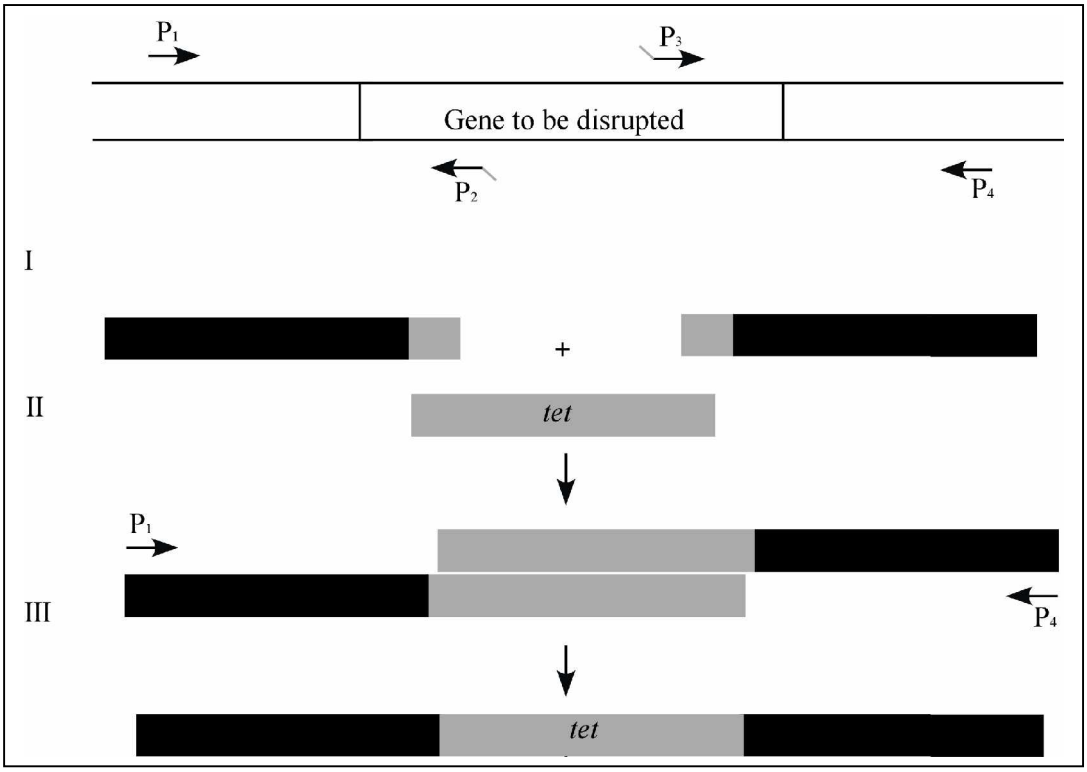
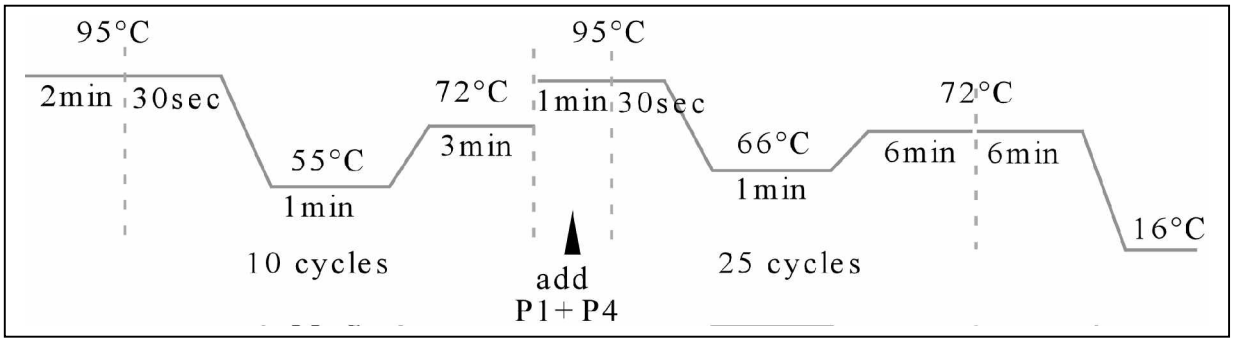


Fig. 8b: Program used for the II and III rounds of PCR reaction:



2.6 Microscopic techniques

2.6.1 Fluorescence microscopy – Principle

Fluorescence microscopy is a useful tool to examine the location or concentration of molecules *in vivo* and can be performed with high sensitivity and specificity. The ability to view activities of proteins within a single living cell began with the discovery of a green fluorescent protein (GFP) from the jellyfish *Aequoria victoria*. The use of this protein has allowed for insight into the microscopic cell world (Gordon *et al*., 1997; Lemon and Grossman, 2000; Margolin, 2000; Phillips, 2001; Li *et al*., 2002; Southward and Surette, 2002). The green fluorescent protein (GFP) has the shape of a cylinder, comprising 11 strands of β-sheet with a α-helix inside and short helical segments on the ends of the cylinder. The fluorophore is protected inside the cylinder and its structures are consistent with the formation of aromatic systems made up of Tyr^{66} with reduction of its C - C bond coupled with cyclization of the neighbouring glycine and serine residues. These side chains, when modified, change the spectral properties of GFP (Yang *et al.*, 1996). The presence of different spectral properties of fluorescence has allowed for simultaneous labelling of different proteins in the cell. Some of the GFP variants used in this work and their spectral details are presented in table below:

Table 11

GFP variants	Excitation /emission (nm)	a.a substitutions in wtGFP
GFPmut1	488/507	2
BFP	380/440	4
YFP	513/527	4
CFP	433/ 475(major) 453/ 501(minor)	6

Fluorescent microscopes are based on the principle that a high-energy, short-wavelength light excites electrons within certain molecules inside a specimen, causing these electrons to shift to an energetically elevated state. When they fall back to their original energy levels, lower-energy, longer-wavelength light in the visible spectrum is emitted. The fluorescent microscope possesses a light source (xenon lamp) that emits a broad spectrum of light. A filter is used to allow only certain wavelengths of

light to pass into the microscope. The light of these wavelengths is focused on the specimen to be studied through a lens. When the proper wavelength of light hits the specimen, the fluorescent protein (e.g. GFP) that might be attached to a cells natural protein begins to glow. The hereby emitted light goes back through the lens, which also contains an emission filter that enables the appropriate image to be seen through the microscope's eyepiece or by a camera that create a digital image on a computer screen. A dichroic beam splitter attached to the microscope allows selecting the proper wavelength to pass through. The emitted light is always produced at a longer wavelength than that originally absorbed (stokes shift). This shift is crucial for the detection of fluorescence as it differentiates the excitation and the emission wavelengths (Emptage, 2001).

The interactions of proteins can be visualized *in vivo* using fluorescence resonance energy transfer (FRET) technique. FRET is a distance-dependent effect between two interacting molecules (e.g. FP-tagged proteins) in which the excitation energy of one molecule (the donor) can be transferred to its partner (acceptor). For FRET to occur molecules must be at close proximity of within 100 Å and the absorption spectrum of the recipient molecule must overlap the emission spectrum of the donor. The use of a pair of proteins of which one is tagged to CFP and the other tagged to YFP is ideal for FRET and allows for colocalization studies (Emptage, 2001).

In this work, fluorescence microscopy was performed on Olympus AX70 microscope. Images were acquired with a digital micromax CCD camera, signal intensities and cell length were measured using the MetaMorph 4.6 program (Universal Imaging). The shape of the cell/nucleoid was examined in bright field light using Nomarski differential interface contrast which resolves different refractive indices.

2.6.2 Vital stains used in fluorescence microscopy

DAPI (4', 6-diamidino-2-phenylindole) is a blue fluorescent nucleic acid stain that preferentially stains double-stranded DNA (dsDNA). This stain was used to visualize the nucleoid. DAPI attaches to AT clusters in the DNA minor groove. Binding of DAPI to dsDNA produces an approximate 20-fold fluorescence

enhancement, apparently caused by the displacement of water molecules from both DAPI and DNA. The excitation maximum for DAPI bound to dsDNA is 358 nm, and the emission maximum is 461 nm. DAPI was used at a final concentration of 0.2 ng/ml to visualize the nucleoid in the cell.

Syto59 is a cell-permeate red fluorescent nucleic acid stain that exhibits bright, red fluorescence upon binding to nucleic acids. It absorbs at 622nm and emits at 645nm which allowed for simultaneous visualization of protein tagged to BFP and the nucleoid.

FM4-64 (N-(3-triethylammoniunpropyl)-4-(p-diethylaminophenylhexatrienyl) pyridinium dibromide) is a lipophilic styryl dye with red fluorescence (excitation/emission spectra approx. 515/640 nm). The stain intercalates into the outer surface of the membrane but is unable to cross the lipid bilayer, therefore only the membrane surface which is directly exposed to the FM 4-64 is stained. FM4-64 was used to simultaneously view the localization of proteins tagged to FP with respect to the cell boundary at the final concentration of 2.5 µg/ml.

2.6.3 Media used for microscopy

The cells were visualized live under the microscope after being grown in a specialized $S7_{50}$ minimal medium that had low level of background fluorescence.

$S7_{50}$ minimal media: The media was prepared fresh, from stock solutions (table12) and sterile filtered

Table 12

Stock solutions	**Final concentration**
10x $S7_{50}$ salts	1x $S7_{50}$ salts
100x metals	1x metals
50% glucose*	1 % glucose*
10 % glutamate	0.1 % glutamate
casamino acids	40 µg/ml
	dH_2O to 1 l

* glucose was substituted with fructose for cultures consisting of strains that had genes with xylose inducible promoters because presence of glucose represses xylose uptake.

10x $S7_{50}$ salts:	MOPS (free acid)	0.5 M (104.7 g)
	$(NH_4)_2SO_4$	100 mM (13.2 g)
	KH_2PO_4	50 mM (6.8 g)
		pH 7 adjusted with KOH dH_2O to 1 l

100x metals:	$MgCl_2$	0.2 M
	$CaCl_2$	70 mM
	$ZnCl_2$	0.1 mM
	$MnCl_2$	5 mM

2.6.4 Preparation of slides for microscopy

In order to visualize live cells under the microscope, 2-3 µl of growing cell culture were applied on the agarose coated slide and covered with a cover slip. Slides were prepared by spreading 800 µl of 1% agarose in $S7_{50}$ medium and covering with another glass slide such that a thin uniform layer is formed between them. After the medium solidified one of the slides were carefully separated, such that the agarose film is retained on one of the slide surface.

3. Results

3.1 Identification of SMC- interacting proteins - Historical observations

As described in more detail in the introduction (1.4), SMC proteins play a crucial cellular role in chromosome condensation and segregation. Disruption of *smc* led to growth impairment, temperature a sensitive phenotype and cells with a decondensed nucleoid. Based on EM studies, SMC proteins were shown to form dimers (Melby *et al.*, 1998). In eukaryotes, SMC formed heterodimers and interacted with other non-SMC components to form a complex. The functional SMC homolog MukB in *E. coli* was also shown to interact with two non-SMC proteins, MukE and MukF. At the beginning of this work, no such interacting proteins were known for SMCs in other bacteria.

Immunoprecipitation experiments employing SMC antibodies first carried out by Alex Strunnikov (NIH, USA), suggested the presence of some SMC-interacting proteins in cell-free extracts prepared from *B. subtilis* (personal communication). However, this observation was not further recognized until the publication of bioinformatic results from Jörg Soppa who, while analyzing the *Halobacterium salinarum* genome, identified a gene encoding a homolog of SMC which was located in an apparent operon with a downstream gene. This encoded protein was highly conserved in all bacterial species containing a putative SMC homolog (Soppa, 2001). The corresponding candidate in *B. subtilis* was identified as a gene that was previously designated *ypuG* by the *B. subtilis* genome consortium and is found in an operon with two downstream genes, *ypuH* and *ypuI* (fig.10):

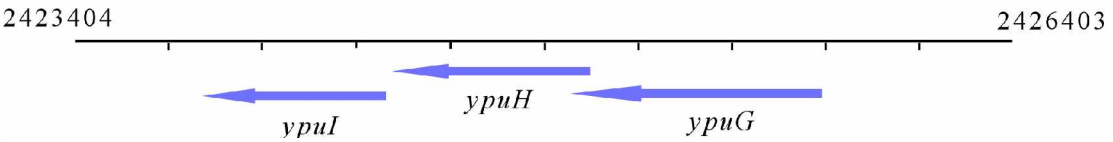


Fig. 10: Genetic organization of the *ypuGHI* operon. Numbers indicate the gene position in *B. subtilis* genome.

The importance of *ypuG* and *ypuH* was initially noted during sequencing of the *B. subtilis* genome. While *ypuI* could be disrupted by single crossover integration of an appropriately constructed plasmid, conditional shut-down of *ypuG* or *ypuH* expression abolished growth of the respective mutants at 37°C which led to the assumption that both of these genes are essential in *B. subtilis* (Vagner *et al.*, 1998). However no additional studies were carried out at that time.

3.2 Phenotypic analysis of ypuG and ypuH

In order to understand the function of proteins coded in the *ypu* operon by the genes *ypuG*, *ypuH*, and *ypuI*, a detailed phenotype analysis of the three individual gene knock-out mutants was initiated. A strategy for systematic deletion of each of the three genes as well as for the construction of a combined *ypuGH* deletion strain was developed. Early attempts to create conditional single-crossover disruption mutants using the pMutin2 vector system (Vagner *et al.*, 1998) resulted in rapid mutant to wild type reversions. Therefore, stable deletions were constructed by replacing 70-80% of the gene of interest with a tetracycline resistance cassette which was integrated into the chromosome by double-crossover using a PCR knockout method (Kuwayama *et al.*, 2002) (see Materials and Methods 2.4.10). The constructed deletion strains were confirmed by PCR using primers locating up and downstream to the genetically modified region (data not shown).

While the *ypuI* null mutant PG31 (*ypuI*::*tet)* grew indistinguishable from wild type cells, the *ypuG* and *ypuH* null mutants JM11 (*ypuG*::*tet)* and JM12 (*ypuH*::*tet*), showed a phenotype quite similar to a *smc* null mutant. This phenotype included temperature sensitivity, i e., cells did not grow above 23°C, and displayed 2-2.5 fold reduced cell doubling times compared to the wild type (table13). In addition to this, both strains grown at 23°C in LB medium contained decondensed nucleoids and formed 12-15% anucleate cells:

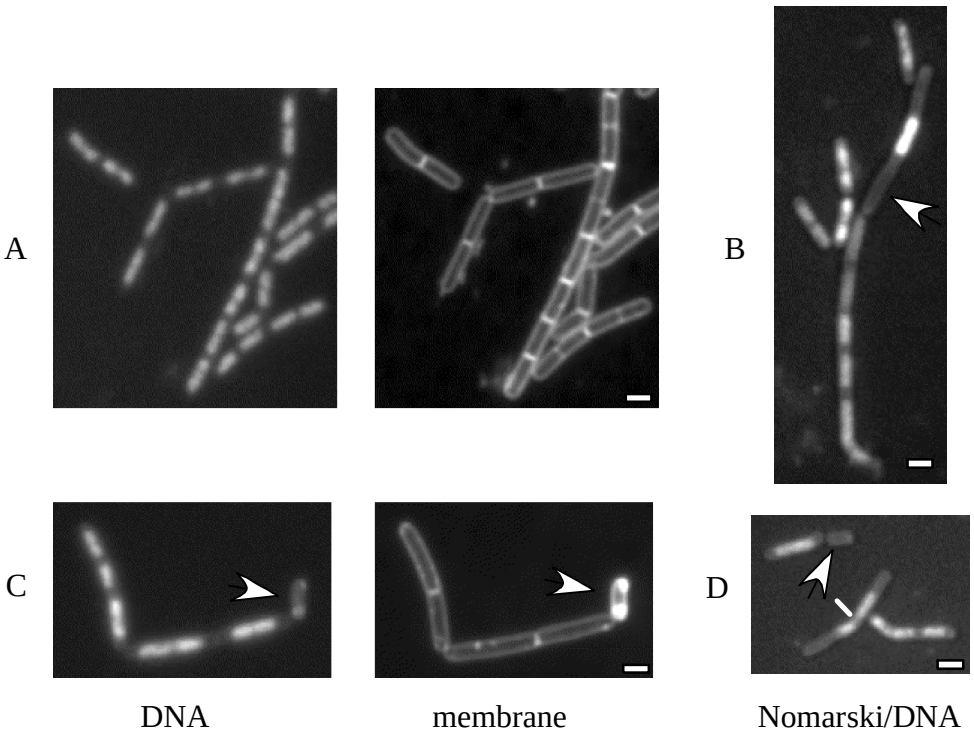


Fig. 11: Fluorescence microscopic images of (A) wild type, (B) JM11 (*ypuG::tet*), (C) PG32 (*ypuH::tet)* and (D) JM12 (*ypuGH::tet*) cells stained with DAPI and FM4-64 stain to view DNA and membrane respectively. Images B and D are DNA stained visualized in Nomarski.

To rule out that the phenotype of deletion of *ypuG* is due to a polar effect on *ypuH*. The strain JM10 (P*xyl ypuH-cfp* at *amyE* locus) was constructed, in which a GFP tagged version of YpuH was expressed under the control of the xylose promoter at the amylase (*amyE*) locus that fully complemented the deletion of the *ypuH* gene when grown in xylose containing medium. Deletion of *ypuG* in JM10 in the presence of xylose still led to a segregation and condensation defect and temperature sensitive slow growth phenotype similar to that of JM11 cells. Showing that both YpuG and YpuH are essential for proper chromosome condensation and segregation.

The combined deletion mutant strain JM13 (*ypuGH*::*tet*), in which both *ypuG* and *ypuH* had been replaced by the tetracycline resistance gene did not further exacerbate the phenotype observed for the single deletion mutations. When the two single deletions, *ypuG* and *ypuH*, were separately combined with a conditional *smc* deletion strain EP58 (*smc*::*kan,* P*spac-smc*: *amyE*), the strains JM19 (*ypuG*::*tet*, *smc*::*kan*, *amyE*::P*spac-smc*) and PG43 (*ypuH*::*tet*, *smc*::*kan*, P*spac-smc*::*amyE*) grew similar to the *smc* deletion strain as long as IPTG was absent. These observations show that like SMC, YpuG and YpuH are involved in chromosome condensation.

It has been reported that combination of *spo0J* (whose deletion results in formation of ~1 % anucleate cells but wildtype like growth) and the *smc* mutant exacerbates the phenotype of the *smc* deletion mutants (Britton *et al.*, 1998). To see if *ypuH* has a similar effect, *ypuH* mutant was combined with *spo0J* in PG39 strain. The cells showed 25-35% increase in anucleate cell formation as well as reduction in growth rate when compared with *ypuH* mutant alone (table 13), suggesting the possibility of partial functional overlap between *smc* and *ypuH*.

It has also been reported that *smc* mutants are synthetically lethal with a *spoIIIE* deletion (Britton and Grossman, 1999). When *ypuG* or *ypuH* mutants where combined with *spoIIIE* mutant, the double mutants of *ypuG* or *ypuH* with *spoIIIE* were temperature sensitive and grew much slower than their single mutants and *smc* mutant (table 13):

strain	doubling time (min)	anucleate cells (%)
PY79 (wt)	92	< 0.01
PG31 (*ypuI*)	98	< 0.01
JM11 (*ypuG*)	196	11
JM12 (*ypuH*)	224	12
JM13 (*ypuGH*)	228	11
PG Δ388 (*smc*)	386	12
JM19 (*smc, ypuG)*	385	11
PG43 (*smc, ypuH*)	388	13
PG39 (*ypuH, spo0J*)	267	28
PG36(*ypuI, spoIIIE*)	96	< 0.01
PG37 (*ypuG, spoIIIE*)	463	< 1
PG38 (*ypuH, spoIIIE*)	448	1

Table 13: Doubling times of wild type PY79, *smc* null mutant, null mutants from *ypuG*, *ypuH*, *ypuI*, *ypuGH* and combinations with *smc*, *spo0J*, and *spoIIIE* null mutants grown at 23°C in LB medium.

In conclusion, all the above results suggest that the gene products of *ypuG* and *ypuH* are involved in a similar cellular function as *smc* and belong to the same epistatic group. However, the influence of SMC seems to be more prominent than YpuG and YpuH.

So far, the 'y'-genes *ypuG* and *ypuH* were of unknown function, based on the observations described above and their apparent role in chromosome condensation and segregation, they were renamed as ScpA and ScpB respectively. 'Scp' stands for 'segregation and condensation protein' or proposed 'SMC complex protein'.

3.3 ScpA and ScpB - A new family of conserved proteins

To analyze the relationship of ScpA (MW: 29.5kDa; pI: 4.8) and ScpB (MW: 22kDa; pI: 4.3) and their occurrence in other organisms, the protein sequences were used to perform a similarity search using a BLASTP internet server at NCBI (Altschul *et al.*, 1997). The results of the BLAST analysis showed that both of the sequences are conserved among bacteria and archaea. ScpA was identified in all bacterial organisms possessing SMC, while ScpB, if present, was found in all organisms possessing ScpA and SMC-like sequences.

The ScpA sequence showed acid-rich sequences between residues 80 to 120 (fig. 12, shown in black bar), and is conserved only in the closely related Bacillus species and in archaea which indicate that ScpA might be involved in interaction with other protein or DNA. The C-terminal region of ScpA showed 40-46% similarity to eukaryotic Rad21, Rec8, and Scc1 families, a subunit of cohesin complex.

Fig. 12:

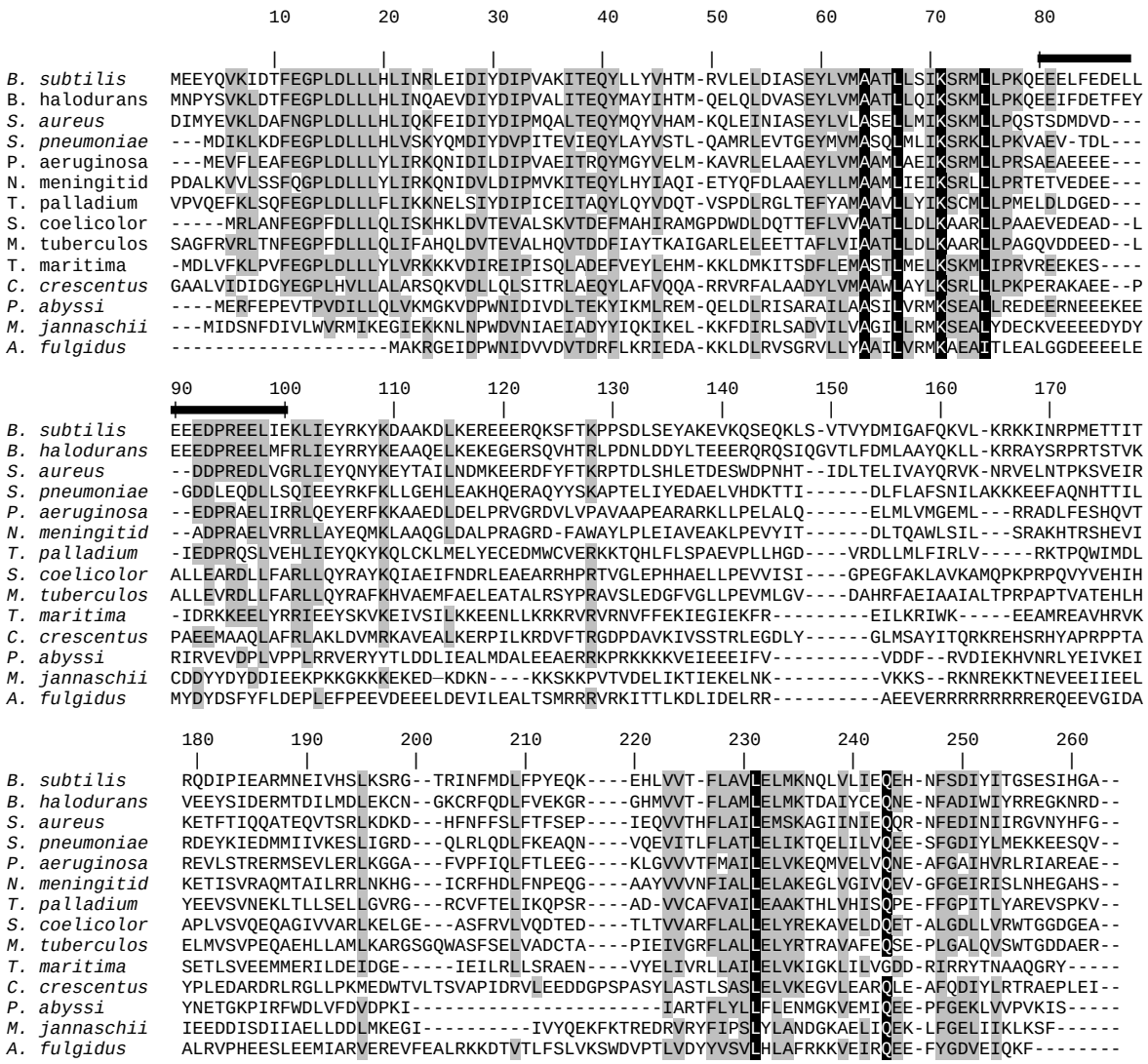


Fig. 12 (above) and 13A (below) are the sequence alignment of ScpA and ScpB from archaea and eubacteria. Organisms: *Bacillus halodurans, Staphylococcus aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa, Neisseria meningitides, Treponema palladium, Streptomyces coelicolor, Mycobacterium tuberculosis, Thermotoga maritima, Caulobacter crescentus, Pyrococcus abyssi, Methanococcus jannaschii, Archaeoglobus fulgidus, Agrobacterium radiobacter, Synechocystis sp.* Invariant residues have black background, conserved residues grey background.

Fig. 13A:

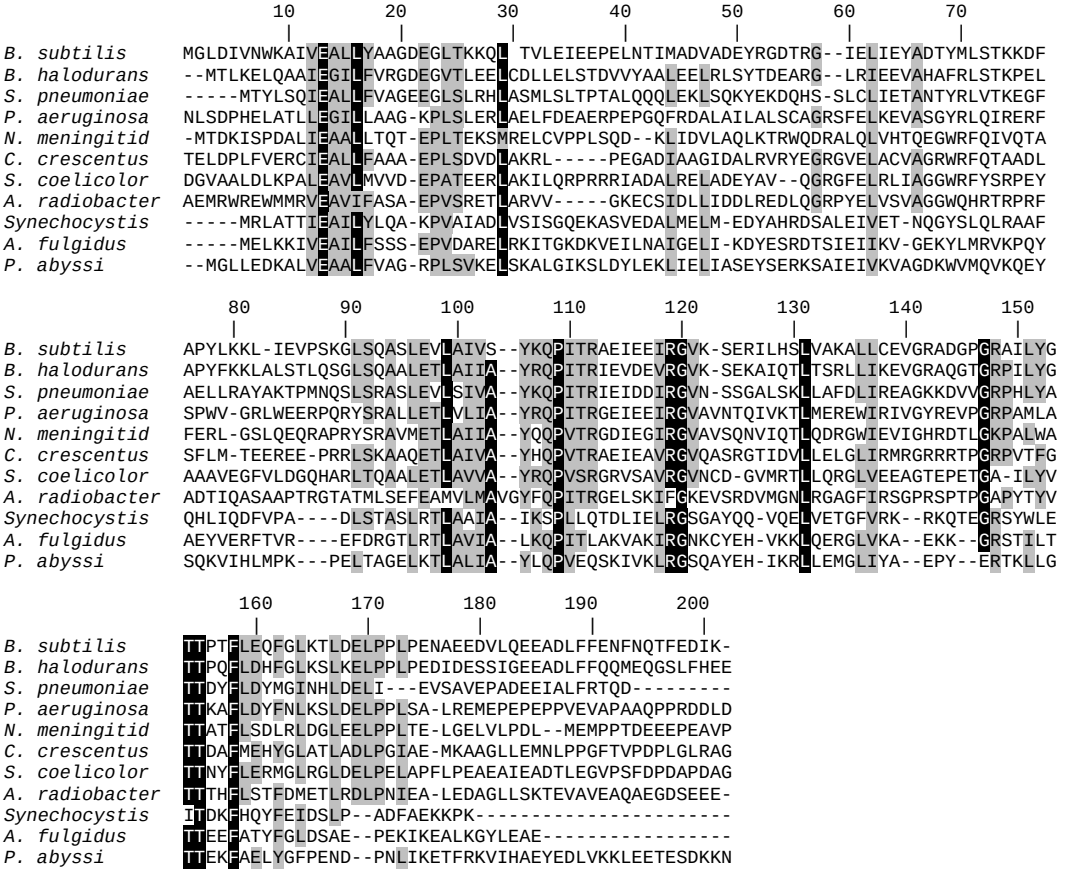


Fig. 13B

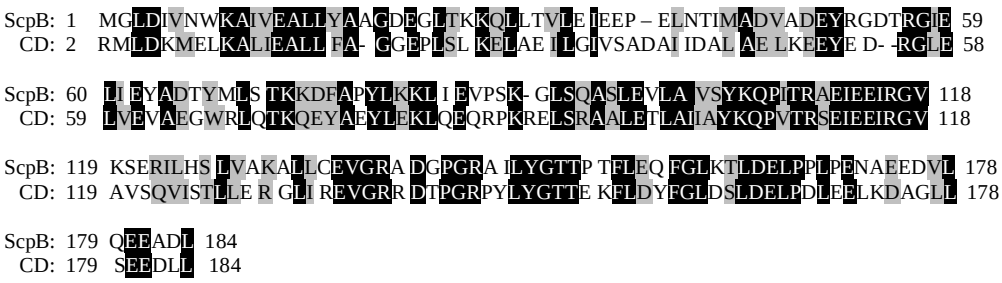


Fig13B: Sequence alignment of ScpB with the predicted transcriptional regulator containing the HTH domain. Conserved residues are with black background.

The conserved domain (CD) search of ScpB using CD-Database showed a sequence similar to the helix-turn-helix (HTH) motif found in other DNA-binding proteins that are conserved among bacteria (fig. 13B). Proteins with HTH belong to the LuxR-FixJ family that constitutes transcriptional activator proteins (Crater and Moran, 2001). The DNA-binding HTH structural motif is composed of an alpha helix,

a turn region, and then a second alpha helix. The second or C-terminal alpha helix of the motif is involved in sequence-specific DNA base interactions and is termed as recognition helix (Pabo and Sauer, 1992).

3.4 Subcellular localization pattern of ScpA, ScpB and SMC

In order to visualize proteins in the living cell, ScpA, ScpB, and SMC were C-terminally tagged to the N-terminus of a fluorescent protein (YFP). To construct the required fusions, the C-terminal region of the *scpA* and *scpB* genes were amplified by PCR and cloned into a plasmid carrying a downstream *yfp* gene. To ensure that tagging of the YFP to ScpA caused no disturbance of transcription of ScpB, an IPTG-inducible P*spac* promoter was cloned into the plasmid upstream of ScpA-YFP. The resulting plasmids were then transformed into PY79, where they integrated at the original gene locus by single-crossover integration.

The strains JM8 (*scpA-yfp*) and JM9 (*scpB-yfp*) were PCR-tested for successful integration of the transformed plasmid into the chromosome and the expression of the respective fusion protein was confirmed by western blot analyses using antibodies against ScpB (fig 14B) and against GFP. Both strains grew in a manner comparable to the wild type, i.e. there were no anucleate cells and growth rate was indistinguishable to the wildtype suggesting that the protein fusions served as functional replacements of their wild type counterparts. Strain JM8 was able to grow in a similar manner with and without IPTG, inferring that tagging of ScpA to a FP did not affect ScpB transcription. Likewise, SMC was tagged to a fluorescent protein in a similar way, except that in this case a glycine linker was introduced between SMC and the FP according to a technique developed in Hiraga's lab for fusing *E. coli*'s functional SMC homolog MukB to GFP (Ohsumi *et al.*, 2001). This method was designed to ensure that the folding of the GFP tag does not interfere with the folding of its N-terminally tagged protein. Integration of this construct into the *smc* locus resulted in strain JM20 (*smc-gfp*) which were temperature sensitive and grew at 25°C but not at 37°C. When the JM20 cells grown at 25°C were examined by microscopy, their nucleoids appeared normal, suggesting that the fusion was partially functional. The observed temperature sensitive phenotype might be due to the process of slowing down folding of the respective proteins or the disruption in transcription of downstream gene due to plasmid integration. To construct a fully functional strain, a

pMutin-YFP vector carrying an IPTG-inducible *spac* promoter designed to drive expression of the downstream gene was used to clone in the *smc* region (Kaltwasser *et al.*, 2002). The resulting strain JM25 (*smc-yfp*, P*spac*) was able to grow at 37°C in the presence but not in the absence of IPTG, indicating that tagging of GFP to SMC abolished the continued transcription of a gene downstream of *smc*, *ftsY*, a signal recognition particle receptor, which serves an important function during the growth (Oguro *et al.*, 1995).

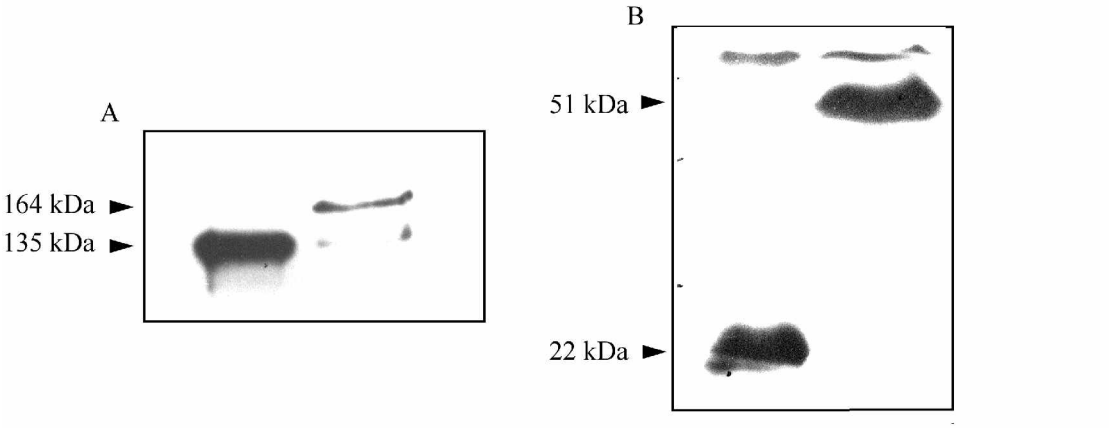


Fig 14:
A. Western blot with SMC antibody. Cell free extracts resolved in 6% SDS-PAGE
lane 1- PY79(wt)
lane 2- JM26 (*smc-yfp*)

B. Western blot with YpuH antibody. Cell free extracts resolved in 15% SDS PAGE
lane 1- PY79 (wt)
lane 2- JM9 (*ypuH-yfp*)

The strains JM8 (*scpA-yfp*), JM9 (*scpB-yfp*), JM20 (*smc-gfp*), and JM25 (*smc-yfp*, P*spac*) showed a similar pattern of fluorescent foci at 25°C in $S7_{50}$ minimal medium. (fig. 15 A, B, C). They localized in a cell cycle-dependent manner where one or two fluorescent foci were present in the middle of small cells, while in larger cells (and thus later in the cell cycle) one or two fluorescent foci were present close to each cell pole of future daughter cell (fig.15). Fluorescence of cells outside of the protein-YFP foci was similar to that seen in cells that did not carry the fusion, indicating that most of the protein molecules are present within the foci. The fluorescence intensity of the foci was brighter in JM8 and JM9 cells when compared to strain JM25. The similar pattern of localization of all the three proteins and the genetic analyses showing similar phenotype upon deletion suggest that the three proteins ScpA, ScpB, and SMC might be involved in a same function and might work together as a complex.

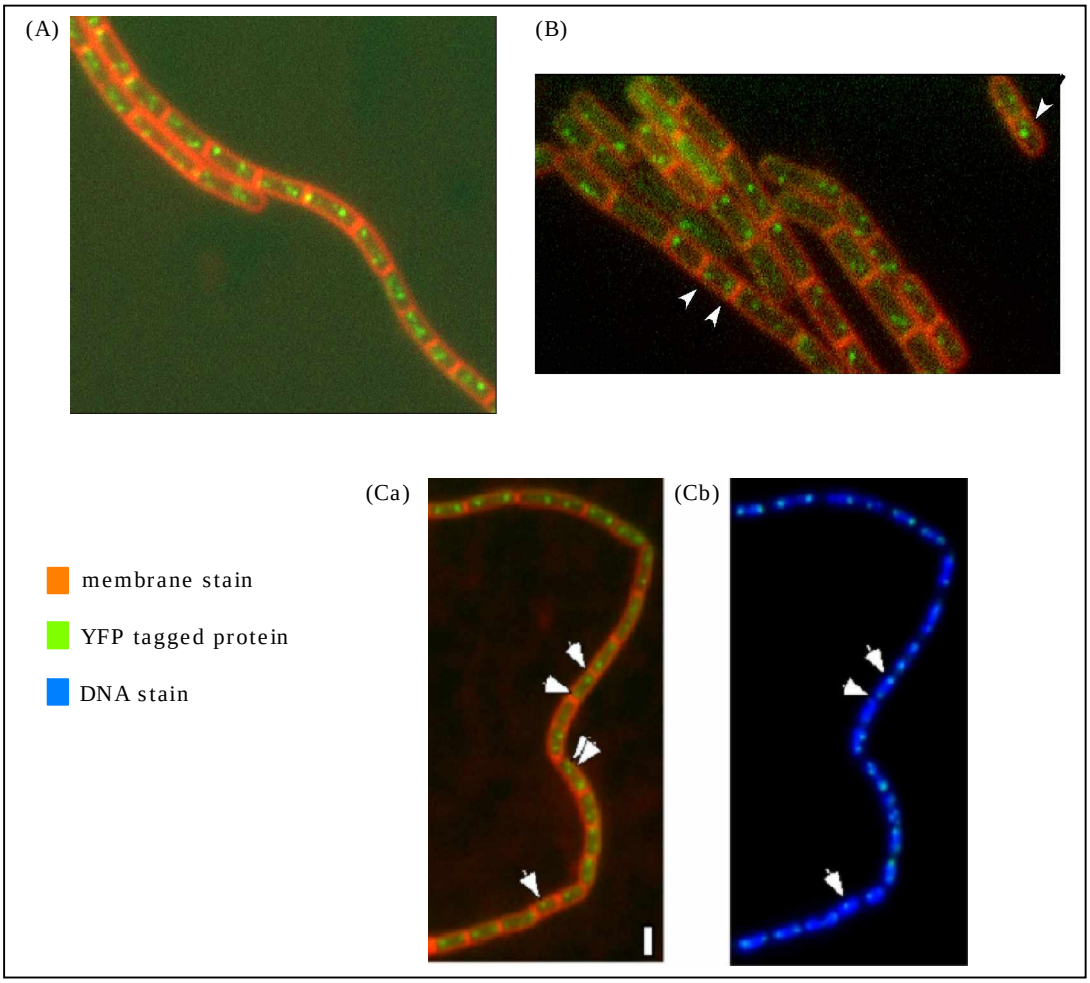


Fig. 15: Fluorescence microscopy. (A) JM8 (*scpA-yfp*), (B) JM9 (*scpB-yfp*), (Ca) JM20 (*smc-yfp*) and (Cb) overlay of JM26 with DAPI stain.

3.5 Dynamic localization of SMC, ScpA, and ScpB

The localization pattern of ScpA, ScpB or SMC proteins seem to differ during the cell growth. The smaller cells showed 1-2 foci at the mid cell and the larger cells had 2-4 bipolar foci. To compare the relative positions of the foci during the cell growth, the location of ScpB was monitored relative to the position of origins of replication during the cell cycle. A strain PG27 which expressed ScpB-YFP and possessed LacI-CFP bound to the tandem repeats of *lacO* cassette near the origin at 359° was used. Cells from this strain showed characteristic bipolar foci of the *Ori's* coloured in green (fig. 16). In most cases, ScpB localized close to the origin regions, often with 2 foci (in red) flanking each origin (fig. 16). In small cells (<1 µm), one or

two central SMC/Scp foci were flanked by two well separated origin signals (fig. 16). Later during cell growth, the origins separated and moved apart towards the poles, which was then followed by SMC/Scp foci movement very close to or coincident with origin regions (fig. 16, between 1 and 1.24 µm). Due to the limited resolution of light microscopy, the foci appeared coincident in some cases. These observations confirm that the SMC complex is not associated with the origin region. *Ori* regions are associated with proteins like Spo0J which might inhibit its association with SMC. The ScpB localization observed under the microscope in the growing cells showed that bipolar movement of ScpB was not synchronized like the Ori-tag movement. One ScpB focus was found very close to the origin, while the other remained close to mid cell, i.e., well separated from the other origin (fig. 16). This observation was vivid with the microscope time lapse snapshots of the cells (data not shown).

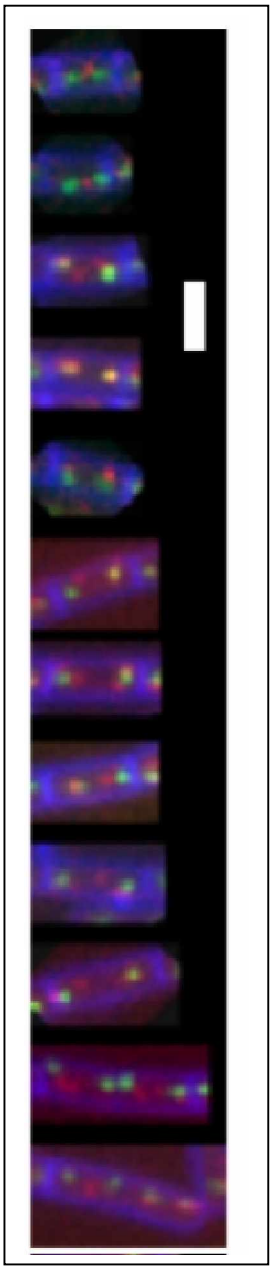


Fig. 16: Fluorescence microscopy of PG27 cells showing localization of ScpB-YFP in red, origin regions in green and membrane in blue. Each panel represents the predominant localization pattern in cells of the respective size. Cells are shown with increasing size, and so according to the state within the cell cycle (Scale bar indicates 2 µm)

After the origins reached the poles, the Scp foci moved away from the origins towards the center of each cells halves (fig. 16). This was visible in the larger cells that had four bipolar origins, while SMC/Scp foci were located towards quarter sites corresponding to the future middle of newborn cells after cell division and flanked by the origin foci which are indicative of new rounds of chromosome replication before cell division (fig. 16). Thus, the mobility of condensation centres appears to be associated with replication process.

To investigate the association of SMC complex with the replication machinery, SMC-YFP encoding plasmid was transformed into strain PG28, carrying a CFP tagged to the C-terminus of the τ subunit of DNA Polymerase III (Lemon and Grossman, 1998). The resulting strains JM27 (*smc-yfp, dnaX-cfp*) showed a centrally located DNA polymerase. SMC foci were adjacent but not coinciding with the DNA polymerase in smaller cells but the foci were not coincident in larger cells (fig. 17):

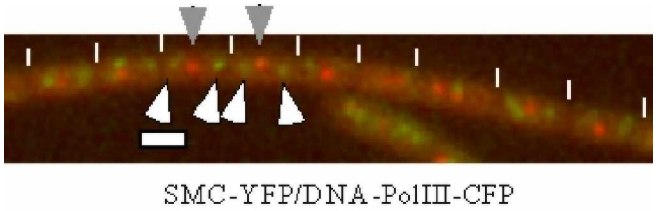


Fig 17: Fluorescence microscopy of JM27 (*smc-yfp, dnaX-cfp*) cells. SMC-YFP is in green (white arrows) and DnaX-CFP in red (grey arrows).

These observations indicate that in smaller cells, the SMC complex might be transiently associated with the replication machinery while organizing the chromosomes for the replication process. Once the chromosomes have started replicating, the SMC foci might be involved in organizing the newly replicated chromosomes. Similar pattern of localization was also observed with ScpB-YFP and DNA Pol III.

3.6 SMC, ScpA, ScpB are associated with DNA

Biochemically, it has been shown that SMC binds to single-stranded (Hirano and Hirano, 1998) as well as double stranded DNA (Volkov *et al.*, 2003). SMC, ScpA, and ScpB were always observed to be present in the nucleoid (fig. 15Cb). To prove the association of SMC and its complex partners with DNA *in vivo*, the chromosomal DNA from JM8 (*scpA-yfp*), JM9 (*scpB-yfp*) or JM25 (*smc-yfp,* P*spac*) were transformed in a *spo0J* mutant strain (*spo0J::spec*) and examined for protein localization in anucleate cells formed as a result of the *spo0J* deficiency (Ireton *et al.*, 1994). SMC, ScpA, and ScpB localized as bipolar foci in cells with DNA but did not show any fluorescence in anucleate cells as seen in fig. 18:

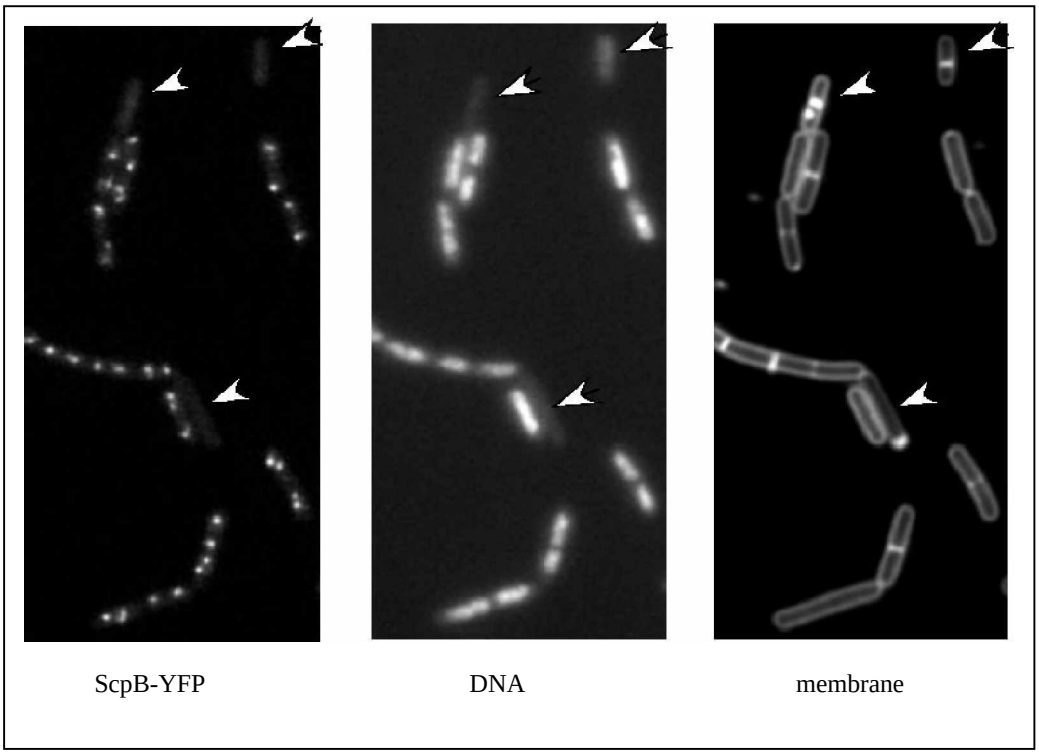


Fig. 18: Fluorescence microscopy of PG39 (*scpB-yfp, spo0J*$^-$) cells. Arrows show the anucleate cells.

This observation demonstrates that SMC, ScpA, and ScpB are associated with cellular DNA.

3.7 Colocalization of ScpA, ScpB, and SMC

In order to examine whether proteins possess the same subcellular address and colocalize with each other, they have to be viewed simultaneously in a cell. To accomplish this, one of the proteins under investigation was tagged to CFP and combined with strains expressing its potential interaction partner fused to YFP (the emission spectra of YFP and CFP do not interfere, which allows the observation of both of the proteins in the cell as long as appropriately selected filters are used). For the construction of N-terminal fusion of ScpB to CFP, *scpB* was cloned in a plasmid that integrated at *amyE* locus and expressed the fusion protein from a xylose-inducible promoter. This strain JM10 (*amy::cfp-scpB,* P*xyl*) was able to complement the *scpB* mutant and also localized in a similar manner as in JM9 cells but the CFP fluorescence was weaker when compared to YFP. The strain JM10 was combined with JM8 (*scpA-yfp*) and JM25 (*smc-yfp*) to yield JM14 (*scpA-yfp, amy::cfp-scpB,* P*xyl*) and PG44 (*smc-yfp, amy::cfp-scpB,* P*xyl*), respectively. When analyzed under microscope, JM14 cells showed foci for both YFP and CFP that were visible in both of the filters. Upon overlapping the images, these foci were coincident:

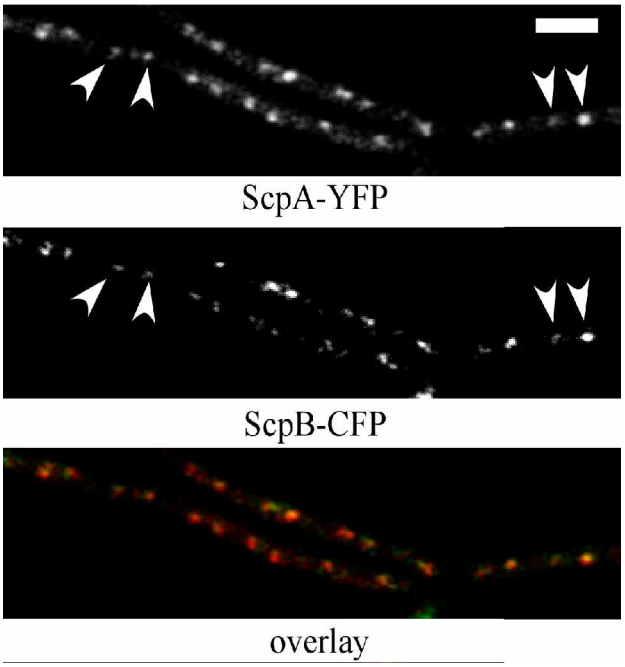


Fig: 19a: Colocalization of ScpA and ScpB in JM14 cells.

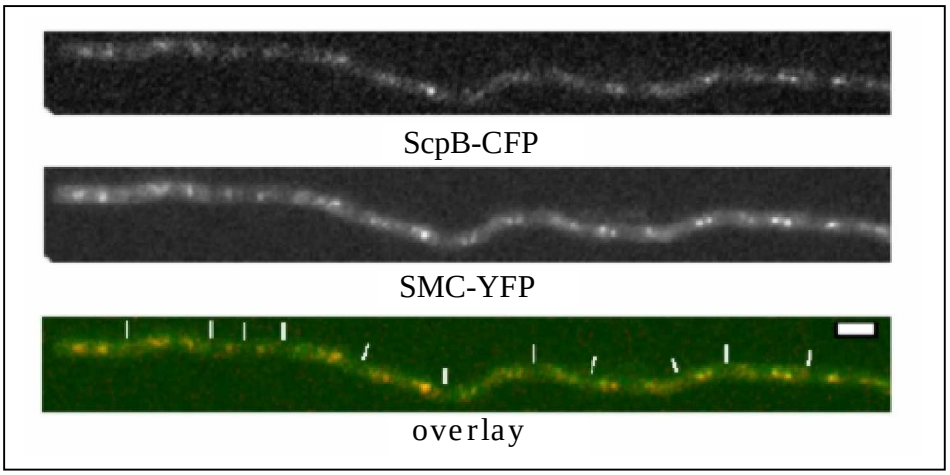


Fig: 19b: Colocalization of ScpB and SMC in JM30 cells.

To colocalize SMC and ScpA, SMC was tagged to CFP in strain JM26 (*smc-cfp*) which showed very faint fluorescence foci in the cell. When combined with ScpA-YFP or ScpB-YFP to yield strains JM29 (*scpA-yfp, smc-cfp*) and JM30 (*scpB-yfp, smc-cfp*) respectively. It was difficult to localize a SMC-CFP fusion in JM29 and in JM30 cells (fig. 19b). In the rare case where clear CFP and YFP foci were visible, they were coincident. Moreover, growth was severely impaired in strain JM29 when compared to JM30. The observation that ScpA and ScpB colocalize with each other and with SMC supports the idea that they function together in a complex. The rather poor visibility of fluorescence foci in PG44, JM29, and JM30 cells might be either due to an interference with proper protein folding or a negative effect mediated by the fluorescent proteins in complex formation, providing a clue that ScpA and ScpB binds at the SMC head region, comprising the N- and the C- terminus of SMC.

3.8 Interaction of ScpA, ScpB, and SMC in vivo

In order to verify whether colocalization is equal to a true interaction of ScpA, ScpB and SMC *in vivo*, a technique called FRET was employed. The FRET effect (fluorescence resonance energy transfer) is distance-dependent and requires interaction between YFP and CFP such that the emission energy of a previously excited CFP (the donor) is transferred and absorbed to excite YFP (the acceptor). Under optimal conditions no emission energy spectra from the donor molecule is seen when examined under the microscope. An efficient energy transfer between YFP and

CFP can occur only if both are at close proximity within 50Å, which requires interaction of the proteins.

The strains PG41 (*scpB::tet*, P*xyl-scpB-cfp*, *scpA-yfp*) and PG44 (*amy::* P*xyl-scpB-cfp, smc-yfp)* were observed through a special FRET filter that specifically excited with a CFP wavelength and allowed the observation of the YFP emission, such that fluorescence was visible only if FRET occurred. When compared to the control strains PG40 (*scpB::tet*, P*xyl-scpB-cfp*) and JM8 (*scpA-yfp*) that did not show any FRET fluorescence (fig. 20 A and B), cells of the combined strain PG41 showed FRET fluorescence which was seen as bipolar foci, confirming the interaction of ScpA and ScpB *in vivo*:

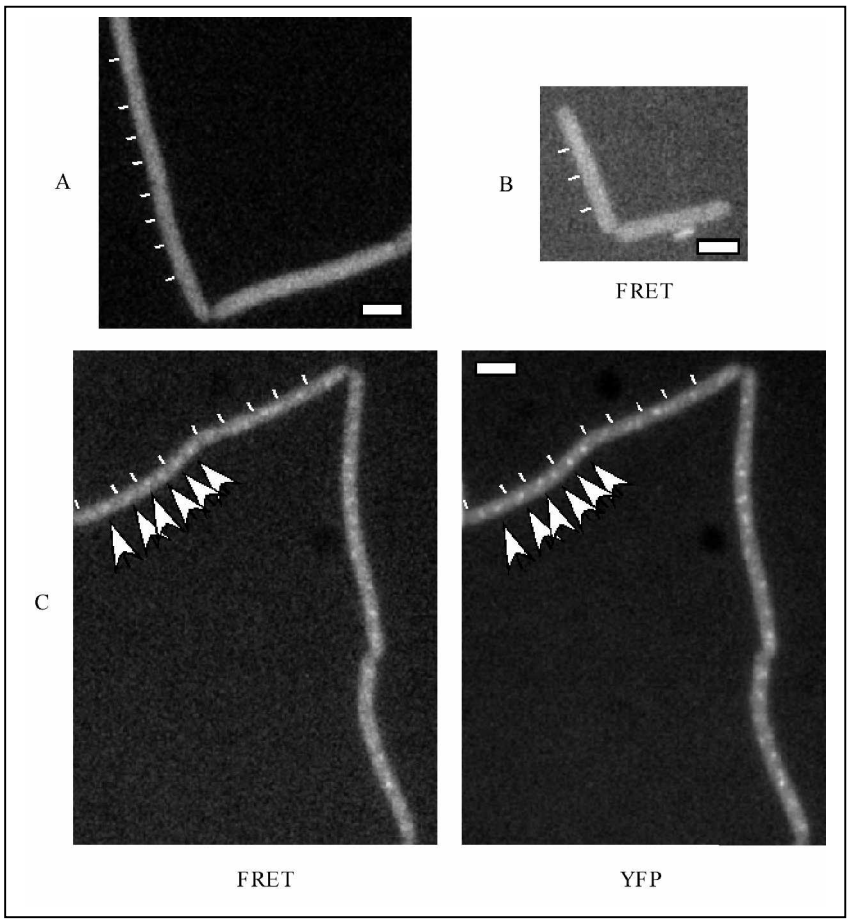


Fig. 20: Fluorescence resonance energy transfer (FRET) analysis.
Fluorescence microscopy of cells grown in the presence of xylose (A) PG40 (*scpB::tet*, P*xyl-scpB-cfp* at *amyE* locus), (B) JM8 (*scpA-yfp*), and (C) PG41 (*scpB::tet*, P*xyl-scpB-cfp* at *Amy* locus, *scpA-yfp*). Cells were observed in FRET and YFP filters. Arrows indicate the ScpA-YFP foci. Short white bars show the ends of cells. Scale bar 2µm.

To confirm the interaction of ScpA and ScpB with SMC, immunoprecipitation experiments were carried out with strains JM8 (*scpA-yfp*) and JM9 (*scpB-yfp*) using a protein A sepharose column saturated with GFP antibodies. These experiments were executed by P. Graumann, who observed that the native SMC was co-precipitatable with JM8 and JM9 cell extracts (Mascarenhas *et al.*, 2002). To investigate whether any other proteins are involved in the interaction, a strain JM24 (*smc-strep*) was constructed in which SMC was tagged C-terminally to a strep tag sequence. Cell lysate of JM24 from the mid log phase was passed through a strep tactin affinity column. The benefit of this column is the one step protein purification from a crude lysate and it also allows the copurification of non-covalently bound ligands. Columns were separately loaded with cell lysates prepared from strains PY79 and JM24. After a wash, the eluates from the respective columns were concentrated and analyzed on a SDS-PAGE gel as well as by western blotting using SMC antibodies. The SDS-PAGE gel showed several bands in the control lane (probably proteins binding the column nonspecifically) but there were two unique bands corresponding to the sizes ScpA and ScpB that were not visible in the control lane, see fig. 21A. The western blot using SMC antibody detected SMC only in the JM24 eluate, fig. 21B:

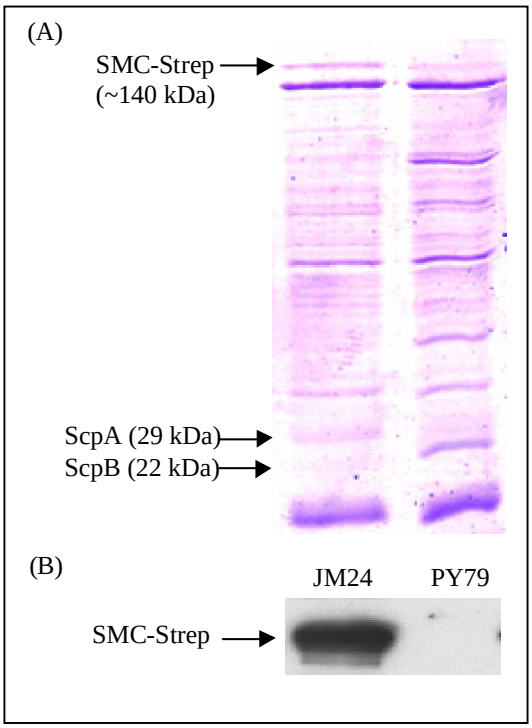


Fig. 21(A): SDS-PAGE gel and (B): Western blot with SMC antibody on the strep column eluates from JM24 (*smc-strep*) and PY79 (wt) cells.

The addition of avidin to the cell lysate before performing elution of the bound material from the column might have subtracted proteins bound with weak affinity,

but this was not performed due to the paucity of the material and time. The results obtained so far from FRET studies and immunoprecipitation experiments confirm that SMC has ScpA and ScpB as its direct interacting partners.

3.9 Specific localization depends on all three proteins of the complex

To investigate the interdependence of SMC, ScpA, and ScpB localization, one of the proteins was viewed in the absence of the others. To do so, the chromosomal DNA from JM8 (*scpA-yfp*) and JM9 (*scpB-yfp*) were transformed into JM16 (*smc*::*kan, amy*::P*spac-smc, cm::tet*) strain that carried SMC under the control of the IPTG-inducible P*spac* promoter. This strain was derived from EP58 (*smc*::*kan, amy*::P*spac smc*) by exchanging the resistance cassette from *cm* to *tet* in order to make it compatible with JM8 or JM9 strain that also carried the *cm* resistance. The corresponding strains JM17 (*scpA-yfp, smc*::*kan, amy*::P*spac-smc*) and PG33 (*scpB-yfp, smc*::*kan, amy*::P*spac-smc*) were grown in the presence and in the absence of IPTG. The strains grown with IPTG behaved like wild type and showed the bipolar foci formation of the ScpA and ScpB proteins, as seen in fig. 22. In the absence of IPTG the cells had decondensed nucleoids and the SMC was not seen in bipolar foci fluorescence but was distributed throughout the cell:

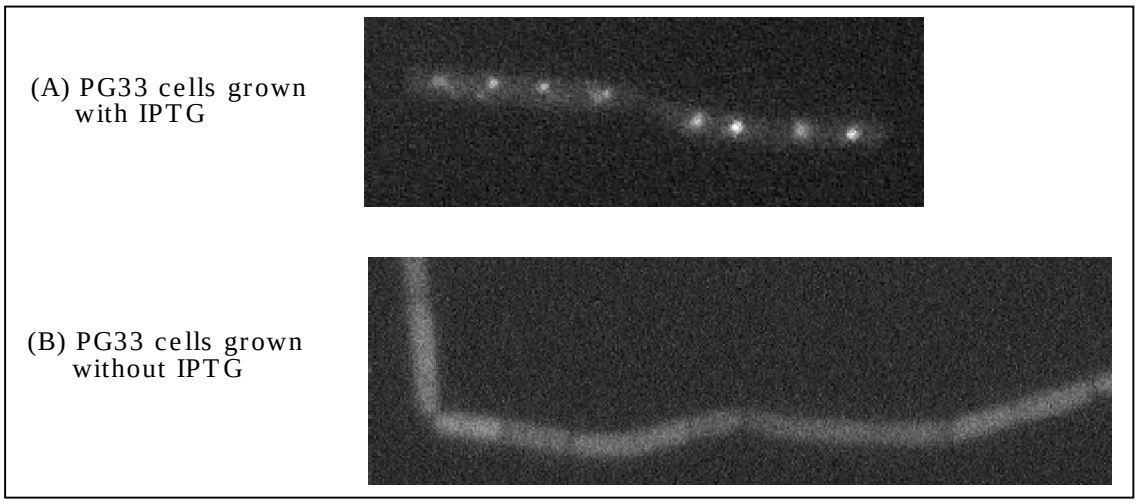


Fig. 22: Fluorescence microscopy of PG33 cells grown (A) with and (B) without IPTG.

To investigate whether SMC can form foci in the absence of ScpA and/or ScpB, strains were derived from JM25 (*smc-yfp*) in which *scpA*, *scpB* or both genes were deleted. A control strain JM33 (*smc-yfp ypuI::tet*) was constructed in which the *ypuI* gene downstream of *scpA* and *scpB* was deleted. As seen in fig. 23A, the control strain JM33 (*smc-yfp ypuI:: tet*) grew like the wild type and showed SMC as bipolar foci. In strains JM31 (*smc-yfp, scpA::tet*), JM32 (*smc-yfp, scpB::tet*) and JM34 (*scpAB::tet, smc-yfp*), SMC was no longer visible as foci but was distributed throughout the cell:

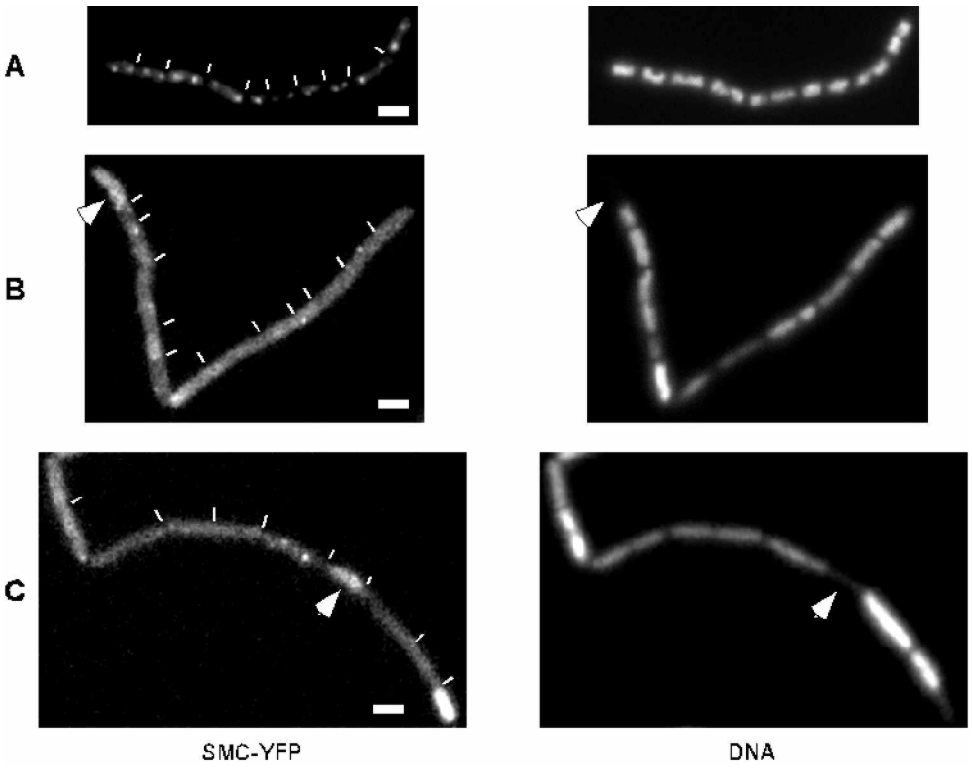


Fig. 23: Fluorescence microscopy of *B. subtilis* cells growing at mid-exponential phase. Left side panel shows fluorescence of SMC-YFP and the right panel, DNA stained by DAPI. A: JM33 (*smc-yfp, ypuI::tet*), B: JM 31(*smc-yfp, scpA::tet*), C: JM32 (*smc-yfp, scpB::tet*).

In most cases, fluorescence was seen distributed throughout the cell (fig. 23B and C), however, some cells had foci that were located on the nucleoids but in an aberrant fashion. In few cases, fluorescence was seen to accumulate near the membrane. Interestingly, some cells showed fluorescence even in anucleate cells (fig. 23B and 23C, indicated by arrows), which was in contrast to anucleate cells containing ScpA and ScpB that carried a *spo0J* mutation. These observations show that the foci represent the active SMC complex. While SMC can still form foci on

DNA, both ScpA and ScpB are required for proper localization on the nucleoids. From this, it can be inferred that ScpA and ScpB play an essential role for the formation of foci which make the condensation centers supporting the ternary complex formation by proteins *in vivo*.

3.10 The SMC complex requires active replication for its bipolar foci segregation

In order to probe the dynamic bipolar movement of the SMC complex foci, the ScpA-YFP fusion was viewed in a strain KL210, which carries a temperature sensitive mutation in *dnaB* that blocks the initiation of replication process upon temperature upshift. JM46 (*scpA-yfp, dnaB*^{ts}) cells were monitored for ScpA localization by shifting the growing cultures from 25°C to 45°C for an hour which arrested the replication process and then back to 25°C to resume replication. At the time of temperature upshift all ongoing replication processes were completed but no new process was initiated. Under the microscope, cells at this stage appeared filamentous and showed a single compact nucleoid (fig 24A). One to three ScpA foci were seen aggregated at the cell center on the nucleoid:

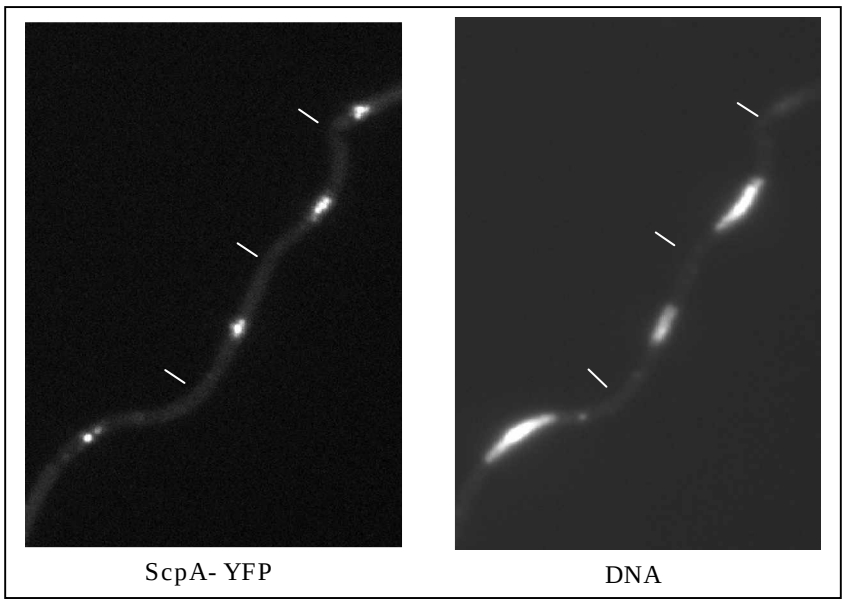
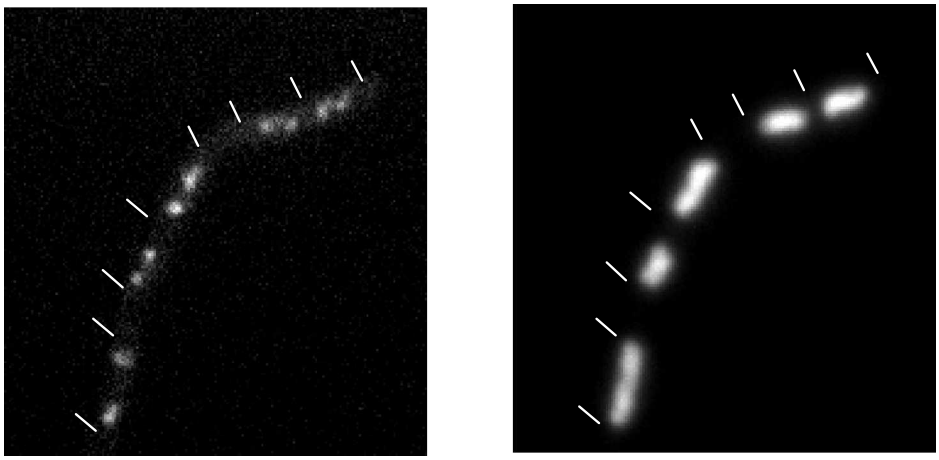


Fig. 24A: Fluorescence microscopy of JM46 cells after 60 min at 45°C (temperature upshift). Left panel showing the ScpA-YFP and the right panel DNA, stained with DAPI. White bars represent the ends of the cell.

When the cultures were shifted from 42°C back to 25°C and grown for an hour, the cells had resumed their replication process, which was visible by the presence of two nucleoids per cell. The cells were no longer filamentous and ScpA was seen as separated bipolar foci, shown in fig. 24B below:



ScpA-YFP DNA

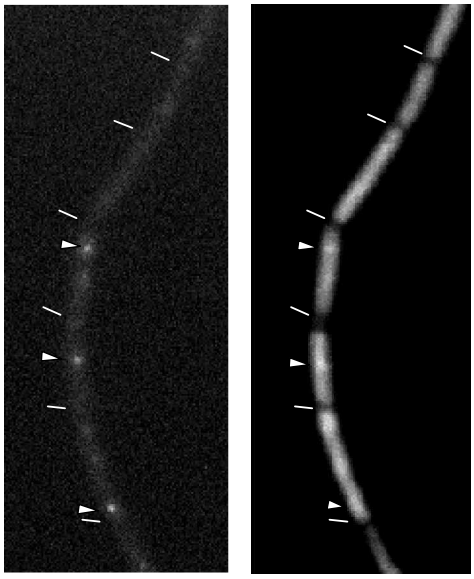
Fig. 24B: Fluorescence microscopy of JM46 cells shifted from 45°C to 25°C (temperature downshift). Left image showing the fluorescence of ScpA-YFP and the right image, the DAPI stained DNA. White lines mark the ends of the cells.

Similar observations were seen even with SMC-YFP strain. These observations show that the active segregation of the replicated chromosomes depends on SMC complex and in turn on active replication process. The SMC complex is initially loaded at the replication center and active replication causes the foci to move apart bidirectionally resulting in condensation and segregation of replicated chromosomes.

3.11 SMC localization depends on DNA topology

Studies have shown that *B. subtilis* SMC affects plasmid topology *in vivo* (Lindow *et al.*, 2002a). Moreover, SMC mutants showed decondensed nucleoids and were hypersensitive to DNA gyrase inhibitors (Lindow *et al.*, 2002a). Depletion of topoisomerase I suppressed the chromosome-partitioning defect of the *smc* null mutant. Thus, SMC seems to function in concert with other topoisomerases as a chromosome organizer. In order to look for the localization behaviour of SMC in the absence of active DNA topology-modulating proteins like gyrase and topoisomerase

IV, JM25 (*smc-yfp*) and JM8 (*scpA-yfp*) cells were treated with drugs that inhibited gyrase activity. Novobiocin belongs to the coumarin drug family that contains natural products originally isolated form *Streptomyces* species. Coumarins inhibit supercoiling and enzyme turnover by preventing the binding and hydrolysis of ATP (Ali *et al.*, 1993). Nalidixic acid is a member of the quinolones drug family that are synthetic drugs that act by forming an inhibiting ternary complex with the topoisomerases in the presence of DNA (Grompone *et al.*, 2003; Kato *et al.*, 1990). JM8 and JM25 cells were treated with novobiocin (10 µg/ml) or nalidixic acid (200 ng/ml) for one hour and examined for changes in the nucleoid structure using DAPI stain and foci localization pattern analyses. The treated cells showed decondensed nucleoids, some of the cells showed a much denser DNA region at the cell center:



ScpA-YFP DNA

Fig. 25: Fluorescence microscopy of JM8 cells treated with nalidixic acid (white bars show the ends of the cells). Arrows indicate dense DNA regions, where a ScpA-YFP focus is formed.

The SMC/ScpA proteins were no longer seen as foci in the cells with decondensed nucleoid, in smaller cells foci were seen at the cell center (fig. 25) which also corresponded to the dense DNA region visualized by DAPI staining. The presence of the central dense DNA region might be due to the secondary effect of gyrase inhibition on replication inhibition. Inhibited gyrase increases the local accumulation of positive supercoils downstream of replication forks and this effect slows down the replication fork progression (to one third) (Khodursky *et al.*, 2000). These observations suggest that SMC requires a defined DNA topology for proper

function. Disturbances exerted by action of the antibiotics novobiocin or nalidixic acid might interfere with proper recognition of the DNA template by SMC and weaken or even abolish SMC-mediated DNA compaction. One of the possible conclusions could be that SMC binds to specific DNA structures and is necessary to maintain the DNA topology originally established by the topoisomerases.

3.12 SMC - A bacterial condensin protein

3.12.1 Effects of overexpression of SMC

To purify SMC from *B. subtilis* for biochemical studies, *smc* was cloned into a plasmid pMW6 that could overexpress the protein as a C-terminal RGS-His tag from the *amyE* locus upon induction with xylose. The resulting strain JM6 (*amy::*P*xyl-smc-RGS-His*$_6$), was able to overproduce SMC-RGS-His$_6$ upon induction (compare lane 1 and 2 in fig. 26), but the western blot with anti-SMC and anti-His antibodies revealed in addition to a band of an expected size, the presence of a second band approximately half of the size of SMC which was seen only in the xylose induced strain (fig. 26, lanes 2). The strain JM6 was not used for further analyses because it also failed to rescue the *smc* null mutant. Instead, another strain CAS4 (*amy::*P*hyperspank-smc-His*$_6$), was used in which SMC could be overproduced using expression driven by an IPTG-inducible *hyperspank* promoter (Volkov *et al.*, 2003). SMC expressed by this system also complemented the *smc* null mutant. Upon induction of SMC with 1 mM IPTG, the nucleoids were more condensed and also formed up to 5% anucleate cells (Volkov *et al.*, 2003). Similar experiments were carried out with overexpression of ScpA or ScpB, which did not exhibit any detectable effect on the cellular chromosome structure suggesting that SMC is the key player in chromosome condensation and the Scp's appear to function only in conjunction with SMC.

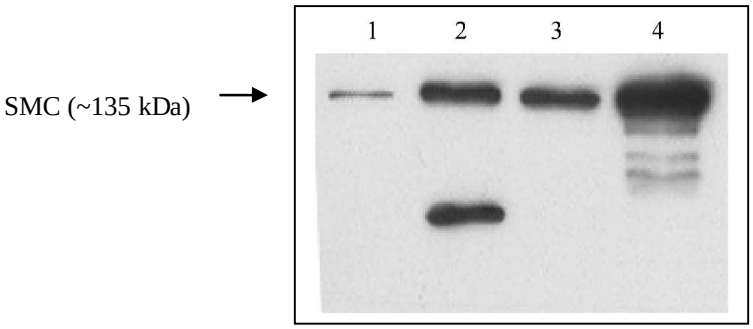


Fig. 26: Western blot analysis using SMC antibody.
Lanes 1 and 2: JM6 cells grown without and with xylose, respectively.
Lanes 3 and 4: CAS4 cells grown without and with IPTG, respectively.

3.12.2 SMC condenses DNA from a single position on the nucleoid

To visualize whether SMC retained its localization pattern upon overproduction, chromosomal DNA from the strain JM25 carrying a functional C-terminal YFP fusion of SMC was transformed into the SMC overexpression strain CAS5 (P*hyperspac-smc*) where *smc* was placed under the control of a *hyperspac* promoter at its original locus. The resulting strain JM35 (*smc::* P*hyperspac-smc-yfp*), grown with 0.025 mM IPTG, showed similar nucleoid morphology from that of wild type cells and SMC-YFP localized in a similar bipolar manner as in the parent strain. With 1 mM IPTG, chromosomes were highly compacted, the SMC-YFP were much brighter but still largely retained in the foci (fig. 27A). Similarly, ScpB-YFP was also retained as foci at 1mM IPTG in JM36 (*scpB-yfp*, P*hyperspac-smc*) cells:

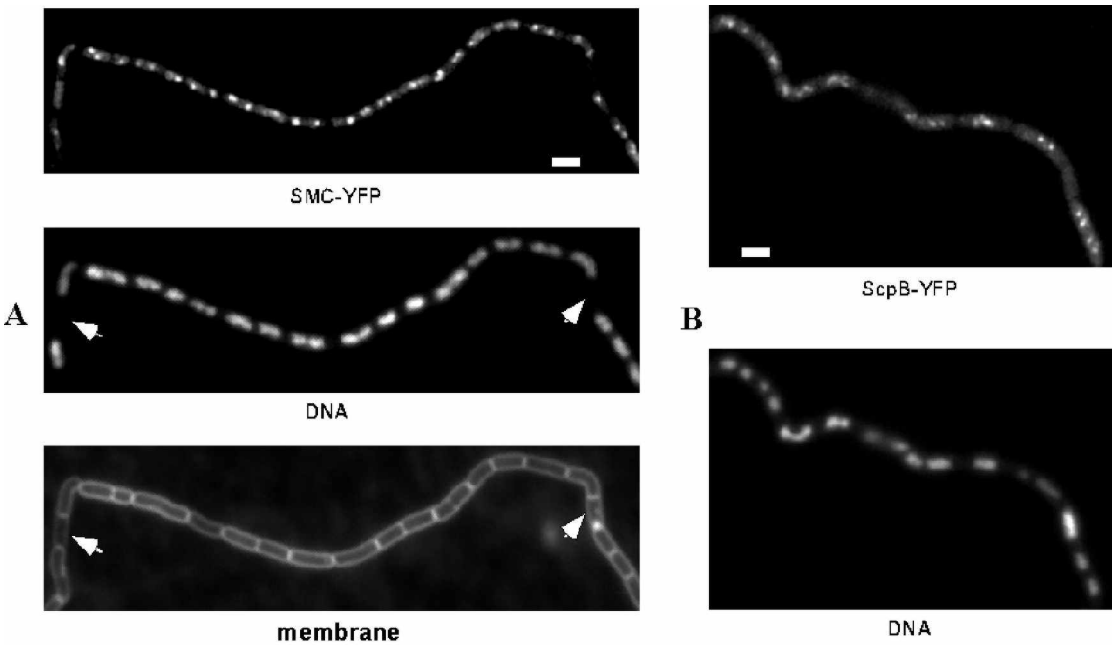


Fig. 27: Fluorescence images showing the effect of SMC overproduction on localization of (A) SMC-YFP in JM35 cells and (B) ScpB-YFP in JM36 cells.

Similar observations were made when SMC-His_6 was overproduced from an ectopic location on the chromosome, while a SMC-YFP fusion was driven by the original promoter at the *smc* locus in PG45 (*smc-yfp*, P*hyperspank, smc-*His_6 at *amyE*). These foci represent the condensed state of the nucleoid and also indicates that the whole SMC complex are retained at its specific location even upon overproduction and brings about global chromosome compaction from a defined position on the nucleoid.

3.13 Regulation of SMC

The rate of gene expression can be modulated in several ways, either through post-transcriptional mechanisms that affect mRNA half lives, translation initiation and/or translation progression (McAdams and Shapiro, 2003) or through translational or post-translational processes operating at the protein level, e.g. by action of proteases. SMC is found in an operon flanked by essential genes with *rncS* located upstream, which is involved in RNA processing (Herskovitz and Bechhofer, 2000) and *ftsY* located downstream and serving as the anchor for the bacterial signal

recognition particle (SRP). FtsY is not only involved in secretion of extracellular proteins, but also participates in proper localization of spore-forming proteins (Kakeshita *et al.*, 2000). In many cases, genes that are co-transcribed are involved in a common functional pathway, which however does not seem to be the case with *smc*. Within the *rncS* operon, all three genes have been shown to be regulated by a σ^A promoter situated upstream of *rnc* (Oguro *et al.*, 1996). In addition, a σ^k promoter sequence was mapped near the 3' end of *smc* that regulates the late stationary phase expression of FtsY (Kakeshita *et al.*, 2000).

Immunoblot and Immunofluorescence studies employing SMC antibodies have demonstrated the absence of SMC in stationary phase cells (Graumann *et al.*, 1998). In order to investigate if SMC is regulated at the transcriptional level, primer extension studies were carried out using total RNA extracts prepared from different growth stages of PY79 cultures (fig. 28B), employing a primer specifically located 150 bp downstream from the start codon of *smc* (fig. 28A). The results of primer extension experiments revealed the presence of a very strong extension signal corresponding to a transcript which was identified in all the growth stages (fig 28C). The size of this transcript corresponds to approx. 480 bp (distance of transcript signal to primer location) and the sequence upstream to the corresponding transcript signal possesses a weak similarity to the consensus of a typical σ^H recognition sequence (AGGA-15 bp-GAAT), although more experiments are required to confirm the existence an independent promoter for *smc* expression. It can of course not be excluded that the transcript points to a post-transcriptional modification site of the mRNA transcribed from the σ^A promoter upstream of *rncS*. The experiment showed the presence of a *smc* transcript in all the growth phases, which strongly suggests that SMC might be regulated at the stage of protein synthesis.

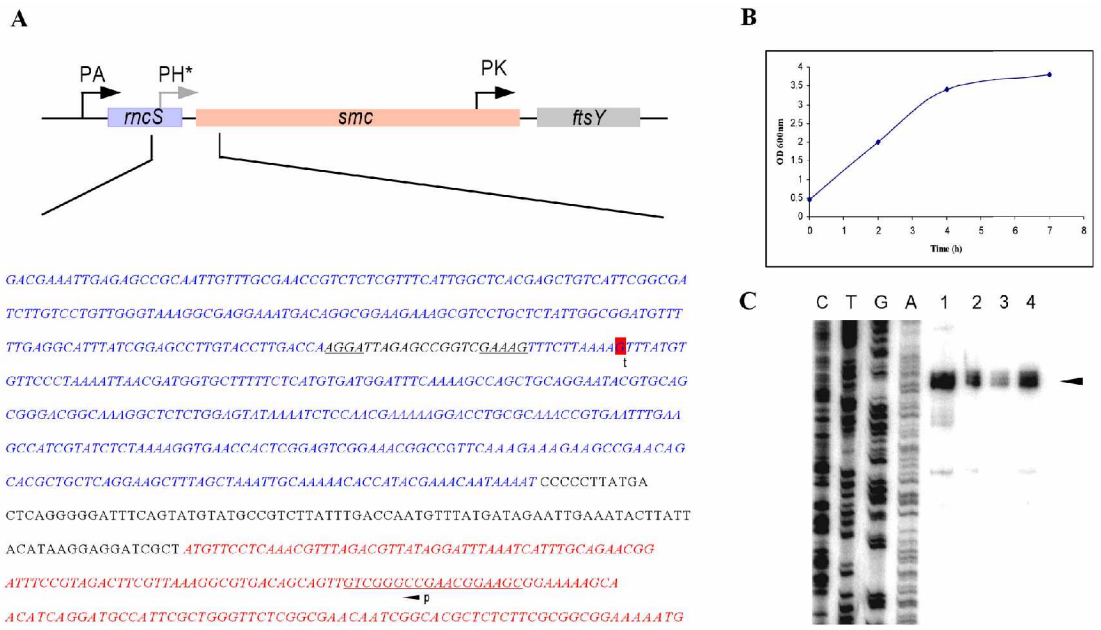


Fig. 28A: Schematic representation of *rnc* operon. The expression of *rncS*, *smc*, and *ftsY* are controlled by PA (σ^A promoter).The expression of *ftsY* in the stationary phase is regulated by the PK (σ^K promoter). PH is the putative promoter site mapped in this experiment from the primer extension analysis.
B: Growth curve of PY79 cells starting from mid exponential phase. The blue dots represent the time points when samples were withdrawn for analyses.
C: Primer extension analysis from total RNA extracts of samples from mid log (lane1), late exponential (lane 2), early stationary (lane 3) and late stationary phase cells (lane 4) (fig.28B). The DNA sequencing ladder to compare the size of the transcript signal (left 4 lanes reading CTGA). Arrow showing the strong transcript signal for *smc*. The sequence region of the *rnc* and *smc* in fig. 28A shows the primer 'p' used to map the *smc* transcript and the transcriptional initiation site 't', leading to the possible promoter sequence PH (black, underlined).

3.13.1 Growth phase dependent expression of SMC and ScpB

To monitor the SMC levels during different growth phases, cell extracts taken at various time points from the growing cultures (fig. 28B) were analyzed by western blotting utilizing SMC antibodies. The results showed the presence of SMC at the mid log phase, a gradually diminishing SMC amount at the onset and progression of the stationary phase, and its absence in the late stationary phase (fig. 29B, lanes 1- 4). The ScpB expression profile was also analyzed by using ScpB polyclonal antibodies (fig. 29C). Interestingly, the ScpB protein profile closely resembled that of SMC which further indicate that both proteins function together and thus might be regulated in a similar manner. Surprisingly, a second band of significantly higher size (~48kDa)

compared to that of ScpB was detected when the ScpB band began to disappear at the entry into the stationary phase. This band might be explained by a cross reaction of ScpB antibody with another protein synthesized at the later stage of growth. Alternatively, although unlikely, it cannot be excluded that this band might correspond to a modified variant of ScpB itself.

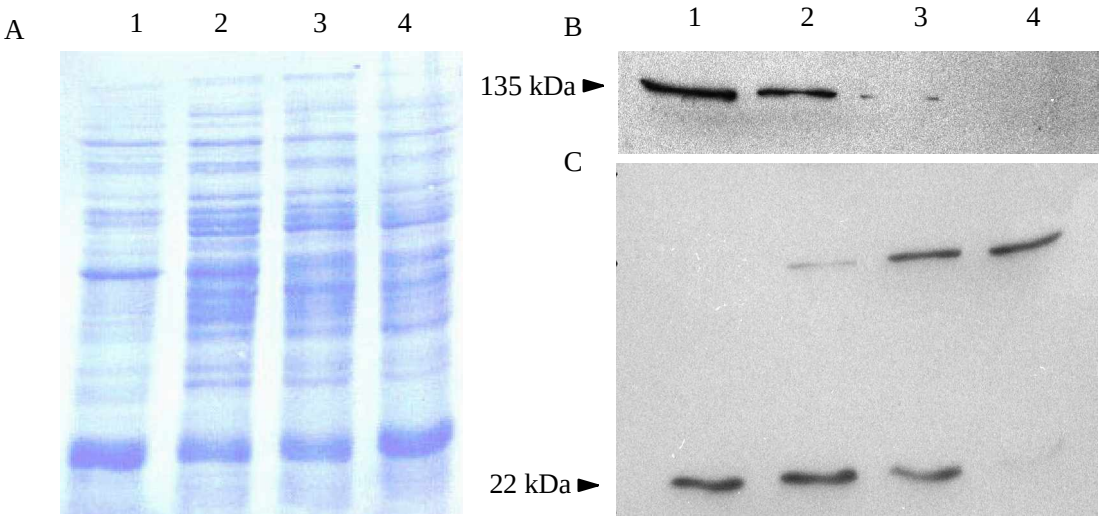


Fig. 29A: SDS-PAGE of the crude cell extracts showing the protein levels. Cells taken from various time points (as in fig. 28B), from mid-exponential phase to stationary phase. B: Western blot with SMC, and C: ScpB antibodies.

3.13.2 Stability of SMC

In order to check the stability of SMC during different growth phases, cultures were treated with chloramphenicol (Cm) to inhibit protein synthesis and the levels of SMC was compared to that of the untreated cultures. Cm (90 µg/mL final concentration) was added to growing cultures at the mid-exponential phase (OD_{600nm}= 0.45) and at the onset of the stationary phase (OD_{600nm}= 2.0). For determining the relative abundance of SMC during the cell cycle, samples were normalized, such that almost equal amounts of total protein were loaded in all lanes and the cellular SMC levels were measured by western analysis employing SMC antibodies. As shown in fig. 30 (lanes 1-4), in untreated cells the levels of SMC decreased during stationary growth. In cultures where Cm was added at mid-exponential growth phase, shown in fig. 30 (lanes 5-7), SMC was present even in the stationary phase cells, even though there were some decrease in their levels, it was not as significant compared to the untreated. In cultures where Cm was added at a later stage of growth, shown in fig. 30 (lanes 8 and 9), SMC levels were similar to the corresponding untreated cultures:

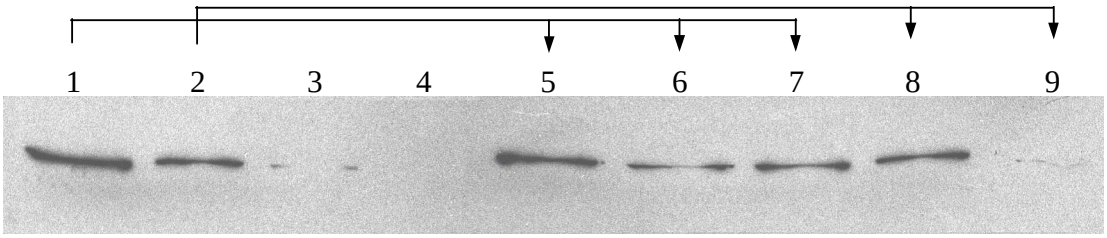


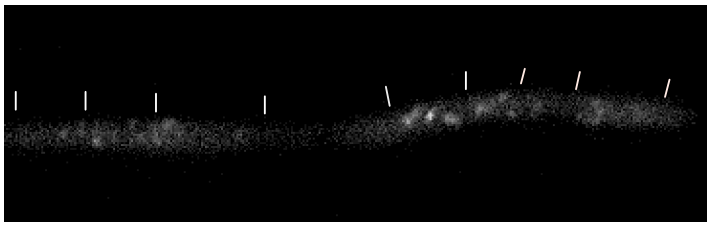
Fig. 30: Stability of SMC: Western blot analyses of cell extracts with SMC antibodies
Lanes 1-4: SMC profile of untreated cells, samples taken at time points similar to previous experiment (fig. 28B)
Lanes 5-6: SMC profile of cells treated with Cm at mid-exponential phase
Lane 5: 2 h after Cm treatment (OD_{600} 0.75)
Lane 6: 4 h after Cm treatment (OD_{600} 0.9)
Lane 7: 7 h after Cm treatment (OD_{600} 1.0)
Lanes 8 and 9: SMC profile of cells treated with Cm at late exponential phase
Lane 8: 1 h after Cm treatment (OD_{600} 2.5)
Lane 9: 5 h after Cm treatment (OD_{600} 3.2)

Together with the experiments described above, these results demonstrate that SMC is specifically degraded in a post-translational manner by a dedicated protease that is expressed exclusively at the transition to the stationary growth phase. In order to identify this protease, the experiments presented here could be repeated with available protease null mutants.

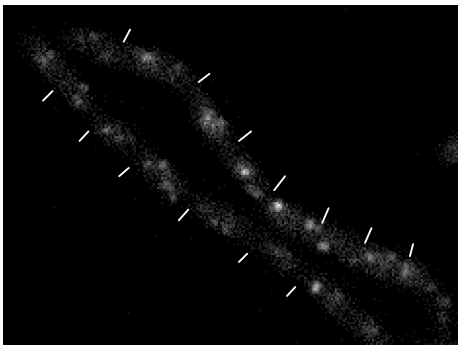
3.14 Involvement of SMC complex in repair

It is known that in eukaryotes SMC proteins like SMC5, SMC6, and Rad50, a member of SMC family play an important role in DNA repair (Introduction 1.4.2). In order to interrogate the involvement of *B. subtilis* SMC in repair, *smc* null mutants were tested for sensitivity to mitomycin C (MMC). MMC, is an antitumor agent isolated from *Streptomyces* cultures which is used in chemotherapy (Paz *et al.*, 1999). MMC acts as a bifunctional or trifunctional alkylating agent which cross-links DNA by formation of adducts to an extent proportional to its content of guanine and cytosine. The excision of adducts triggers the SOS response, a concerted induction of several DNA repair and recombination activities that has been studied in *E. coli*, where the response is controlled by the *lexA*-specified repressor (Wei *et al.*, 2001).

The strains PGΔ388, JM11, and JM13 carrying deletion of *smc*, *scpA*, and *scpB* genes, respectively, and the wild type PY79, were streaked on LB plates containing 0, 50, 100 and 200 ng/ml of MMC and were incubated at 23°C because of the temperature sensitivity of the SMC and ScpAB mutants. All four strains grew on LB plates without MMC. While the wild type cells grew on plates containing 50 and 100 ng/ml MMC, the deletion strains of *smc* and also its interaction partners *scpA* and *scpB* did not show any growth even on a plate with 50 ng/ml MMC. The sensitivity of the mutants towards MMC suggests a direct or an indirect involvement of SMC, ScpA, and ScpB in DNA repair. Furthermore, the localization patterns of SMC and ScpB were examined after the treatment of the cells with MMC (100 ng/ml) for 30 minutes. The MMC treated cells possessed a slightly decondensed nucleoid and anamalous foci:



SMC-YFP



ScpB-YFP

Fig. 31: Fluorescence microscopic images showing the effect of mitomycin on localization of SMC and ScpB in JM25 (*smc-yfp*) and JM9 (*scpB-yfp*) cells respectively. White bars represent the ends of the cell.

Under normal conditions there are 2-4 foci in the cell occupying the polar positions, whereas MMC-treated cells (200 ng/ml) showed foci distribution throughout the cellular space. Some cells contained two foci at the cell center rather than at the poles (fig. 31). These foci rearrangements after the MMC treatment might

indicate an incomplete process of recombination where the SMC/ScpA/ScpB complex might play a crucial role mediating the repair process.

3.15 Identification and examination of other SMC-like proteins in Bacillus subtilis

Since eukaryotic cells possess more than just one SMC-like protein, it was interesting to investigate for other SMC homologs in *B. subtilis*. A BLASTP search performed at the SubtiList server using SMC from *B. subtilis* as a query sequence revealed a number of homologs with the two most significant having E-values of $3 \cdot 10^{-12}$ and $6 \cdot 10^{-7}$. These corresponded to YirY (1130 a.a; MW 128.69 kDa) which was identified in the course of genome sequencing project as an SbcC homolog (Medina *et al.*, 1997) and RecN (576 a.a; MW 64.3 kDa) which is involved in DNA repair and genetic recombination (Alonso *et al.*, 1993).

In *B. subtilis*, YirY/SbcC is located in an operon flanked by *addA*, *addB* (ATP-dependent DNAses and analogs of RecBCD in *E. coli*), and *sbcD* genes upstream and a functionally unknown *yisB* downstream of it:

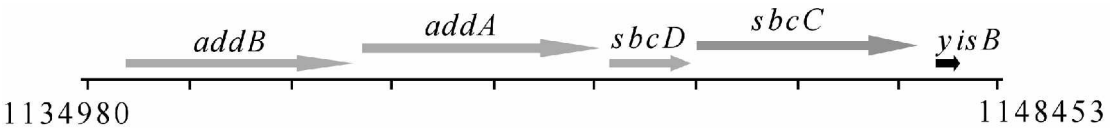


Fig. 32: Organization of *add* operon in *B. subtilis* genome.

The proteins AddAB and SbcCD are DNA associated and homologs of these proteins have been shown to play a role in recombination and repair in various organisms (Alonso *et al.*, 1993; Cromie *et al.*, 2001). The SbcC-SbcD complex in *E. coli* are similar to the eukaryotic Rad50-Mre11 complex involved in double strand break repair (Cromie *et al.*, 2001). Rad50 is a member of the SMC protein family possessing the conserved ABC ATPases and a N-terminal 'FKS' (or FRS) motif located near the Walker A site, conserved in most of the SMC proteins and also in SbcC and RecN (Cobbe and Heck, 2003).

3.15.1 Analysis of YirY/SbcC function

In order to experimentally probe the SbcC (YirY) function and its proposed involvement in DNA repair, a *sbcC* disruption strain JM42 (*sbcC*::pJQ43) was constructed by a Campbell integration of a plasmid carrying an internal fragment (~1 kb) of *sbcC*. The strain JM42 did not show any obvious phenotype and grew like the wild type under optimal growth conditions. To test for the role of SbcC in DNA repair, the strain JM42 was streaked on LB plates supplemented with MMC (that induces double stranded breaks). When compared to wild type cells that survived on the plate containing 50 and 100 ng/ml MMC, JM42 grew only poorly in the presence of 50 ng/ml of MMC and did not show any growth on a plate with 100 ng/ml of MMC confirming its role in the DNA damage related stress response.

To localize SbcC in cells and to observe the effect of DNA damage stress, SbcC was tagged to a fluorescent protein in a plasmid with a xylose promoter that assured the transcription of the down stream genes. The resulting strain JN6 (*sbcC-yfp*, P*xyl*) grew only in the presence of xylose but slowed down its growth when xylose was removed from the medium, indicating an important cellular role for *yisB*. When analyzed under the microscope, the JN6 cells showed low level fluorescence visible throughout the cell. 30 min after addition of MMC, nucleoids appeared slightly condensed and SbcC-YFP foci were detectable in 5% of the cells. After 2 h of induction with mitomycin, 1 or 2 foci were observed in 45% of the cells (fig. 33). These foci might be interpreted as locations where DNA repair takes place. In contrast to the foci formation of SbcC, SbcD-YFP localized through out the cell (data not shown).

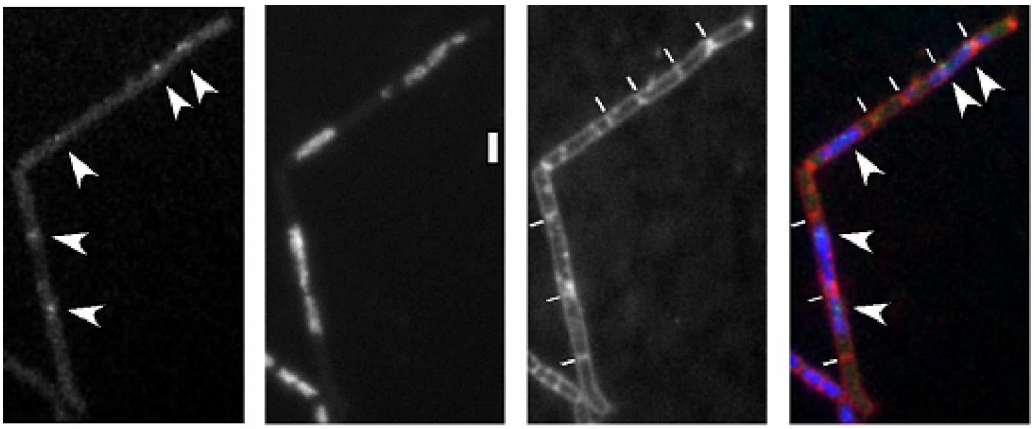


Fig. 33: Fluorescence microscopy of JN6 cells 2 h after addition of MMC. Cells were stained for DNA and membrane. Image to the extreme right shows the overlay of the images with membrane in red, DNA in blue and the SbcC-YFP foci in green showed by arrows.

RecN, the second SMC-like protein in *B. subtilis* that functions in association with RecF, RecL, RecO, RecR, and RecA proteins in homologous recombination during transformation (Alonso *et al.*, 1993), also showed foci formation upon induction with MMC. The RecN disruption mutant behaved similar to *sbcC* mutant on MMC plates (Kidane *et al*, in press).

3.15.2 Localization of AddAB

The *addA* and *addB* genes encode different subunits of the nuclease-helicase AddAB (also termed exonuclease V or RecBCD in *E. coli*) involved in initial stages of recombination (Haijema *et al.*, 1996). AddB was discussed as an interacting protein of ScpA, a subunit of SMC complex (Noirot P, 2003). If AddB and ScpA would interact, one would also expect them to colocalize. To verify this idea, the proteins AddA and AddB were C-terminally tagged to fluorescence proteins in plasmids with inducible promoters that assured the transcription of downstream genes upon integration into operon. Fluorescence microscopic investigations of strains JN7 (*addA-gfp,* P*xyl*) or JM41 (*addB-yfp,* P*spac*) did not show any foci formation like ScpA, instead a weak fluorescence was uniformly distributed through out the cell. The observed fluorescence increased between 30 min and 2 h after addition of MMC:

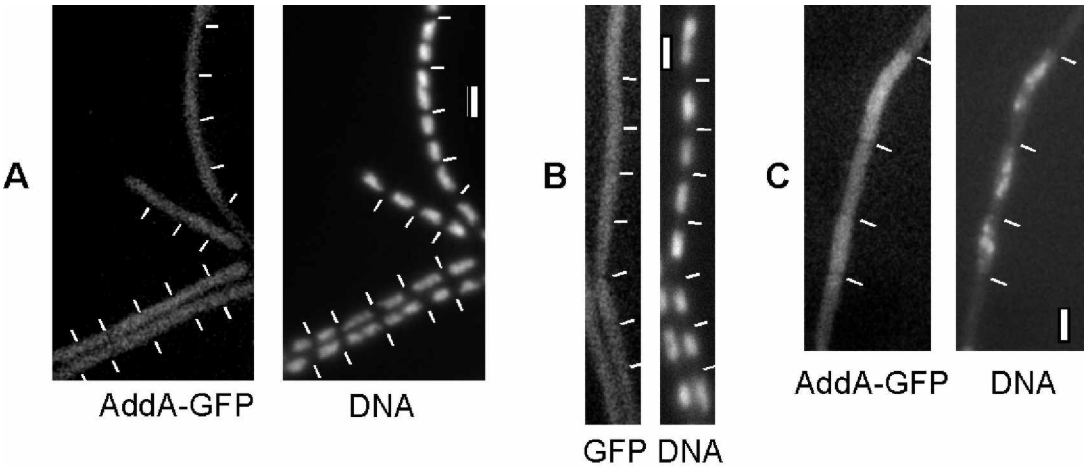


Fig. 34: Fluorescence microscopy of JN7 (*addA-gfp*) cells after MMC induction. A: untreated, B: after 1 h, C: after 2 h. White bars show the ends of the cell.

These observations show that proteins acting at specific sites on the DNA (i.e. DNA breaks) do not necessarily form foci after induction of breaks or are not necessarily associated with the nucleoid.

3.16 Topoisomerase IV - A chromosome segregator

So far, SMC has been thought to play a key role in chromosome segregation, however, the studies presented in this work show that SMC is more a condensing than a segregation factor. Other proteins that are also involved in chromosome segregation are topoisomerase IV (Huang *et al.*, 1998), CodV and RipX (Sciochetti *et al.*, 1999). Topoisomerase IV is primarily responsible for unlinking the catenates that are generated at the end of replication when the two replication forks converge (Zechiedrich and Cozzarelli, 1995). Topoisomerase IV is a heterodimeric enzyme belonging to the type II topoisomerase family and is formed of two subunits- ParC and ParE, similar to the ParC and ParE of *E. coli.* Temperature sensitive mutants of topoisomerase IV in *E. coli* and *Salmonella typhimurium* formed long filamentous cells defective in nucleoid segregation that divided frequently to produce anucleate cells (Luttinger *et al.*, 1991). *B. subtilis* also showed a similar phenotype at the non-permissive temperature (Huang *et al.*, 1998). Topoisomerase IV mutants of *C. crescentus* are highly pinched at multiple sites, a typical cell separation phenotype,

but the cells do not divide to produce cells lacking DNA (Ward and Newton, 1997). Similar to overproduction of gyrase, ParC and ParE showed enhanced relaxation activity of supercoiled plasmid and were able to suppress a *topA* mutation (Kato *et al.*, 1990).

In *E. coli*, ParC is associated with the replication machinery and was seen to form foci that colocalized with DNA polymerase III while ParE was not present at the replication factory but localized as foci at the poles and at the cell center (Espeli *et al.*, 2003). In *B. subtilis*, ParE-GFP was shown to localize uniformly throughout the cell in actively growing cells, while the ParC-GFP fusion protein showed bipolar localization (Huang *et al.*, 1998) in a manner which looked similar to SMC.

In order to verify whether ParC and SMC colocalize, the protein ParC was tagged to YFP to yield the strain JM43 (*parC-yfp*). Contrary to what was reported previously (Huang *et al.*, 1998), ParC-YFP localized throughout the nucleoids and the fluorescence distribution was similar to the DAPI stain:

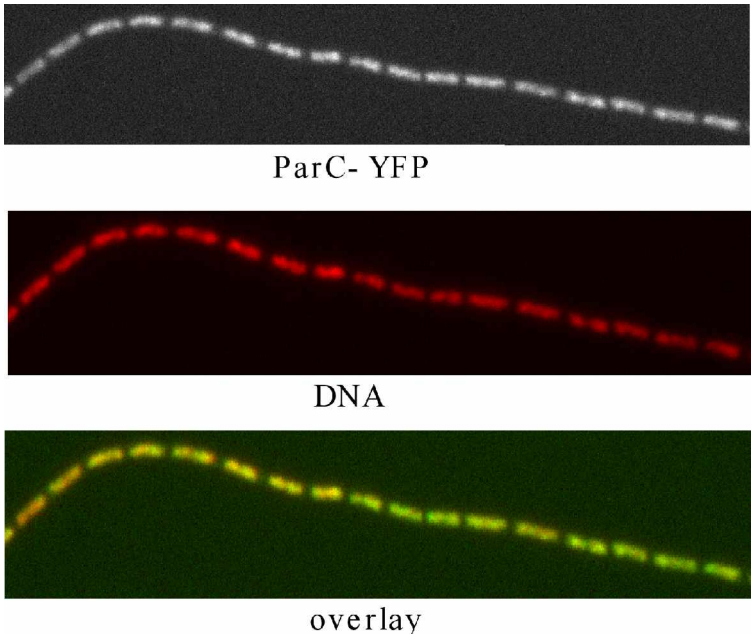


Fig. 35: Fluorescence microscopy of JM43 cells (A) ParC-YFP (B) DNA stained with DAPI, (C) overlay of image (A) and (B).

Thus, ParC unlike SMC does not act on a specific site but bind throughout the nucleoids. In the meantime, another work group presented results demonstrating that ParE-GFP fusion was present on the nucleoid similar to that of ParC (Meile C.J, 2003), suggesting that they function together in maintaining the DNA topology.

In *E. coli*, a *topA* mutant was found to be one of the suppressors of *mukB* deletion strain (Sawitzke and Austin, 2000). It was also reported that mutations in *topA* could be rescued by overproduction of ParC and ParE or the gyrases (Kato *et al.*, 1990). In *B. subtilis*, the partitioning defect of the *smc* null mutant was significantly suppressed by the depletion of topA (Lindow *et al.*, 2002a). These observations led to the question whether the depletion of topoisomerase IV could rescue the segregation defect of *smc* null mutant. To answer this, strain JM45 (P*hyperspank-parE*) was constructed in which ParE and ParC expression was controlled by an IPTG-inducible *hyperspac* promoter. Initial experiments with JM45 cells upon induction with 1 mM IPTG showed more condensed nucleoids. Further analyses of this strain could not be carried out due to the paucity of time. However, the effect of ParC and ParE overproduction or depletion on *smc* phenotype could give us important insight towards a better understanding of the networked action of proteins involved in chromosome organization.

4. Discussion

In this work, a novel protein complex termed the SMC complex has been identified and characterized. The evolutionary conserved SMC protein forms a complex with two proteins, ScpA and ScpB, formerly known as YpuG and YpuH respectively, which have been analyzed and assigned a new role in chromosome condensation. The deletion/disruption of these genes was demonstrated to affect chromosome organization and each null mutant showed a phenotype similar to that of a *smc* deletion strain, with temperature sensitive slow growth and decondensed nucleoids showing a strong segregation defect (Britton *et al.*, 1998; Graumann *et al.*, 1998; Moriya *et al.*, 1998). Depletion of *smc* in the *scpA* or *scpB* deletion strains, or deletion of both *scpA* and *scpB*, also resulted in the same phenotype, indicating that SMC, ScpA, and ScpB function in the same pathway for chromosome condensation and thus belong to the same epistatic group. ScpA and ScpB form a new family of proteins that is conserved in most of the eubacterial and archaeal species that possess a SMC homolog. ScpA also shows homology to the eukaryotic SMC-interacting protein Scc1 and has lately been assigned as a member of the kleisin protein family (Schleiffer *et al.*, 2003). The resemblance of ScpA to Scc1 and the presence of a HTH motif in ScpB provides us with some insights about how these proteins might be associated to function with SMC. Because biochemical and structural studies of these proteins are not available, at the moment, several possible roles can be assigned to them ranging from a cofactor in the mechanical functioning of the SMC molecule to a regulator of SMC protein that directs the SMC complex to bind DNA in a specific manner by mediating its interaction with other proteins.

A key observation in this work has been the finding that SMC, ScpA, and ScpB form discrete foci on the DNA, unlike other chromosome-associated proteins that are bound throughout the nucleoids. This striking finding is a novel pattern of localization and suggests that SMC, ScpA, and ScpB affect chromosome compaction and segregation from a defined region on the nucleoids and the foci represented the functional SMC complex and thus the condensed state of the nucleoid. Attempts to localize SMC was previously made by (Britton *et al.*, 1998), who observed discrete foci with partially functional SMC-GFP fusion. Immunolabeling with SMC antibodies also showed bipolar foci which were independent of nucleoid as reported

by (Graumann *et al.*, 1998). Similar patterns for SMC and ScpB were later reported by (Lindow *et al.*, 2002b) which was consistent with the observations described here. The SMC counterpart in *E. coli*, MukB also showed foci formation (den Blaauwen *et al.*, 2001). In *Caulobacter*, SMC showed 2-3 foci in swarmer cells that do not replicate their DNA, while stalked cells which actively replicate DNA possessed 3-4 foci randomly distributed in the cell (Jensen and Shapiro, 2003). The observed localization pattern of the SMC/ScpA/ScpB complex in form of foci within the nucleoid might indicate a recruitment of the proteins to a specific nucleoid structure. In the eukaryote *Saccharomyces cerevisiae*, the SMC1 and SMC2 was demonstrated to bind to secondary structures, preferentially to AT rich sequences (Akhmedov *et al.*, 1998; Gregson *et al.*, 2002; Laloraya *et al.*, 2000) and SMC4p-GFP localized to rDNA regions (Freeman *et al.*, 2000). Surprisingly, *B. subtilis* SMC has been shown to bind to DNA in a non-specific manner *in vivo* (Lindow *et al.*, 2002b) and *in vitro* (Volkov *et al.*, 2003). In bacteria, it is known that chromosomal DNA is divided into topologically independent domains (Sinden and Pettijohn, 1981); (Drlica, 1986). Therefore, it can be speculated that the presence of repetitive patches of T/AT-rich sequences within the *B. subtilis* genome might be of some significance in providing a defined chromosomal structure that could represent a binding location for the SMC complex. In fact, as shown in this work, the observed specific subcellular localization of the SMC complex requires a well-defined chromosome topology since SMC foci formation was abolished upon addition of the gyrase inhibitor novobiocin, which disturbs the DNA supercoiling status.

The bipolar localization pattern of SMC, ScpA, and ScpB suggests that all three proteins function together in a complex, especially since the interaction was also confirmed *in vivo* using FRET technique. The SMC strep tag affinity purification and immunoprecipitation experiments also confirmed that SMC appears to have ScpA and ScpB as interacting partners. The formation of a ternary complex of SMC/ScpA/ScpB has also been biochemically confirmed using surface plasmon resonance technique with purified proteins. ScpA was able to bind to SMC head domains only in the presence of ScpB (Volkov *et al.*, 2003), although the binding was not influenced by DNA in this case. In *E. coli*, the subunits of MukB complex, MukE and MukF, were shown to bind to the C-terminus of MukB (Yamazoe *et al.*, 1999). In yeast, it has been shown that a cohesins non-SMC subunit Scc1 binds to the SMC head domain (Yoshimura *et al.*, 2002) by bridging the head regions of a SMC3 and SMC1

heterodimer (Gruber *et al.*, 2003). The interactions between SMC, ScpA, and ScpB were also independently confirmed by yeast two hybrid screenings reported by D. Ehrlich's lab (Soppa *et al.*, 2002).

SMC appears to act as a true condensing factor, since the overproduction of SMC alone led to more condensed nucleoids (Volkov *et al.*, 2003), while overproduction of ScpA or ScpB did not affect the nucleoid structure (unpublished data). This infers that SMC is the major condensing factor and that ScpA/ScpB act as cofactors in SMC function. The overproduction of SMC did not disturb the bipolar localization pattern of the SMC complex, but the fluorescence was retained as brighter foci in the cell. These findings support the idea that the foci represent chromosome condensation centers, where SMC actively condenses the nucleoid from a single specific position.

So far, a few biochemical studies have tried to explain how SMC might function at the mechanistic level. Hirano *et al.*, studied biochemical properties of SMC by constructing various mutations in the hinge domain of the SMC molecule as well as in its ATPase cassette. More specifically, in their studies focusing on the ATPase function of SMC, they introduced point mutations in (i) the Walker A motif changing the lysine at position 37 to isoleucine (K37I), (ii) the Walker B motif changing aspartic acid 1117 to alanine (D1117A), and (iii) the signature C motif where the conserved serine 1090 residue was mutated to arginine (S1090R). While the Walker A and Walker B mutants were unable to bind ATP, the C motif mutant could bind ATP but was unable to hydrolyse it. All the three mutants showed no DNA binding activity, suggesting the importance of the ATPase region in the DNA binding function (Hirano *et al.*, 2001). Attempts to study the behaviour of ATPase mutants *in vivo* were initiated during this work. Point mutations identical to those described above on an YFP plasmid carrying a C-terminal or N-terminal SMC fragment. The constructed plasmids were sequenced for confirmation of the presence of the desired mutation and were then transformed into PY79 cells to yield JM37 (K37I, P*hyperspac-smc-yfp*), JM38 (D1155G, *smc-yfp),* and JM39 (S1090R, *smc-yfp*). Surprisingly, all the three mutants did not show any growth defect and grew similar to wildtype cells. Microscopic observations, however, revealed 10-15% of the cells with anamalous nucleoids where the fluorescence was distributed through out the cell. Although the expression of SMC as mutant protein *in vivo* needs to be confirmed, these preliminary observations suggest that the specific foci formation is strictly

dependent on DNA binding, which in turn is facilitated by ATPase activity. However, it cannot be ruled out that the ATPase activity might also be necessary for some sort of mechanical action of SMC during its role in chromosome condensation. Furthermore, it remains to be explained why strains harbouring mutated ATPase SMC variants grow like the wild-type. This discovery might be a first clue to that the observed growth defect and the aberrant nucleoid structure of *smc* null mutants might be a result of at least two different, i.e. independent effects of SMC's cellular functions.

Several studies have tried to address the role of ATP binding and hydrolysis in SMC proteins. In Rad50, a member of SMC family, ATP binding and hydrolysis are connected to DNA binding and end processing by Mre11 (Hopfner *et al.*, 2000). In *B. subtilis* SMC, the addition of DNA stimulated the ATPase activity by 2- to 4-fold and also its DNA binding (Hirano *et al.*, 2001; Yoshimura *et al.*, 2002). Studies using atomic force microscopy (AFM) have shown that a single condensin complex is able to trap two positive supercoils of DNA in an ATP hydrolysis-dependent manner (Yoshimura *et al.*, 2002). Based on biochemical results, Haering *et al* proposed that cohesin holds the two sister chromatids together by 'embracing' them within its arms (Haering *et al.*, 2002) by forming a closed ring-like structure. Biochemical studies with SMC proteins and DNA using surface plasmon resonance supported this model and showed that SMC does not bind to DNA through its head domains alone, but requires the coiled coil regions, therefore the entire SMC molecule takes part in DNA binding (Hirano and Hirano, 2002; Volkov *et al.*, 2003). It was speculated that ATPase activity is required for initial loading of SMC on DNA, whereby ATP binding either induces the formation of the closed loops or is required for temporary opening of the rings in the loading process (Hopfner, 2003). Recent reports on eukaryotic cohesin (SMC1 and SMC3) have shown that ATP hydrolysis is required for its stable association with chromosomes (Arumugam *et al.*, 2003).

Nucleoids undergo several conformational changes during cell growth. At the onset of stationary phase and of sporulation, the compact central nucleoid transforms to an elongated axial filament and the chromosome is stored as a condensed donut structure in spores (Pogliano *et al.*, 1995). The SMC complex is responsible for the compact chromosome organization in rapidly replicating cells. As demonstrated in this work, at the onset of stationary phase, *B. subtilis* cells contain progressingly reduced amounts of SMC which is reflected by a decondensed nucleoid. In spores, the

chromosome has to be accommodated into a much smaller space. This extreme compaction/conformational change is brought about by HBsu and SASPs that are nucleoid-binding proteins abundantly expressed in spores (Ross and Setlow, 2000). Even in *E. coli*, during the early stationary growth phase, average fluorescence of MukB-GFP foci decreased (Ohsumi *et al.*, 2001). Moreover, the levels of major nucleoid associated proteins, Fis, Hfq, StpA, H-NS, HU, and DnaA are reduced in the stationary phase, while there is an increased level of IHF, Dps and other DNA-binding proteins (Ali Azam *et al.*, 1999). Such variation in the levels of DNA binding proteins affect the DNA topology, and in turn change the transcriptional profile, which is required for adaptation of the bacterium to the changed stationary phase environment.

During the transition state of exponential to stationary phase, *B. subtilis* changes its protein expression profile and induces various extracellular proteases and other degradative enzymes to maximize utilization of nutrients and also by altering its metabolic pathways (Phillips and Strauch, 2002). SMC might be regulated at the transcriptional or at the protein level due to the effect of stationary phase-induced proteases, since the primer extension studies showed the presence of *smc* transcript throughout the various growth phases. Instability of SMC at stationary phase could also explain the observation that overproduction of SMC during the stationary phase had no effect on chromosome condensation (P. Graumann, personal communication).

Functionally, SMC might act as a coupling factor between replication and chromosomal condensation. SMC stability assays showed that SMC activity is essential only in the actively replicating/growing cells. Also the movement of the SMC foci was dependent on active replication. SMC is necessary for the proper alignment of chromosomes in the cell, since the *smc* mutants showed aberrant location of origin regions (Graumann, 2000). The process of sporulation is initiated when the origins are at the poles and chromosomes extend into an axial filament. A proper positioning of the *Ori* region is disturbed in the absence of SMC which is reflected in its spore forming deficiency (only 0.4% cells sporulate in *smc* null strain when compared to 70% in wildtype cells). Recent observations with MreB and Mbl mutants showed disturbances in chromosome alignment and mislocalization of the SMC complex foci (Soufo and Graumann, 2003). MreB and Mbl are actin-like skeletal proteins in *B. subtilis*, involved in cell shape determination and localize as helical filaments around the circumference of the rod-shaped cells *in vivo* (Jones *et*

al., 2001). MreB and Mbl could mediate the interaction with certain regions of chromosomal DNA to the membrane, thus orienting the chromosome in the cell. These observations support the long-standing notion of DNA being attached to the membrane, in this case mediated by MreB and Mbl. One of the future aspects would be to see if MreB and Mbl mediate the interaction of SMC to DNA.

The hypersensitivity of SMC or ScpA/B mutants to Mitomycin C (MMC) and the disturbance of the localization pattern in response to MMC treatment demonstrates their role in DNA repair, either by acting as a cohesion factor through bringing together the sister chromosomes to repair the damaged strand by homologous recombination or making the damaged strands accessible for the repair proteins. Other proteins that showed similarity to SMC, SbcC and the RecN in *B. subtilis* are also crucial for cell function during DNA damage stress. The localization as 'foci' in these proteins infers that they have common mode of DNA binding while performing their respective functions. So far, no data have been available on the *in vivo* role of proteins involved in DSB repair, and the findings in this work provide a base to answer further questions. It will be interesting to attempt colocalization of SMC and RecN or SbcC, which form repair centers (Kidane *et al*, in press), and attempt to co-immunoprecipitate to detect any possible protein/protein interactions. Also in this work, the discovery that *yisB* is important for growth has opened a new prospects for further investigation and our lab has already discovered that YisB possesses a HNH motif typical for endonulceases and indeed shows DNA endonuclease activity *in vitro* (V. Kaiser, personal communication).

Model for SMC function

The results acquired during this study could be combined into a model schematically shown in fig.37. The SMC complex can be imagined to possess a ring conformation that can open and close. The closed form mediated by the binding of ScpA/ScpB (fig37A) and the ring opening caused by ATP hydrolysis, DNA binding or proteolysis. The cycle of opening and closing of the ring facilitates the traping of more and more DNA, leading to the DNA condensation process. The foci formation of SMC complex proteins can be explained by the aggregation of the SMC proteins in the form of a 'rosette' by protein-protein interactions mediated by the ScpA/B

proteins and DNA topology (fig. 37B). The condensation foci representing several SMC complexes were seen close to the replication foci in smaller cells. The bidirectional movement of the condensing foci followed after the separation of the origins towards the poles. It was reported that SMC does not influence the bipolar separation of origins (Graumann, 2000), so one can surmise the involvement of an unknown motor-like protein in the bidirectional movement of the origins and the condensing foci, one possible candidate would be MreB. In young cells, the SMC complex is initially loaded at the cell center near the replication machinery, which is supported by the observation that SMC foci were at the cell center upon inhibition of replication. When replication commences, the SMC complex brings together loops of the newly replicated chromosomes and compacts them. As replication progresses, the extrusion force of replicating chromosome or an additional mechanism that directs the replicated origins also drives the condensing foci outwards close to the poles. Thus the progressive condensation of chromosomes by the SMC complex further eases the segregation of chromosomes.

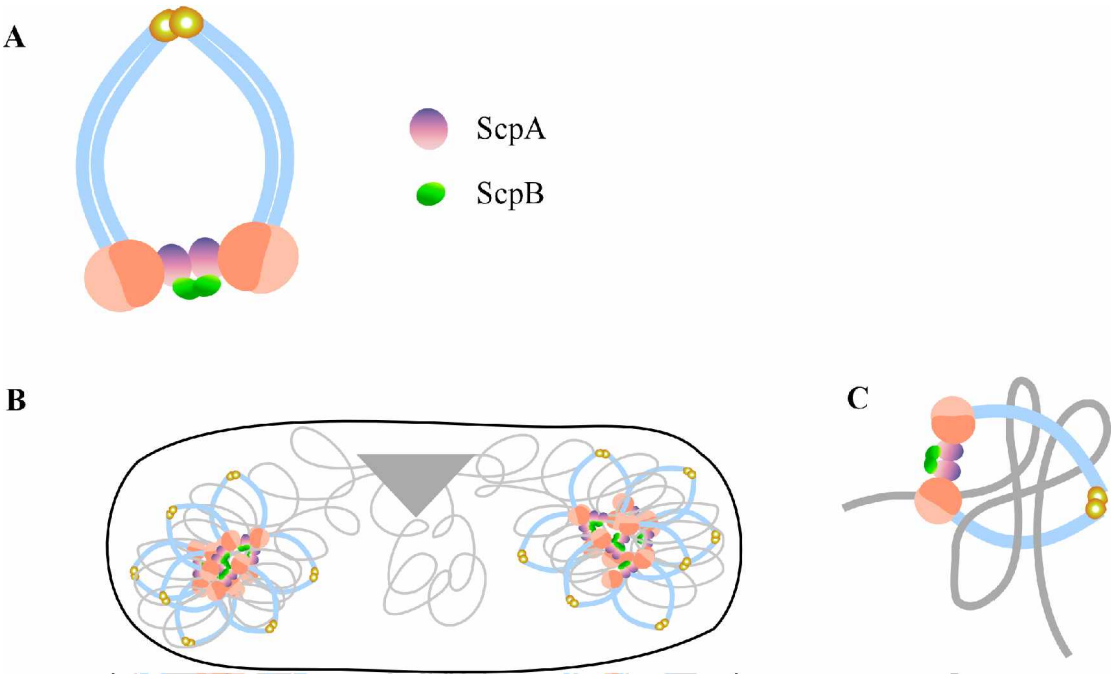


Fig 37: Models representing, A: SMC complex, B: Aggregation of SMC complex in the replicating cell. DNA polymerase (grey triangle) replicating DNA (grey lines) and C: Mode of DNA binding by SMC complex.

The existence of SMC and SMC-like proteins in prokaryotes and eukaryotes shows that SMC proteins have an ancient origin, reflecting their fundamental role in chromosome dynamics. The genetic and microscopic studies in *B. subtilis* SMC has given an insight to the *in vivo* functioning of prokaryotic SMC. Its mechanism of action and its coordination with various proteins, involved in DNA topology, replication and repair needs to be addressed in the future. Investigation of suppressors to SMC mutant might unravel the whole network of regulatory genes involved in chromosome organization. In eukaryotes, the SMC protein family has been of special relevance, since its members play a central role in several cell cycle regulatory networks, and hence they have been proposed as a promising target for therapeutic drugs affecting cell proliferation (Strunnikov, 2003). Consequently, even in bacteria, disruption of the SMC complex formation might represent a promising drug target, especially since ScpA and ScpB are not present in eukaryotic cells, but vital for the function of the complex.

4.1 Fluorescence microscopy – changing the view of prokaryotes

Unlike eukaryotes that contain separated compartments designed to fulfil specialized cellular functions, bacterial cells have long been thought of resembling a more or less simple bag in which the proteins freely diffuse. However, in recent years, with the help of advanced microscopic techniques and the knowledge of entire genome sequences and characterization of unknown proteins, it has become clear that bacteria are precursors of highly evolved eukaryotes and therefore possess a compartmentalized cell architecture as well, in which even cytosolic proteins carry out their function(s) at well-defined places by interactions with specific partners.

Intracellular distribution of proteins

Fluorescence microscopy has proved as a valuable *in vivo* tool to spot proteins and provide us with a better knowledge about the probable functioning of these proteins in the cell.

Proteins associated with the nucleoid that has been observed so far, showed two kinds of localization patterns, either they are uniformly distributed throughout the nucleoids or they form specific foci on the DNA. The major DNA-binding protein HBsu and the type II topoisomerase proteins GyrA and GyrB localized all over on the nucleoid (Huang *et al.*, 1998), similar pattern was observed for ParC (this work) and ParE (Meile C.J, 2003). DnaB and Spo0J are the proteins that form foci, bind to the origin region (Lewis and Errington, 1997). SMC, ScpA, ScpB proteins that also localized as bipolar foci, might bind to specific topological structures (this work). Various DNA repair proteins like the SbcC, RecA, RecN, RecO, RecF formed foci upon DNA damage induction (Kidane et al, in press and submitted). These repair foci might represent the repair centers, where the damaged DNA might be recruited for repair. UvrA, a base excitation repair protein, localizes throughout the nucleoid, indicating that the protein probably constantly scans the genome searching for lesions in the DNA (Smith *et al.*, 2002). PolA, (DNA polymerase I involved in replication and repair) localized throughout the nucleoid (Kidane et al., submitted), while AddAB, required for homologous recombination, did not show any specific localization but was present throughout the cell (this work). Several different proteins constituting the DNA replication machinery localized in a single focus located at about the mid-cell in actively growing *B. subtilis* cells.

The septal protein FtsZ, assembles as a ring at the mid cell in actively growing cells and repositions itself near the pole through a spiral intermediate during sporulation (Lutkenhaus, 2002), thus assigning its role as a major divisome protein. Other division proteins DivIVA, MinC and MinD localize to the poles (Marston and Errington, 1999b), whereas the Min proteins in *E. coli* (MinC, D, E), are organized into extended membrane-associated spiral structures that wind around the cell between the two poles, preventing the formation of the division septum by FtsZ at the poles (Shih *et al.*, 2003). Soj, which is distantly related to MinD but is involved in chromosome segregation and transcriptional regulation, also undergoes dynamic, co-operative movement (Marston and Errington, 1999a). Bacterial cells also possess cytoskeletal proteins- MreB and Mbl that are homologs of actin and essential for cell shape in bacilli. MreB and Mbl were shown to localize as spirals or helical filaments along the long axis of the cell in *E. coli* and *B. subtilis* (Jones *et al.*, 2001). It is still unclear how most of the above mentioned proteins achieve their specific localization within the cell.

The localization of proteins associated with ribosomes and RNA provided evidence of existence of transcription and translational compartmentalization in bacteria. The RNA polymerase localized in concentrated regions on the nucleoid called transcription foci corresponding to the sites of rRNA synthesis, and ribosomes were present in the cytoplasmic spaces surrounding the nucleoid (Davies and Lewis, 2003; Lewis *et al.*, 2000). Cold shock proteins (CSP) that are induced in the cells upon temperature downshift are thought to bind to the nascent mRNA preventing the secondary structure formation (Graumann *et al.*, 1997) also colocalized with ribosomes (Weber *et al.*, 2001). A part of this work has dealt with this aspect (see appendix, Pg. 103). Observing the localization of ribosomes, CspB, and DNA simultaneously in the cell, has shown that CspB might mediate coupling of transcription and translation, because it was seen to overlap between the peripheries of nucleoid and ribosomes.

In conclusion, the era of fluorescence microscopic technique has given a new approach to characterize cellular proteins in three dimensional context of the cell. The release of the *Bacillus* genome sequence in 1998 showed that out of 4225 coding genes, approx. 2000 represent yet uncharacterized genes encoding proteins of unknown function (called ‘y’ genes). The systematic characterization of these ‘y’ genes will surely provide a better knowledge of the organism. Together with genetic, biochemical and fluorescence microscopic techniques and with the anticipated further advances of these techniques one can expect additional important applications and discoveries that will give us a more detailed insight into the microbial cell.

5. Appendix

Specific polar localization of ribosomes in Bacillus subtilis depends on active transcription

http://www.nature.com/cgi-taf/DynaPage.taf?file=/embor/journal/v2/n8/full/embor360.html&filetype=pdf

5.2.1 Strains used in this work

Table 13

B. subtilis strains	genotype	references
EP58	*smc::kan*, P*spac-smc* at *amy* locus	Gift from R. Losick's lab
PGΔ388	*smc::kan*	(Graumann *et al.*, 1998)
PG25	*lacI-cfp* at *thr* locus	(Lemon *et al.*, 2001)
PG26	*lacO-cassette at359°, lacI cfp* at *thr* locus	P. Graumann
PG27	*scpB*-yfp, *lacO-cassette at359°, lacI cfp* at *thr* locus	P. Graumann
PG28	*dnaX-cfp*	(Lemon and Grossman, 2001)
AG1468	*spo0J::spec*	(Ireton *et al.*, 1994)
PL412	s*poIIIE::spec*	Gift from R. Losick's lab
KL210	dnaBts	Gift from A. Grossman's lab
MW2	*cspB-gfp*	M. Weber
PG31	*ypuI::tet*	P. Graumann
PG29	*scpA-yfp, dnaX-cfp*	P. Graumann
PG30	*scpB*-yfp, *dnaX-cfp*	P. Graumann
PG33	*scpB-yfp, smc::kan*, P*spac-smc* at *amy* locus	P. Graumann
PG34	*scpA-yfp, spo0J::spec*	P. Graumann
PG35	*scpB-yfp, spo0J::spec*	P. Graumann
PG36	*ypuI::tet,* s*poIIIE::spec*	P. Graumann
PG37	*scpA::tet, spoIIIE::spec*	P. Graumann
PG38	*scpB::tet, spoIIIE::spec*	P. Graumann
PG39	*scpB::tet, spo0J::spec*	P. Graumann
PG40	*scpB::tet*, P*xyl-scpB-cfp* at *amy* locus	P. Graumann
PG41	*scpB::tet*, P*xyl-scpB-cfp* at *amy* locus, *scpA-yfp*	P. Graumann
PG43	*scpB::tet, smc::kan*, P*spac-smc* at *amy* locus	P. Graumann

5.2.2 List of plasmids and strains constructed in this work

E. coli plasmids constructed for general use:

Table 14a

Modified plasmid	Cloning description (Plasmid/primers used)	Cloning sites used	Original plasmid	Unique cloning sites
pBsk Kan	Subcloned *kan* from pDG1	*Hind*III-*Eco*RV	pBskII(+)	*Acc*I, *Apa*I, *Cla*I, *Eco*RI, *Kpn*I, *Hinc*II, *Pst*I, *Sal*I, *Sph*I, *Xho*I- *Bam*HI, *Sac*I, *Spe*I
pBsk tet	Subcloned *tet* from pDG1515	*Bam*HI, *Hind*III	pBskII(+)	*Bam*HI, *Not*I, *Pst*I, *Sac*I, *Sma*I, *Spe*I, *Xba*I, *Xma*I-*Apa*I, *Bst*EII, *Eco*RI, *Hind*III, *Kpn*I, *Sal*I, *Sma*I, *Xho*I
pBsk Mls	Subcloned *erm* from pDG646	*Hind*III, *Cla*I	pBskII(+)	*Eco*RI, *Eco*RV, *Hind*III, *Not*I, *Sma*I, *Spe*I, *Sph*I, *Xma*I-*Apa*I, *Cla*I, *Kpn*I, *Xho*I
pBsk Cm	Subcloned *cat* from pΔTE (K. Eppelmann)	*Bam*HI, *Pst*I	pBskII(+)	*Sac*I, *Not*I, *Xba*I, *Spe*I, *Bam*HI-*Sph*I, *Pst*I, *Eco*RV, *Hind*III, *Cla*I, *Xho*I, *Apa*I, *Kpn*I,
p1164YFP/CFP	Subcloned *yfp/cfp* from pDG1187/1186	*Apa*I, *Spe*I	pSG1164	*Kpn*I, *Apa*I, *Xho*I, *Cla*I, *Eco*RV, *Eco*RI, *Pst*I
p1151 strep	Strep sequence from (162, 164) Primer annealing cloning method	*Eco*RI, *Spe*I	pSG1151	*Kpn*I, *Apa*I, *Xho*I, *Cla*I, *Eco*RV, *Eco*RI, *Pst*I
PMutinYFP/CFPmcs	*mcs* (228, 229) Primer annealing cloning method	*Hind*III, *Xma*III	pMutin-YFP/CFP	*Hind*III, *Sal*I, *Kpn*I, *Cla*I, *Bgl*II, *Xma*III
pJQhyperspank	Subcloned P*hyperspank* from pDr111	*Hind*III, *Eco*RI	pJQ43	*Hind*III, *Sal*I, *Nhe*I, *Sph*I

Bacillus subtilis strains constructed in this work:

Table 14b

B. subtilis Strain	Genotype	Modified *E. coli* plasmid transformed in *B. subtilis*	*E. coli* plasmid/ Strain	Cloning details	Primers used (table 15)
JM1	*rplA-bfp, spec*^{r}	pBrsp	pBFP2 (Clontech) pDG1726 (BGSC)	Cloned *spec*^{r} from pDG1726 at *Hind*III-*Bam*HI, and PCR product of C-terminal *rplA* at *Bam*HI-*Kpn*I of pBFP2	3, 4
JM3	*rplA-bfp, csp-gfp, spec*^{r}		MW2 (M. Weber)	Chromosomal DNA from JM1 transformed in MW2 cells	
JM6	*amy::smc*-His P*xyl, cm*^{r}	pSMX	pMW6 (M. Weber)	PCR product of *smc* digested with *XbaI* and *BamHI* and cloned in *Spe*I-*Bam*HI of pMW6	101, 102
JM8	(*ypuG*) *scp*A-*yfp,cm*^{r}	pyG	pKL184 (A. Grossman)	PCR product of C-terminal *ypuG* cloned at *Eco*RI-*Xho*I in pKL184	64, 65
JM9	(*ypuH*) *scpB-yfp, cm*^{r}	pyH	pSG1187 (P. Lewis)	PCR product of C-terminal *ypuH* cloned at *Kpn*I-*Eco*RI in pSG1187	68, 69
JM10	*amy*::Pxyl *cfp-scp*B, *spec*^{r}	pCHamy	pSG1192 (P. Lewis)	PCR product of *ypuH* cloned at *Kpn*I-*Eco*RI in pSG1192	127, 69
JM11	(*ypuG*) *scp*A::*tet*			PCR knockout method	P_1 : 72; P_2: 107 P_3: 108; P_4: 73
JM12	(*ypuGH*) *scp*AB::*tet*			PCR knockout method	P_1: 72; P_2: 107 P_3: 93; P_4: 73
JM13	(*ypuH*) *scpB::tet*			PCR knockout method	P_1=72; P_2: 92 P_3: 93; P_4: 73
JM14	*scp*A-*yfp, cm*^{r}, *amy*::P*xyl cfp-scp*B, *spec*^{r}			Chromosomal DNA from JM10 transformed in JM8 cells	
JM15	*scp*B-*yfp* (*cm::tet*)			JM9 transformed with pCm::tet	
JM16	*smc::kan, amy*::P*spac smc(cm::tet)*	pCm::tet		Plasmid pCm::tet transformed in EP58 cells	
JM17	*scpA-yfp, smc::kan,amy*::P*spac smc(cm::tet)*			Chromosomal DNA from JM8 transformed in JM16 cells	
JM18	*scp*A::*tet*, *amy*::P*xyl cfp-scp*B, *spec*^{r}			Chromosomal DNA from JM11 transformed in JM10 cells	
B. subtilis Strain	Genotype	Modified *E. coli* plasmid transformed	*E. coli* plasmid/ Strain	Cloning details	Primers used (table 15)

		in *B. subtilis*			
JM19	*scpA::tet, smc::kan, amy*::P*spac smc(cm::tet)*			Chromosomal DNA from JM11 transformed in JM16 cells	
JM20	*smc-gfp, cm*^{r}	Psmc-gfp	pSG1151	PCR product of C-terminal *smc* cloned at *Apa*I-*Cla*I in pSG1151	103, 105
JM24	*smc-strep, cm*^{r}	Psmc-strep	pSG1151strep	PCR product of C-terminal *smc* cloned at *Apa*I-*Cla*I in pSG1151strep	103, 105
JM25	*smc-yfp,* P*spac, mls*^{r}	psY mls	pMutinYFP	PCR product of C-terminal *smc* cloned at *Kpn*I-*Cla*I in pMutinYFP	194, 105
JM26	*smc-cfp,* P*spac, mls*^{r}	psC mls	pMutinCFP	PCR product of C-terminal *smc* cloned at *Kpn*I-*Cla*I in pMutinCFP	194, 105
JM27	*dnaX-cfp, spec*^{r}, *smc-yfp,* P*spac, mls*^{r}	pJCL61	pJCL61 (A. Grossman)	pJCL61 transformed in JM25	
JM28	*spoOJ::spec*, *smc-yfp,* P*spac, mls*^{r}			Chromosomal DNA from JM25 transformed in AG1468	
JM29	*scpA-yfp, cm*^{r}, *smc-cfp,* P*spac, mls*^{r}			Chromosomal DNA from JM27 transformed in JM8	
JM30	*scpB-yfp, cm*^{r} , *smc-cfp,* P*spac, mls*^{r}			Chromosomal DNA from JM27 transformed in JM9	
JM31	*scpA::tet, smc-yfp,* P*spac, mls*^{r}			Chromosomal DNA from JM11 transformed in JM25	
JM32	*scpB::tet, smc-yfp,* P*spac, mls*^{r}			Chromosomal DNA from JM13 transformed in JM25	
JM33	*ypuI::tet, smc-yfp,* P*spac, mls*^{r}			Chromosomal DNA from PG31 transformed in JM25	
JM34	*scpAB::tet, smc-yfp,* P*spac, mls*^{r}			Chromosomal DNA from JM12 transformed in JM25	
JM35	P*hyperspac smc-yfp,* P*spac, mls*^{r} *cm*^{r}			Chromosomal DNA from JM25 transformed in CAS4	
JM36	*scpB-yfp,* P*hyperspac smc-yfp,* P*spac, mls*^{r} *cm*^{r}			Chromosomal DNA from JM35 transformed in JM9	
JM37	K37I, P*hyperspack-smc-yfp, cm*^{r}, *mls*^{r}, IPTG	pA*S	pCAS5 (C. Andrei-Selmer)	Primer generated site-directed mutagenesis on pCAS5 and transformed in JM25	230, 231

B. subtilis Strain	Genotype	Modified *E. coli* plasmids transformed in *B. subtilis*	*E. coli* Plasmid/ Strain	Cloning details	Primers used (table 15)
JM38	D1155G, *smc-yfp, mls*^{r}, IPTG	pB*Sy	psY mls	Primer generated site-directed mutagenesis on psY mls	222, 223
JM39	S1090R, *smc-yfp, mls*^{r}, IPTG	pC*Sy	psY mls	Primer generated site directed mutagenesis on psY mls	232, 234
JM40	p148*yisB, kan*^{r}	pYisB148	pDG148	PCR product of *yisB* cloned at *Hin*dIII-*Sph*I in pDG148	201, 202
JN1	sbcC-yfp, *cm*^{r}	pC87	pSG1187	PCR product of C-terminal *sbcC* cloned at *Eco*RI- *Hin*dIII in pSG1187	140, 141
JN2	sbcC-cfp, *cm*^{r}	pC86	pSG1186	PCR product of C-terminal *sbcC* cloned at *Eco*RI- *Hin*dIII in pSG1186	140, 141
JN3	*sbcD-yfp, cm*^{r}	pD87	pSG1187	PCR product of C-terminal *sbcD* cloned at *Eco*RI- *Hin*dIII in pSG1187	142, 143
JN4	*sbcD-cfp, cm*^{r}	pD86	pSG1186	PCR product of C-terminal *sbcD* cloned at *Eco*RI- *Hin*dIII in pSG1186	142, 143
JN5	*sbcD.gfp, cm*^{r}	pD1164	pSG1164	PCR product of C-terminal *sbcD* cloned at *Apa*I-*Eco*RI in pSG1164	148, 143
JN6	*sbcC-yfp*, P*xyl*	pC87xyl	pSG1164	subcloned *Eco*RI-*Spe*I *sbcC-yfp* fragment from pC87	
JN7	*addA-gfp, cm*^{r}	pA1164	pSG1164	PCR product of C-terminal *addA* cloned at *Apa*I-*Eco*RI in pSG1164	150, 151
JM41	*addB-yfp, mls*^{r}	pBy	pMutin-YFP	PCR product of C-terminal *addB* cloned at *Cla*I in pMutin-YFP	213, 214
JM42	*sbc*C::*sbc*C-*gfp, cm*^{r}	pSbcKO	pJQ43	PCR product of internal fragment of *sbcC* cloned at *Hin*dIII- *Sph*I in pJQ43	284, 285
JM43	*parC-yfp, cm*^{r}	pParCYFP	pKL184	PCR product of C-terminal *parC* cloned at *Eco*RI- *Xho*I in pKL184	30, 31
JM44	*parE-yfp, mls*^{r}	pParEYFP	PMutin-YFPmcs	PCR product of C-terminal *parE* cloned at *Hin*dIII- *Kpn*I in pMutin-YFPmcs	270, 286
JM45	P*hyperspank -parE*	pJQhyperspank ParE	pJQhyperspank	subcloned *Hin*dIII- *Nhe*I P*hyperspank* fragment from pDr111	272, 273

5.2.3 List of primers used

Table 15

Primers	Sequence (5'-3')
3 (rplAup)	c*gg atc c*cg ttc tcg tcg ttt tcg caa aag g
4(rplAdw)	gg*g gta cc*c cac ctt tta cgt taa aag ttg aag agt c
30(parCyfpdw)	cgg *ctc gag* acc tcc ttg ttc tgt atg aag gcg cc
31(parCyfpup)	cg*g aat tc*g ata aaa ttt gat ccg tc
64(ypuGcfpup)	cg*g aat ttc* ccg aag caa gag gag g
65(ypuGcfpdw)	ccg *ctc gag* agc ccc atg aat gga ttc ac
68 (ypuHyfpup)	cgg a*gg tac c*ct tct tta tgc ggc agg
69 (ypuHyfpdw)	ccg *gaa ttc* ggt ttt tat atc ttc gaa ggt ttg g
72 (ypuP1)	acg tgg tac cgc tca ttt tca taa tag atc gg
73 (ypuP4)	atc gcc gcg ggg ctt ctt tcg ttt atg ccg
93 (ypuHP3)	*ttg atc ctt ttt tta taa cag gaa ttc* gcc ctt cat cgc ctg ccg c
92 (ypuHP2)	*gaa caa cct gca caa ttg caa ga*g aaa act tta acc aaa cct tcg aag
101 (smx5)	cga *ttc tag a*tt cct caa acg ttt aga cg
102 (smx3)	ata t*gg atc c*tc tct gaa cga att ctt ttg
103 (sm-Cgfpup)	gcg c*gg gcc c*ga aca aaa aga aga ttt aac aga
105 (smgfpcla)	cc*a tcg a*ta ccg cct ccc tga acg aat tct ttt gtt tct tc
107 (ypuGP2)	*gaa caa cct gca cca ttg caa ga*g ctg atg aaa aat cag ctg gtc c
108 (ypuGP3)	*ttg atc ctt ttt tta taa cag gaa ttc* gta tca att ttc act tga tat tct tc
117 (ypuHaup)	ccc *aag ctt* aca tta cgg gga gtg aat cc
118 (ypuhadw)	cg*g gat cc*t tct att tta tat ctt cga ag
122 (ypuGP2new)	*gaa caa cct gca cca ttg caa ga*g aaa act tta acc aaa cct tcg aag
123 (ypuGP1)	gca ccg tca ata atg atc gcg c
127 (cH-amyup)	tag *ggtacc* ggg gct tga tat cgt gaa ttg
140 (SbcCyfpup)	att c*aa gct t*gc aaa ctt gaa aac gag
141 (SbcCyfpdw)	tcg *gaa ttc* acc acc gcc cat caa ctc aag tga tac ccg
143 (SbcDdw)	tcg *gaa ttc* acc gcc ttt cgc atc ctc ctc ctc ttg aac

Primers	Sequence (5'-3')
148 (SbcDupn)	att c*gg gcc c*tt tgc atc gcc cgc aaa cg
150 (addAup)	att c*gg gcc c*ag ctg agc tgg acc tac
151 (addAdw)	tcg *gaa ttc* acc acc gcc taa tgt cag aat gtg ccc
162 (strepup)	att tca ctg ctt gga gcc acc cgc agt tcg aaa aat aag cat gca
164 (strepdwn)	gtg acg aac ctc ggt ggg cgt caa gct ttt tat tcg tac gtg atc
193 (69cla)	cc*a tcg at*g gtt ttt ata tct tcg aag gtt tgg
194 (103kpn)	gg*g gta cc*g aac aaa aag aag att taa cag a
198 (smc CNco)	cat g*cc atg g*gc ttt aac gac aca ttc gtc
201 (yisBhind3)	atc *aag ctt* gag ttg atg taa ggg ag
202 (yisBSph1)	gct t*gc atg c*ga caa att ata tag acc cc
213 (AddBdw)	cca tcg ata ccg cct ccg gaa tgt tca ttg cca tc
214 (AddBup)	ttg att tat cga tta cac att c
209 (fpn)	agg gtg ggc cag ggc acg ggc (downstream primer ~180bp inside of GFP)
222 (mutSMC)	gtgccgttttgcgtccttgccgcagtagaggctgcgctcgac
223 (mutSMC3)	gtcgagcgcagcctctactgcggcaaggacgcaaaacggcac
228 (mcs5)	agc ttgtcg acg aat tcg gta ccc cat ggg gat cca atc gat aga tct ggg gga ggt c
229 (mcs3)	ggc cga cct ccc cca gat cta tcg atg gat ccc cat ggg gta ccg aat tcg tcg aca
230 (K37Iup)	gtcgggccgaacggaagcggaataagcaacatcacggatgcc
231 (K37Iup-r)	ggcatccgtgatgttgcttattccgcttccgttcggcccgac
232 (S1099Rup)	caaaacttaaacctcctgacgaggcggagacgtgcg
234 (S1099Rup-r)	cgcacgctctccgcctcgcaggaggtttaagttttg
270 (ParEGFPup)	tac c*aa gct t*tt at gat ca tt cat gcg atc
271 (ParEGFPdw)	cgc *gga tcc* tta aac ctc ctc agc gac
272 (ParE-Nup)	tca t*aa gct t*tg aaa ggg gtt tgt acg ttt g
273 (ParE-Nup)	cag *gct agc* taa caa aaa cgc cag act ctc
284 (SbcKOdw)	aca t*gc atg c*aa tct gcc ctt cgc ctc ttg
285 (SbcKOup)	tca t*aa gct t*ca aac agg aac agc ttt cac g
259 (S-Exn1)	acc gga tcc cgc ttc cgt tcg gcc cga c (primer extension)

5.2.4 Primer annealing temperatures

Table displaying the annealing temperatures of oligonucleotides, calculated as described in 2.1.5

Table16

primer length (L)

ΣG + C ↓	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
1	31,24	33,48	35,47	37,25	38,85	40,30	41,62	42,82	43,93	44,94	45,88	46,74	47,55	48,30	49,00	49,65	50,27	50,85	51,39	51,90	52,38	52,84	53,27	53,68	54,08
2	33,80	35,89	37,74	39,41	40,90	42,25	43,48	44,60	45,63	46,58	47,45	48,26	49,01	49,71	50,37	50,98	51,55	52,09	52,59	53,07	53,52	53,95	54,35	54,74	55,10
3	36,36	38,30	40,02	41,56	42,95	44,20	45,35	46,39	47,34	48,22	49,03	49,78	50,48	51,13	51,73	52,30	52,83	53,33	53,80	54,24	54,66	55,06	55,43	55,79	56,13
4	38,93	40,71	42,30	43,72	45,00	46,16	47,21	48,17	49,05	49,86	50,61	51,30	51,94	52,54	53,10	53,62	54,11	54,57	55,01	55,41	55,80	56,16	56,51	56,84	57,15
5	41,49	43,12	44,58	45,88	47,05	48,11	49,07	49,95	50,76	51,50	52,18	52,82	53,41	53,96	54,47	54,95	55,39	55,82	56,21	56,59	56,94	57,27	57,59	57,89	58,18
6	44,05	45,54	46,86	48,04	49,10	50,06	50,94	51,73	52,47	53,14	53,76	54,34	54,87	55,37	55,83	56,27	56,68	57,06	57,42	57,76	58,08	58,38	58,67	58,94	59,20
7	46,61	47,95	49,13	50,19	51,15	52,01	52,80	53,52	54,18	54,78	55,34	55,86	56,34	56,78	57,20	57,59	57,96	58,30	58,62	58,93	59,22	59,49	59,75	59,99	60,23
8	49,18	50,36	51,41	52,35	53,20	53,97	54,66	55,30	55,88	56,42	56,92	57,37	57,80	58,20	58,57	58,91	59,24	59,54	59,83	60,10	60,36	60,60	60,83	61,04	61,25
9	51,74	52,77	53,69	54,51	55,25	55,92	56,53	57,08	57,59	58,06	58,49	58,89	59,26	59,61	59,93	60,24	60,52	60,78	61,04	61,27	61,49	61,71	61,91	62,09	62,28
10	54,30	55,18	55,97	56,67	57,30	57,87	58,39	58,87	59,30	59,70	60,07	60,41	60,73	61,02	61,30	61,56	61,80	62,03	62,24	62,44	62,63	62,81	62,98	63,15	63,30
11	56,86	57,59	58,24	58,83	59,35	59,82	60,25	60,65	61,01	61,34	61,65	61,93	62,19	62,44	62,67	62,88	63,08	63,27	63,45	63,61	63,77	63,92	64,06	64,20	64,33
12	59,43	60,01	60,52	60,98	61,40	61,78	62,12	62,43	62,72	62,98	63,22	63,45	63,66	63,85	64,03	64,20	64,36	64,51	64,65	64,79	64,91	65,03	65,14	65,25	65,35
13	61,99	62,42	62,80	63,14	63,45	63,73	63,98	64,21	64,43	64,62	64,80	64,97	65,12	65,27	65,40	65,53	65,64	65,75	65,86	65,96	66,05	66,14	66,22	66,30	66,38
14	64,55	64,83	65,08	65,30	65,50	65,68	65,85	66,00	66,13	66,26	66,38	66,49	66,59	66,68	66,77	66,85	66,93	67,00	67,06	67,13	67,19	67,25	67,30	67,35	67,40
15	67,11	67,24	67,36	67,46	67,55	67,63	67,71	67,78	67,84	67,90	67,95	68,00	68,05	68,09	68,13	68,17	68,21	68,24	68,27	68,30	68,33	68,35	68,38	68,40	68,43
16	69,68	69,65	69,63	69,62	69,60	69,59	69,57	69,56	69,55	69,54	69,53	69,52	69,51	69,51	69,50	69,49	69,49	69,48	69,48	69,47	69,47	69,46	69,46	69,45	69,45
17	72,24	72,06	71,91	71,77	71,65	71,54	71,44	71,34	71,26	71,18	71,11	71,04	70,98	70,92	70,87	70,82	70,77	70,72	70,68	70,64	70,61	70,57	70,54	70,51	70,48
18	74,80	74,48	74,19	73,93	73,70	73,49	73,30	73,13	72,97	72,82	72,68	72,56	72,44	72,33	72,23	72,14	72,05	71,97	71,89	71,81	71,74	71,68	71,62	71,56	71,50
19	77,36	76,89	76,47	76,09	75,75	75,44	75,16	74,91	74,68	74,46	74,26	74,08	73,91	73,75	73,60	73,46	73,33	73,21	73,09	72,99	72,88	72,79	72,69	72,61	72,53
20	79,93	79,30	78,74	78,25	77,80	77,40	77,03	76,69	76,38	76,10	75,84	75,60	75,37	75,16	74,97	74,78	74,61	74,45	74,30	74,16	74,02	73,89	73,77	73,66	73,55

7. References

Adachi, S., and Hiraga, S. (2003) Mutants suppressing novobiocin hypersensitivity of a mukB null mutation. *J Bacteriol* **185**: 3690-3695.

Akhmedov, A.T., Frei, C., Tsai-Pflugfelder, M., Kemper, B., Gasser, S.M., and Jessberger, R. (1998) Structural maintenance of chromosomes protein C-terminal domains bind preferentially to DNA with secondary structure. *J Biol Chem* **273**: 24088-24094.

Ali Azam, T., Iwata, A., Nishimura, A., Ueda, S., and Ishihama, A. (1999) Growth phase-dependent variation in protein composition of the Escherichia coli nucleoid. *J Bacteriol* **181**: 6361-6370.

Ali, J.A., Jackson, A.P., Howells, A.J., and Maxwell, A. (1993) The 43-kilodalton N-terminal fragment of the DNA gyrase B protein hydrolyzes ATP and binds coumarin drugs. *Biochemistry* **32**: 2717-2724.

Alonso, J.C., Stiege, A.C., and Luder, G. (1993) Genetic recombination in Bacillus subtilis 168: effect of recN, recF, recH and addAB mutations on DNA repair and recombination. *Mol Gen Genet* **239**: 129-136.

Alonso, J.C., Gutierrez, C., and Rojo, F. (1995) The role of chromatin-associated protein Hbsu in beta-mediated DNA recombination is to facilitate the joining of distant recombination sites. *Mol Microbiol* **18**: 471-478.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389-3402.

Arumugam, P., Gruber, S., Tanaka, K., Haering, C.H., Mechtler, K., and Nasmyth, K. (2003) ATP hydrolysis is required for cohesin's association with chromosomes. *Curr Biol* **13**: 1941-1953.

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

Bignell, C., and Thomas, C.M. (2001) The bacterial ParA-ParB partitioning proteins. *J Biotechnol* **91**: 1-34.

Bohrmann, B., Villiger, W., Johansen, R., and Kellenberger, E. (1991) Coralline shape of the bacterial nucleoid after cryofixation. *J Bacteriol* **173**: 3149-3158.

Bouet, J.Y., and Funnell, B.E. (1999) P1 ParA interacts with the P1 partition complex at parS and an ATP-ADP switch controls ParA activities. *EMBO J* **18**: 1415-1424.

Brill, S.J., DiNardo, S., Voelkel-Meiman, K., and Sternglanz, R. (1987) DNA topoisomerase activity is required as a swivel for DNA replication and for ribosomal RNA transcription. *NCI Monogr*: 11-15.

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

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

Broccoli, S., Phoenix, P., and Drolet, M. (2000) Isolation of the topB gene encoding DNA topoisomerase III as a multicopy suppressor of topA null mutations in Escherichia coli. *Mol Microbiol* **35**: 58-68.

Bussiere, D.E., and Bastia, D. (1999) Termination of DNA replication of bacterial and plasmid chromosomes. *Mol Microbiol* **31**: 1611-1618.

Bylund, J.E., Haines, M.A., Piggot, P.J., and Higgins, M.L. (1993) Axial filament formation in Bacillus subtilis: induction of nucleoids of increasing length after addition of chloramphenicol to exponential-phase cultures approaching stationary phase. *J Bacteriol* **175**: 1886-1890.

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

Cobbe, N., and Heck, M.M. (2000) Review: SMCs in the world of chromosome biology- from prokaryotes to higher eukaryotes. *J Struct Biol* **129**: 123-143.

Cobbe, N., and Heck, M.M. (2003) The Evolution of SMC Proteins: Phylogenetic Analysis and Structural Implications. *Mol Biol Evol*.

Crater, D.L., and Moran, C.P., Jr. (2001) Identification of a DNA binding region in GerE from Bacillus subtilis. *J Bacteriol* **183**: 4183-4189.

Cromie, G.A., Connelly, J.C., and Leach, D.R. (2001) Recombination at double-strand breaks and DNA ends: conserved mechanisms from phage to humans. *Mol Cell* **8**: 1163-1174.

Davies, K.M., and Lewis, P.J. (2003) Localization of rRNA synthesis in Bacillus subtilis: characterization of loci involved in transcription focus formation. *J Bacteriol* **185**: 2346-2353.

Declais, A.C., de La Tour, C.B., and Duguet, M. (2001) Reverse gyrases from bacteria and archaea. *Methods Enzymol* **334**: 146-162.

den Blaauwen, T., Lindqvist, A., Lowe, J., and Nanninga, N. (2001) Distribution of the Escherichia coli structural maintenance of chromosomes (SMC)-like protein MukB in the cell. *Mol Microbiol* **42**: 1179-1188.

Draper, G.C., and Gober, J.W. (2002) Bacterial chromosome segregation. *Annu Rev Microbiol* **56**: 567-597.

Dubnau, D. (1991) Genetic competence in Bacillus subtilis. *Microbiol Rev* **55**: 395-424.

Dworkin, J. (2003) Transient genetic asymmetry and cell fate in a bacterium. *Trends Genet* **19**: 107-112.

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

Emptage, N.J. (2001) Fluorescent imaging in living systems. *Curr Opin Pharmacol* **1**: 521-525.

Errington, J. (2001) Septation and chromosome segregation during sporulation in Bacillus subtilis. *Curr Opin Microbiol* **4**: 660-666.

Errington, J., Bath, J., and Wu, L.J. (2001) DNA transport in bacteria. *Nat Rev Mol Cell Biol* **2**: 538-545.

Espeli, O., Levine, C., Hassing, H., and Marians, K.J. (2003) Temporal regulation of topoisomerase IV activity in E. coli. *Mol Cell* **11**: 189-201.

Fernandez, S., Sorokin, A., and Alonso, J.C. (1998) Genetic recombination in Bacillus subtilis 168: effects of recU and recS mutations on DNA repair and homologous recombination. *J Bacteriol* **180**: 3405-3409.

Feucht, A., and Lewis, P.J. (2001) Improved plasmid vectors for the production of multiple fluorescent protein fusions in Bacillus subtilis. *Gene* **264**: 289-297.

Freeman, L., Aragon-Alcaide, L., and Strunnikov, A. (2000) The condensin complex governs chromosome condensation and mitotic transmission of rDNA. *J Cell Biol* **149**: 811-824.

Fujioka, Y., Kimata, Y., Nomaguchi, K., Watanabe, K., and Kohno, K. (2002) Identification of a novel non-structural maintenance of chromosomes (SMC)

component of the SMC5-SMC6 complex involved in DNA repair. *J Biol Chem* **277**: 21585-21591.

Gordon, G.S., and Wright, A. (2000) DNA segregation in bacteria. *Annu Rev Microbiol* **54**: 681-708.

Graumann, P., Wendrich, T.M., Weber, M.H., Schroder, K., and Marahiel, M.A. (1997) A family of cold shock proteins in Bacillus subtilis is essential for cellular growth and for efficient protein synthesis at optimal and low temperatures. *Mol Microbiol* **25**: 741-756.

Graumann, P.L., Losick, R., and Strunnikov, A.V. (1998) Subcellular localization of Bacillus subtilis SMC, a protein involved in chromosome condensation and segregation. *J Bacteriol* **180**: 5749-5755.

Graumann, P.L. (2000) Bacillus subtilis SMC is required for proper arrangement of the chromosome and for efficient segregation of replication termini but not for bipolar movement of newly duplicated origin regions. *J Bacteriol* **182**: 6463-6471.

Gregson, H.C., Van Hooser, A.A., Ball, A.R., Jr., Brinkley, B.R., and Yokomori, K. (2002) Localization of human SMC1 protein at kinetochores. *Chromosome Res* **10**: 267-277.

Grompone, G., Ehrlich, S.D., and Michel, B. (2003) Replication restart in gyrB Escherichia coli mutants. *Mol Microbiol* **48**: 845-854.

Gruber, S., Haering, C.H., and Nasmyth, K. (2003) Chromosomal cohesin forms a ring. *Cell* **112**: 765-777.

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

Guillen, N., and Bohin, J.P. (1986) Folded chromosome in Bacillus subtilis after rifamycin treatment. *Biol Cell* **56**: 41-48.

Haering, C.H., Lowe, J., Hochwagen, A., and Nasmyth, K. (2002) Molecular architecture of SMC proteins and the yeast cohesin complex. *Mol Cell* **9**: 773-788.

Hagstrom, K.A., and Meyer, B.J. (2003) Condensin and cohesin: more than chromosome compactor and glue. *Nat Rev Genet* **4**: 520-534.

Haijema, B.J., Venema, G., and Kooistra, J. (1996) The C terminus of the AddA subunit of the Bacillus subtilis ATP-dependent DNase is required for the ATP-dependent exonuclease activity but not for the helicase activity. *J Bacteriol* **178**: 5086-5091.

Harmon, J.M., and Taber, H.W. (1977) Some properties of a membrane-deoxyribonucleic acid complex isolated from Bacillus subtilis. *J Bacteriol* **129**: 789-795.

Harvey, S.H., Krien, M.J., and O'Connell, M.J. (2002) Structural maintenance of chromosomes (SMC) proteins, a family of conserved ATPases. *Genome Biol* **3**: REVIEWS3003.

Herskovitz, M.A., and Bechhofer, D.H. (2000) Endoribonuclease RNase III is essential in Bacillus subtilis. *Mol Microbiol* **38**: 1027-1033.

Hiraga, S., Niki, H., Imamura, R., Ogura, T., Yamanaka, K., Feng, J., Ezaki, B., and Jaffe, A. (1991) Mutants defective in chromosome partitioning in E. coli. *Res Microbiol* **142**: 189-194.

Hiraga, S. (1992) Chromosome and plasmid partition in Escherichia coli. *Annu Rev Biochem* **61**: 283-306.

Hiraga, S. (2000) Dynamic localization of bacterial and plasmid chromosomes. *Annu Rev Genet* **34**: 21-59.

Hirano, M., and Hirano, T. (1998) ATP-dependent aggregation of single-stranded DNA by a bacterial SMC homodimer. *EMBO J* **17**: 7139-7148.

Hirano, M., Anderson, D.E., Erickson, H.P., and Hirano, T. (2001) Bimodal activation of SMC ATPase by intra- and inter-molecular interactions. *EMBO J* **20**: 3238-3250.

Hirano, M., and Hirano, T. (2002) Hinge-mediated dimerization of SMC protein is essential for its dynamic interaction with DNA. *EMBO J* **21**: 5733-5744.

Hirano, T., Kobayashi, R., and Hirano, M. (1997) Condensins, chromosome condensation protein complexes containing XCAP-C, XCAP-E and a Xenopus homolog of the Drosophila Barren protein. *Cell* **89**: 511-521.

Hirano, T. (2002) The ABCs of SMC proteins: two-armed ATPases for chromosome condensation, cohesion, and repair. *Genes Dev* **16**: 399-414.

Hobot, J.A., Villiger, W., Escaig, J., Maeder, M., Ryter, A., and Kellenberger, E. (1985) Shape and fine structure of nucleoids observed on sections of ultrarapidly frozen and cryosubstituted bacteria. *J Bacteriol* **162**: 960-971.

Hoffmann, T., Troup, B., Szabo, A., Hungerer, C., and Jahn, D. (1995) The anaerobic life of Bacillus subtilis: cloning of the genes encoding the respiratory nitrate reductase system. *FEMS Microbiol Lett* **131**: 219-225.

Hopfner, K.P., Karcher, A., Shin, D.S., Craig, L., Arthur, L.M., Carney, J.P., and Tainer, J.A. (2000) Structural biology of Rad50 ATPase: ATP-driven conformational control in DNA double-strand break repair and the ABC-ATPase superfamily. *Cell* **101**: 789-800.

Hsieh, T., and Brutlag, D. (1980) ATP-dependent DNA topoisonmerase from D. melanogaster reversibly catenates duplex DNA rings. *Cell* **21**: 115-125.

Huang, W.M., Libbey, J.L., van der Hoeven, P., and Yu, S.X. (1998) Bipolar localization of Bacillus subtilis topoisomerase IV, an enzyme required for chromosome segregation. *Proc Natl Acad Sci U S A* **95**: 4652-4657.

Imai, Y., Ogasawara, N., Ishigo-Oka, D., Kadoya, R., Daito, T., and Moriya, S. (2000) Subcellular localization of Dna-initiation proteins of Bacillus subtilis: evidence that chromosome replication begins at either edge of the nucleoids. *Mol Microbiol* **36**: 1037-1048.

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

Jensen, R.B., and Shapiro, L. (1999) The Caulobacter crescentus smc gene is required for cell cycle progression and chromosome segregation. *Proc Natl Acad Sci U S A* **96**: 10661-10666.

Jensen, R.B., Wang, S.C., and Shapiro, L. (2002) Dynamic localization of proteins and DNA during a bacterial cell cycle. *Nat Rev Mol Cell Biol* **3**: 167-176.

Jensen, R.B., and Shapiro, L. (2003) Cell-cycle-regulated expression and subcellular localization of the Caulobacter crescentus SMC chromosome structural protein. *J Bacteriol* **185**: 3068-3075.

Jinno, S., Suto, K., Nagata, A., Igarashi, M., Kanaoka, Y., Nojima, H., and Okayama, H. (1994) Cdc25A is a novel phosphatase functioning early in the cell cycle. *EMBO J* **13**: 1549-1556.

Jones, L.J., Carballido-Lopez, R., and Errington, J. (2001) Control of cell shape in bacteria: helical, actin-like filaments in Bacillus subtilis. *Cell* **104**: 913-922.

Kakeshita, H., Oguro, A., Amikura, R., Nakamura, K., and Yamane, K. (2000) Expression of the ftsY gene, encoding a homologue of the alpha subunit of mammalian signal recognition particle receptor, is controlled by different

promoters in vegetative and sporulating cells of Bacillus subtilis. *Microbiology* **146 (Pt 10)**: 2595-2603.

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

Kato, J., Nishimura, Y., Imamura, R., Niki, H., Hiraga, S., and Suzuki, H. (1990) New topoisomerase essential for chromosome segregation in E. coli. *Cell* **63**: 393-404.

Kellenberger, E. (1991) Functional consequences of improved structural information on bacterial nucleoids. *Res Microbiol* **142**: 229-238.

Khodursky, A.B., Peter, B.J., Schmid, M.B., DeRisi, J., Botstein, D., Brown, P.O., and Cozzarelli, N.R. (2000) Analysis of topoisomerase function in bacterial replication fork movement: use of DNA microarrays. *Proc Natl Acad Sci U S A* **97**: 9419-9424.

Kido, M., Yamanaka, K., Mitani, T., Niki, H., Ogura, T., and Hiraga, S. (1996) RNase E polypeptides lacking a carboxyl-terminal half suppress a mukB mutation in Escherichia coli. *J Bacteriol* **178**: 3917-3925.

Kimura, K., and Hirano, T. (1997) ATP-dependent positive supercoiling of DNA by 13S condensin: a biochemical implication for chromosome condensation. *Cell* **90**: 625-634.

Klein, W., and Marahiel, M.A. (2002) Structure-function relationship and regulation of two Bacillus subtilis DNA-binding proteins, HBsu and AbrB. *J Mol Microbiol Biotechnol* **4**: 323-329.

Kohler, P., and Marahiel, M.A. (1997) Association of the histone-like protein HBsu with the nucleoid of Bacillus subtilis. *J Bacteriol* **179**: 2060-2064.

Koshland, D., and Strunnikov, A. (1996) Mitotic chromosome condensation. *Annu Rev Cell Dev Biol* **12**: 305-333.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A.M., Alloni, G., Azevedo, V., Bertero, M.G., Bessieres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S.C., Bron, S., Brouillet, S., Bruschi, C.V., Caldwell, B., Capuano, V., Carter, N.M., Choi, S.K., Codani, J.J., Connerton, I.F., Danchin, A., and et al. (1997) The complete genome sequence of the gram-positive bacterium Bacillus subtilis. *Nature* **390**: 249-256.

Kuwayama, H., Obara, S., Morio, T., Katoh, M., Urushihara, H., and Tanaka, Y. (2002) PCR-mediated generation of a gene disruption construct without the use of DNA ligase and plasmid vectors. *Nucleic Acids Res* **30**: E2.

Laloraya, S., Guacci, V., and Koshland, D. (2000) Chromosomal addresses of the cohesin component Mcd1p. *J Cell Biol* **151**: 1047-1056.

Larionov, V.L., and Strunnikov, A.V. (1987) [Accumulation of artificial minichromosomes in a Saccharomyces cerevisiae mutant with impaired segregation process]. *Dokl Akad Nauk SSSR* **294**: 1493-1497.

Laurent, S.J., and Vannier, F.S. (1973) Temperature-sensitive initiation of chromosome replication in a mutant of Bacillus subtilis. *J Bacteriol* **114**: 474-484.

Le Hegarat, F., Salti-Montesanto, V., Hauck, Y., and Hirschbein, L. (1993) Purification and characterization of the HU-like protein HPB9 from the Bacillus subtilis nucleoid. *Biochim Biophys Acta* **1172**: 101-107.

Lemon, K.P., and Grossman, A.D. (1998) Localization of bacterial DNA polymerase: evidence for a factory model of replication. *Science* **282**: 1516-1519.

Lemon, K.P., and Grossman, A.D. (2001) The extrusion-capture model for chromosome partitioning in bacteria. *Genes Dev* **15**: 2031-2041.
Levin, P.A., and Losick, R. (1996) Transcription factor Spo0A switches the localization of the cell division protein FtsZ from a medial to a bipolar pattern in Bacillus subtilis. *Genes Dev* **10**: 478-488.
Levin, P.A., and Grossman, A.D. (1998) Cell cycle: the bacterial approach to coordination. *Curr Biol* **8**: R28-31.
Lewis, P.J., and Errington, J. (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.
Lewis, P.J., and Marston, A.L. (1999) GFP vectors for controlled expression and dual labelling of protein fusions in Bacillus subtilis. *Gene* **227**: 101-110.
Lewis, P.J., Thaker, S.D., and Errington, J. (2000) Compartmentalization of transcription and translation in Bacillus subtilis. *EMBO J* **19**: 710-718.
Li, Y., and Austin, S. (2002) The P1 plasmid in action: time-lapse photomicroscopy reveals some unexpected aspects of plasmid partition. *Plasmid* **48**: 174-178.
Lin, D.C., and Grossman, A.D. (1998) Identification and characterization of a bacterial chromosome partitioning site. *Cell* **92**: 675-685.
Lindow, J.C., Britton, R.A., and Grossman, A.D. (2002a) Structural maintenance of chromosomes protein of Bacillus subtilis affects supercoiling in vivo. *J Bacteriol* **184**: 5317-5322.
Lindow, J.C., Kuwano, M., Moriya, S., and Grossman, A.D. (2002b) Subcellular localization of the Bacillus subtilis structural maintenance of chromosomes (SMC) protein. *Mol Microbiol* **46**: 997-1009.
Lopez-Garcia, P., and Forterre, P. (1999) Control of DNA topology during thermal stress in hyperthermophilic archaea: DNA topoisomerase levels, activities and induced thermotolerance during heat and cold shock in Sulfolobus. *Mol Microbiol* **33**: 766-777.
Losick, R., and Shapiro, L. (1999) Changing views on the nature of the bacterial cell: from biochemistry to cytology. *J Bacteriol* **181**: 4143-4145.
Lowe, J., Cordell, S.C., and van den Ent, F. (2001) Crystal structure of the SMC head domain: an ABC ATPase with 900 residues antiparallel coiled-coil inserted. *J Mol Biol* **306**: 25-35.
Lutkenhaus, J. (2002) Unexpected twist to the Z ring. *Dev Cell* **2**: 519-521.
Luttinger, A.L., Springer, A.L., and Schmid, M.B. (1991) A cluster of genes that affects nucleoid segregation in Salmonella typhimurium. *New Biol* **3**: 687-697.
Marczynski, G.T., and Shapiro, L. (2002) Control of chromosome replication in caulobacter crescentus. *Annu Rev Microbiol* **56**: 625-656.
Marston, A.L., Thomaides, H.B., Edwards, D.H., Sharpe, M.E., and Errington, J. (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.
Marston, A.L., and Errington, J. (1999a) Dynamic movement of the ParA-like Soj protein of B. subtilis and its dual role in nucleoid organization and developmental regulation. *Mol Cell* **4**: 673-682.
Marston, A.L., and Errington, J. (1999b) Selection of the midcell division site in Bacillus subtilis through MinD-dependent polar localization and activation of MinC. *Mol Microbiol* **33**: 84-96.
Mascarenhas, J., Soppa, J., Strunnikov, A.V., and Graumann, P.L. (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.

Mason, J.M., and Setlow, P. (1987) Different small, acid-soluble proteins of the alpha/beta type have interchangeable roles in the heat and UV radiation resistance of Bacillus subtilis spores. *J Bacteriol* **169**: 3633-3637.

McAdams, H.H., and Shapiro, L. (2003) A bacterial cell-cycle regulatory network operating in time and space. *Science* **301**: 1874-1877.

Medina, N., Vannier, F., Roche, B., Autret, S., Levine, A., and Seror, S.J. (1997) Sequencing of regions downstream of addA (98 degrees) and citG (289 degrees) in Bacillus subtilis. *Microbiology* **143 (Pt 10)**: 3305-3308.

Meima, R., Haan, G.J., Venema, G., Bron, S., and de Jong, S. (1998) Sequence specificity of illegitimate plasmid recombination in Bacillus subtilis: possible recognition sites for DNA topoisomerase I. *Nucleic Acids Res* **26**: 2366-2373.

Melby, T.E., Ciampaglio, C.N., Briscoe, G., and Erickson, H.P. (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.

Mengiste, T., Revenkova, E., Bechtold, N., and Paszkowski, J. (1999) An SMC-like protein is required for efficient homologous recombination in Arabidopsis. *EMBO J* **18**: 4505-4512.

Micka, B., and Marahiel, M.A. (1992) The DNA-binding protein HBsu is essential for normal growth and development in Bacillus subtilis. *Biochimie* **74**: 641-650.

Mohr, S.C., Sokolov, N.V., He, C.M., and Setlow, P. (1991) Binding of small acid-soluble spore proteins from Bacillus subtilis changes the conformation of DNA from B to A. *Proc Natl Acad Sci U S A* **88**: 77-81.

Moriya, S., Fukuoka, T., Ogasawara, N., and Yoshikawa, H. (1988) Regulation of initiation of the chromosomal replication by DnaA-boxes in the origin region of the Bacillus subtilis chromosome. *EMBO J* **7**: 2911-2917.

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

Nakamura, K., Yahagi, S., Yamazaki, T., and Yamane, K. (1999) Bacillus subtilis histone-like protein, HBsu, is an integral component of a SRP-like particle that can bind the Alu domain of small cytoplasmic RNA. *J Biol Chem* **274**: 13569-13576.

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

Oguro, A., Kakeshita, H., Honda, K., Takamatsu, H., Nakamura, K., and Yamane, K. (1995) srb: a Bacillus subtilis gene encoding a homologue of the alpha-subunit of the mammalian signal recognition particle receptor. *DNA Res* **2**: 95-100.

Oguro, A., Kakeshita, H., Takamatsu, H., Nakamura, K., and Yamane, K. (1996) The effect of Srb, a homologue of the mammalian SRP receptor alpha-subunit, on Bacillus subtilis growth and protein translocation. *Gene* **172**: 17-24.

Ohsumi, K., Yamazoe, M., and Hiraga, S. (2001) Different localization of SeqA-bound nascent DNA clusters and MukF-MukE-MukB complex in Escherichia coli cells. *Mol Microbiol* **40**: 835-845.

Pabo, C.O., and Sauer, R.T. (1992) Transcription factors: structural families and principles of DNA recognition. *Annu Rev Biochem* **61**: 1053-1095.

Paz, M.M., Das, T.A., and Tomasz, M. (1999) Mitomycin C linked to DNA minor groove binding agents: synthesis, reductive activation, DNA binding and cross-linking properties and in vitro antitumor activity. *Bioorg Med Chem* **7**: 2713-2726.

Pedersen, L.B., and Setlow, P. (2000) Penicillin-binding protein-related factor A is required for proper chromosome segregation in Bacillus subtilis. *J Bacteriol* **182**: 1650-1658.

Phillips, Z.E., and Strauch, M.A. (2002) Bacillus subtilis sporulation and stationary phase gene expression. *Cell Mol Life Sci* **59**: 392-402.

Pogliano, K., Harry, E., and Losick, R. (1995) Visualization of the subcellular location of sporulation proteins in Bacillus subtilis using immunofluorescence microscopy. *Mol Microbiol* **18**: 459-470.

Ragkousi, K., Cowan, A.E., Ross, M.A., and Setlow, P. (2000) Analysis of nucleoid morphology during germination and outgrowth of spores of Bacillus species. *J Bacteriol* **182**: 5556-5562.

Rigden, D.J., Setlow, P., Setlow, B., Bagyan, I., Stein, R.A., and Jedrzejas, M.J. (2002) PrfA protein of Bacillus species: prediction and demonstration of endonuclease activity on DNA. *Protein Sci* **11**: 2370-2381.

Robinson, K.M., and Kadonaga, J.T. (1998) The use of chromatin templates to recreate transcriptional regulatory phenomena in vitro. *Biochim Biophys Acta* **1378**: M1-6.

Ross, M.A., and Setlow, P. (2000) The Bacillus subtilis HBsu protein modifies the effects of alpha/beta-type, small acid-soluble spore proteins on DNA. *J Bacteriol* **182**: 1942-1948.

Rouviere-Yaniv, J., Yaniv, M., and Germond, J.E. (1979) E. coli DNA binding protein HU forms nucleosomelike structure with circular double-stranded DNA. *Cell* **17**: 265-274.

Ryter, A., and Chang, A. (1975) Localization of transcribing genes in the bacterial cell by means of high resolution autoradiography. *J Mol Biol* **98**: 797-810.

Saitoh, N., Goldberg, I.G., Wood, E.R., and Earnshaw, W.C. (1994) ScII: an abundant chromosome scaffold protein is a member of a family of putative ATPases with an unusual predicted tertiary structure. *J Cell Biol* **127**: 303-318.

Sawitzke, J., and Austin, S. (2001) An analysis of the factory model for chromosome replication and segregation in bacteria. *Mol Microbiol* **40**: 786-794.

Sawitzke, J.A., and Austin, S. (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.

Schleiffer, A., Kaitna, S., Maurer-Stroh, S., Glotzer, M., Nasmyth, K., and Eisenhaber, F. (2003) Kleisins: a superfamily of bacterial and eukaryotic SMC protein partners. *Mol Cell* **11**: 571-575.

Sciochetti, S.A., Piggot, P.J., Sherratt, D.J., and Blakely, G. (1999) The ripX locus of Bacillus subtilis encodes a site-specific recombinase involved in proper chromosome partitioning. *J Bacteriol* **181**: 6053-6062.

Sciochetti, S.A., and Piggot, P.J. (2000) A tale of two genomes: resolution of dimeric chromosomes in Escherichia coli and Bacillus subtilis. *Res Microbiol* **151**: 503-511.

Setlow, B., and Setlow, P. (1995) Small, acid-soluble proteins bound to DNA protect Bacillus subtilis spores from killing by dry heat. *Appl Environ Microbiol* **61**: 2787-2790.

Setlow, P. (1988) Small, acid-soluble spore proteins of Bacillus species: structure, synthesis, genetics, function, and degradation. *Annu Rev Microbiol* **42**: 319-338.

Shapiro, L., and Losick, R. (2000) Dynamic spatial regulation in the bacterial cell. *Cell* **100**: 89-98.

Shih, Y.L., Le, T., and Rothfield, L. (2003) Division site selection in Escherichia coli involves dynamic redistribution of Min proteins within coiled structures that extend between the two cell poles. *Proc Natl Acad Sci U S A* **100**: 7865-7870.

Sikora-Borgula, A., Slominska, M., Trzonkowski, P., Zielke, R., Mysliwski, A., Wegrzyn, G., and Czyz, A. (2002) A role for the common GTP-binding protein in coupling of chromosome replication to cell growth and cell division. *Biochem Biophys Res Commun* **292**: 333-338.

Sinden, R.R., and Pettijohn, D.E. (1981) Chromosomes in living Escherichia coli cells are segregated into domains of supercoiling. *Proc Natl Acad Sci U S A* **78**: 224-228.

Smith, B.T., Grossman, A.D., and Walker, G.C. (2002) Localization of UvrA and effect of DNA damage on the chromosome of Bacillus subtilis. *J Bacteriol* **184**: 488-493.

Smith, T. (2002) Picture story. Double, double coils or trouble. *Nat Struct Biol* **9**: 645.

Soppa, J. (2001) Prokaryotic structural maintenance of chromosomes (SMC) proteins: distribution, phylogeny, and comparison with MukBs and additional prokaryotic and eukaryotic coiled-coil proteins. *Gene* **278**: 253-264.

Soppa, J., Kobayashi, K., Noirot-Gros, M.F., Oesterhelt, D., Ehrlich, S.D., Dervyn, E., Ogasawara, N., and Moriya, S. (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., and Graumann, P.L. (2003) Actin-like Proteins MreB and Mbl from Bacillus subtilis Are Required for Bipolar Positioning of Replication Origins. *Curr Biol* **13**: 1916-1920.

Steinmetz, M., and Richter, R. (1994) Plasmids designed to alter the antibiotic resistance expressed by insertion mutations in Bacillus subtilis, through in vivo recombination. *Gene* **142**: 79-83.

Stragier, P., and Losick, R. (1996) Molecular genetics of sporulation in Bacillus subtilis. *Annu Rev Genet* **30**: 297-241.

Strunnikov, A.V., Larionov, V.L., and Koshland, D. (1993) SMC1: an essential yeast gene encoding a putative head-rod-tail protein is required for nuclear division and defines a new ubiquitous protein family. *J Cell Biol* **123**: 1635-1648.

Strunnikov, A.V. (2003) Condensin and biological role of chromosome condensation. *Prog Cell Cycle Res* **5**: 361-367.

Taylor, E.M., Moghraby, J.S., Lees, J.H., Smit, B., Moens, P.B., and Lehmann, A.R. (2001) Characterization of a novel human SMC heterodimer homologous to the Schizosaccharomyces pombe Rad18/Spr18 complex. *Mol Biol Cell* **12**: 1583-1594.

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

Vagner, V., Dervyn, E., and Ehrlich, S.D. (1998) A vector for systematic gene inactivation in Bacillus subtilis. *Microbiology* **144 (Pt 11)**: 3097-3104.

van den Ent, F., Lockhart, A., Kendrick-Jones, J., and Lowe, J. (1999) Crystal structure of the N-terminal domain of MukB: a protein involved in chromosome partitioning. *Structure Fold Des* **7**: 1181-1187.

van den Ent, F., Moller-Jensen, J., Amos, L.A., Gerdes, K., and Lowe, J. (2002) F-actin-like filaments formed by plasmid segregation protein ParM. *EMBO J* **21**: 6935-6943.

Volkov, A., Mascarenhas, J., Andrei-Selmer, C., Ulrich, H.D., and Graumann, P.L. (2003) A prokaryotic condensin/cohesin-like complex can actively compact chromosomes from a single position on the nucleoid and binds to DNA as a ring-like structure. *Mol Cell Biol* **23**: 5638-5650.

Walker, J.E., Saraste, M., Runswick, M.J., and Gay, N.J. (1982) Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J* **1**: 945-951.

Ward, D., and Newton, A. (1997) Requirement of topoisomerase IV parC and parE genes for cell cycle progression and developmental regulation in Caulobacter crescentus. *Mol Microbiol* **26**: 897-910.

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

Weber, M.H., Volkov, A.V., Fricke, I., Marahiel, M.A., and Graumann, P.L. (2001) Localization of cold shock proteins to cytosolic spaces surrounding nucleoids in Bacillus subtilis depends on active transcription. *J Bacteriol* **183**: 6435-6443.

Weber, M.H., and Marahiel, M.A. (2003) Bacterial cold shock responses. *Sci Prog* **86**: 9-75.

Wei, Y., Vollmer, A.C., and LaRossa, R.A. (2001) In vivo titration of mitomycin C action by four Escherichia coli genomic regions on multicopy plasmids. *J Bacteriol* **183**: 2259-2264.

Weitao, T., Nordstrom, K., and Dasgupta, S. (1999) Mutual suppression of mukB and seqA phenotypes might arise from their opposing influences on the Escherichia coli nucleoid structure. *Mol Microbiol* **34**: 157-168.

Weitao, T., Dasgupta, S., and Nordstrom, K. (2000) Plasmid R1 is present as clusters in the cells of Escherichia coli. *Plasmid* **43**: 200-204.

Welch, T.J., Farewell, A., Neidhardt, F.C., and Bartlett, D.H. (1993) Stress response of Escherichia coli to elevated hydrostatic pressure. *J Bacteriol* **175**: 7170-7177.

Wheeler, R.T., Gober, J.W., and Shapiro, L. (1998) Protein localization during the Caulobacter crescentus cell cycle. *Curr Opin Microbiol* **1**: 636-642.

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

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

Yamanaka, K., Ogura, T., Niki, H., and Hiraga, S. (1992) Identification and characterization of the smbA gene, a suppressor of the mukB null mutant of Escherichia coli. *J Bacteriol* **174**: 7517-7526.

Yamanaka, K., Mitani, T., Ogura, T., Niki, H., and Hiraga, S. (1994) Cloning, sequencing, and characterization of multicopy suppressors of a mukB mutation in Escherichia coli. *Mol Microbiol* **13**: 301-312.

Yamanaka, K., Ogura, T., Niki, H., and Hiraga, S. (1996) Identification of two new genes, mukE and mukF, involved in chromosome partitioning in Escherichia coli. *Mol Gen Genet* **250**: 241-251.

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

Yang, F., Moss, L.G., and Phillips, G.N., Jr. (1996) The molecular structure of green fluorescent protein. *Nat Biotechnol* **14**: 1246-1251.

Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**: 103-119.

Yoshimura, S.H., Hizume, K., Murakami, A., Sutani, T., Takeyasu, K., and Yanagida, M. (2002) Condensin architecture and interaction with DNA: regulatory non-SMC subunits bind to the head of SMC heterodimer. *Curr Biol* **12**: 508-513.

Zechiedrich, E.L., and Cozzarelli, N.R. (1995) Roles of topoisomerase IV and DNA gyrase in DNA unlinking during replication in Escherichia coli. *Genes Dev* **9**: 2859-2869.

Zhu, Q., Pongpech, P., and DiGate, R.J. (2001) Type I topoisomerase activity is required for proper chromosomal segregation in Escherichia coli. *Proc Natl Acad Sci U S A* **98**: 9766-9771.

Zimmerman, S.B. (2003) Underlying regularity in the shapes of nucleoids of Escherichia coli: Implications for nucleoid organization and partition. *J Struct Biol* 142: 256-265.

Books and conferences

Drlica, K. (1996)" The Nucleoid" In *Escherichia coli* and *Salmonella*". Cellular and Molecular Biology, American Society for Microbiology, Washington, DC. pp 91-103

Harwood, R.C and Cutting, M.S (1990) Molecular Biological Methods for *Bacillus*. A Wiley- Interscience Publication.

Meile, C.J. (2003). Oral presentation at 'Functional Genomics of Gram-positive Microorganisms, .12th international conference on Bacilli'.

Messer, W., and Weigel, C. (1996) Initiation of chromosome replication. In *Escherichia coli* and *Salmonella*. Cellular and Molecular Biology, American Society for Microbiology, Washington, DC. pp. 1579–1601.

Neidhard, F. C., Curtiss, R., III, Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M., and Umbarger, H. E., Eds. (1996) "Timing of synthetic activities in the cell cycle. In *Escherichia coli* and *Salmonella*". Cellular and Molecular Biology, American Society for Microbiology, Washington, DC. pp. 1627–1639.

Noirot, P (2003). Oral presentation at 'Functional Genomics of Gram-positive Microorganisms, .12th international conference on Bacilli'.

Papers published or submitted in the course of this work:

Mascarenhas, J., Weber, M.H.W, and Graumann, P.L. (2001) Specific polar localization of ribosomes in *Bacillus subtilis* depends on active transcription. *EMBO Rep* **2**: 685-689.

Mascarenhas, J., Soppa, J., Strunnikov, A.V., and Graumann, P.L. (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.

Volkov, A., Mascarenhas, J., Andrei-Selmer, C., Ulrich, H.D., and Graumann, P.L. (2003) A prokaryotic condensin/cohesin-like complex can actively compact chromosomes from a single position on the nucleoid and binds to DNA as a ring-like structure. *Mol Cell Biol* **23**: 5638-5650.

Kaiser, V. , J. Mascarenhas, and P. L. Graumann (2003) *Bacillus subtilis yisB* encodes a stand alone HNH-like nuclease, HlpB, that is associated with stress induced chromosome degradation. Submitted.

Kidane, D., J. Mascarenhas, and P. L. Graumann (2003) Differential localization of *Bacillus subtilis* Rad50, SMC and DNA polymerase I after induction of DNA double strand breaks. Submitted.

Erklärung

Ich versichere, daß ich meine Dissertation '**Chromosome dynamics in *Bacillus subtilis* – Characterization of the Structural Maintenance of Chromosomes (SMC) complex**' selbständig, ohne unterlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrüklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzeigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, den

Acknowledgements

First of all I would like to thank my supervisor, Dr. Peter Graumann, for offering me a Ph.D. position and steering me through the project. I would like to thank him for his expert help with microscopy, his patience, constructive comments and encouragement with my work and constant concern and support for my personal needs.

I wish to thank Prof. Dr. E. Bremer, Prof. Dr. A. Batschauer and Prof. Dr. M. Bölker for their participation in the examination commission.

I am grateful to Prof. Dr. M. Marahiel, Dr. T. Stachelhaus, their lab members and all my colleagues for their helpful discussions and readiness to help me especially during the initial stages of my work and for providing a wonderful work atmosphere. I thank Michael and Richard for their critical corrections of my thesis.

I am thankful to Prof. Georgi Muskhelishvili erstwhile at MPI, Marburg, for allowing me to work in his lab and Ramesh for all the help with my RNA and primer extension project.

Many thanks to Prof Umesh Varshney and his lab members at IISc, Bangalore, for giving me a base to start up my profession in research.

I would like to heartily thank my dear friends, Suresh, Su-Mei, Nicole, Lalmohan, Naganand and all 'Desi' people here and at home for being there for me and encouraging me at all times.

Special thanks to Michael for giving me a positive attitude, for all the help with techniques, for helpful discussions and corrections, and for being a great companion during my stay in Marburg.

Most deeply, I thank my families for their love, support and encouragement in doing what I have always wanted to do.

Curriculum vitae

Judita Mascarenhas

Born on 26th October 1975 in Bhadravathi, India.

Education:

1980-1991	Primary and high school, St. Charles High School, Bhadravathi, India.
1991-1993	Pre-university examination in science, Silver Jubilee Government College, Bhadravathi, India.
1993-1996	Bachelors of science in chemistry, botany and zoology, Kuvempu University, Shimoga, India.
1996-1998	Master of science in biotechnology, Mysore University, Mysore, India.
1997	M.Sc project under the guidance of Prof. M. S. Shaila in the Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore, India.
1998-2000	Project assistant at Prof. Umesh Varshney in the Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore, India.
2000 -2004	Ph.D thesis in 'Characterization of the Structural Maintenance of Chromosomes (SMC) complex' under the guidance of Dr. Peter Graumann, Fachberiech Chemie, Philipps Universität Marburg, Germany.