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Anatomy of the pneumococcal nucleoid

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Anatomy of the pneumococcal nucleoid

Visualizing replication, chromosome segregation and
chromosome condensation dynamics in
Streptococcus pneumoniae

Renske van Raaphorst



The research described in this thesis was carried out at the department of Molecular Genetics (MolGen), Groningen Biochemistry and Biotechnology Institute (GBB), Faculty of Science and Engineering (FSE), University of Groningen, the Netherlands, and at the Veening Lab of the Department of Fundamental Microbiology (DMF), Faculty of Biology and Medicine (FBM), University of Lausanne, Switzerland.

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Visualizing replication, chromosome segregation and
chromosome condensation dynamics in *Streptococcus
pneumoniae*

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Chapter 1:

General Introduction

The pneumococcus: the accidental pathogen

The story of the pneumococcus as a pathogen is not a very logical one. The natural niche of the pneumococcus, or *Streptococcus pneumoniae*, is the human nasopharynx. It colonizes approximately 50% of children under five years old and about 10% of the adult population (Regev-Yochay *et al.* 2004; Southern *et al.* 2018). The pneumococcus is a commensal bacterium, causing no disease in most carriers, and its life cycle does not require pathogenicity for transmission to new hosts (Bogaert, de Groot, and Hermans 2004). Even more so: killing its host without increasing the chance of transmission will have a biological cost rather than being beneficial for the pneumococcus (Weiser 2010). Against the odds, the pneumococcus is a pathogen nonetheless. It can cause pneumonia, inner ear infections, meningitis or sepsis when it moves from the nasopharynx into the respiratory tract. In this way, the pneumococcus causes over one million deaths worldwide annually, especially in young children, the elderly and the immunocompromised (Troeger *et al.* 2018). Yet, how the pneumococcus transforms from a quiet inhabitant of the nasopharynx to a notorious killer is still one of the greater mysteries in pneumococcal research.

Pneumococcal conjugate vaccines were introduced in the national immunization program of 142 countries (Troeger *et al.* 2018) since the beginning of this century. This lowered the global mortality rate of lower respiratory tract infections caused by *S. pneumoniae* by 7.24%. The two vaccines on the market target respectively ten or thirteen of the most dangerous variants, or serotypes, of the pneumococcus. When an infection occurs, medical guidelines advise to use amoxicillin, doxycycline, macrolides or, as a last resort, fluoroquinolones (Metlay *et al.* 2019). Many of these antibiotics target essential processes of the bacterial cell cycle, like formation of the cell wall (such as the penicillins), protein synthesis (such as erythromycin) or chromosome organization (such as the quinolones).

While it might be an involuntary pathogen, the pneumococcus is a shape-shifter and an escape artist (Slager *et al.* 2019) that masters the evasion of the immune system. The pneumococcus can change its genome by taking up DNA from its environment and incorporating it into its genome, a process called competence.

By this, and by acquiring mutations, the pneumococcus can change the composition of its polysaccharide capsule, the target of antibodies. Even without any change into its genome, it can temporarily stop the expression of the capsule, making the pneumococcus invisible to the immune system. According to the CDC (Centre for Disease Control in the United States), in 30% of the severe *S. pneumoniae* infection cases in the United States, the pneumococci are resistant to one or more antibiotics, mainly to penicillins and erythromycins (CDC Antibiotic Threats Report, CS298822-2, 2019).

More than a bag of molecules

For a long time, bacterial cell biology was a bit of an ugly duckling. While in eukaryotic cells, cell growth, DNA replication, chromosome segregation and cytokinesis are strictly separated processes, bacterial cells do all at the same time. Bacteria do not have organelles and are so small, that it might seem hard to imagine that there is much regulation going on in this 'bag of molecules'. Still, already since the nineteen-thirties, researchers were trying to identify and explain what is going on inside bacterial cells. In particular, *Streptococcus pneumoniae* has a claim to fame in these early days of bacteriology. In 1929, Frederick Griffith reported the discovery of the transforming principle in the pneumococcus, nowadays known as bacterial competence (Griffith 1928). Fifteen years later, a variant of his experiment was the basis for the first evidence by Avery, MacLeod, and McCarty (1944) that DNA is the carrier of genetic information.

Between these paradigm-shifting experiments, the invention of the first nucleic acid stains made it possible to see DNA inside bacterial cells. In the end of the nineteen-thirties, Berthe Delaporte, Gerhard Piekarski, and others started to image these nucleal-positive bodies and speculated that these bodies were propagating inside cells during cell growth (Delaporte 1939; Piekarski 1937; Stille 1937). Piekarski was the first to coin the term nucleoid and, while not knowing the role of these nucleoids in bacterial cells, he observed a difference in nucleoid-propagation in fast-growing and slow-growing bacilli (**Figure 1.A**).

Early pneumococcal cell biology

In the next decade, the first scientific papers on pneumococcal cell biology were published, deploying both nucleic acid staining and electron microscopy. Some of the conclusions in these early papers were based on sample preparation artefacts – there are reports of membrane-enclosed organelles as well as the existence of pneumococcal mitochondria (McLean, Mudd, and Davis 1955; Tomasz, Jamieson, and Ottolenghi 1964). The first proposition of the cell cycle of the related species *S. faecalis* included the fusion of nuclei as an intermediate stage (**Figure 2.A**). In the meantime, paradigm after paradigm was built: by the end of the decade, the structure of DNA was deciphered, DNA polymerases were discovered and the central dogma of molecular biology proposed. This set the foundation for understanding bacterial growth, leading to a new uplift in bacterial cell biology.

While studies on the cell cycles of *B. subtilis*, *E. coli* and *Caulobacter crescentus* were getting off the ground (for instance Donachie *et al.* 1979; Kolter and Helsinki 1979; Shapiro 1976), *S.*

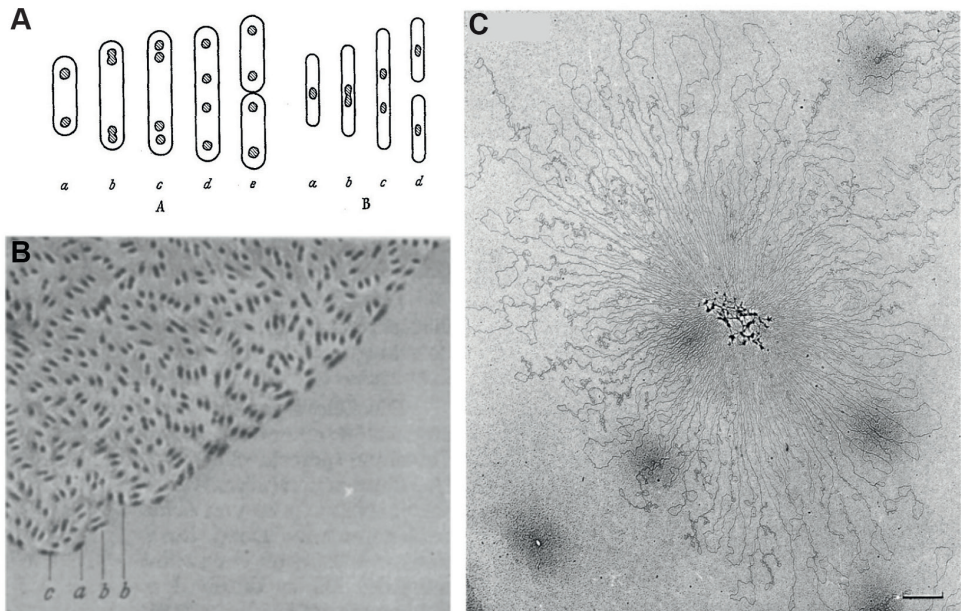
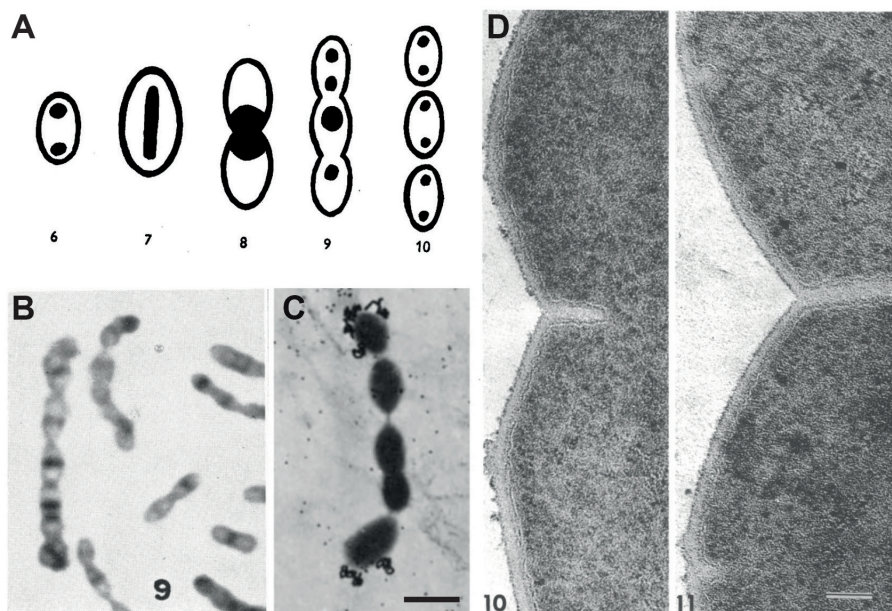


Figure 1. A. Deduction of the propagation of “nucleal-positive bodies” during the cell cycle of fast (**A.A**) and slow (**A.B**) growing bacteria by Gerhard Piekarski (1937). **B.** Micrograph of slow growing ‘paraphytus bacilli’, hydrolyzed and stained with Giemsa-staining, imaged by Gerhard Piekarski. The indicated *a*, *b* and *c* correspond to the cell cycle stages depicted in **A.A**. **A** and **B** reprinted with permission from Piekarski 1937, licence nr. 4744770845916. **C.** An electron micrograph of an isolated *E. coli* nucleoid bound to cytochrome C. Scale bar represents 1 μ m. Reprinted with permission from Kavenoff and Bowen, 1976.

Figure 2. **A.** Model of the chromosome cycle of *S. faecalis*, a member of the same clade as *S. pneumoniae*, by Bisset (1948) based on Giemsa stained cells. Reprinted with permission. **B.** Early imaging of the cell wall of the pneumococcus using Carol Hale's cell wall staining procedure (Hale 1953). Reprinted from (McLean, Mudd, and Davis 1955). **C.** Electron microscopic image, scale bar: 1 μ m. Silver grains on both ends of the cell chain of *S. pneumoniae* switched from choline- 3 H medium to ethanolamine medium show the localization of the old cell poles. Reprinted with permission from Briles and Tomasz, 1970. **D.** Electron micrographs showing a nearly divided (10) and a divided (11) *S. faecalis* cell. Scale bar: 0.1 μ m. Reprinted with permission from Higgins and Shockman 1970.



pneumoniae and related species were mostly studied to find ways to eradicate them. There are some exceptions. Studies as Higgins and Shockman, 1970 depicted beautiful electron micrographs to support their theory that both septal and peripheral peptidoglycan is formed from mid-cell in *S. faecalis* (Figure 2.D). In the same year, Briles and Tomasz, 1970 elegantly showed the localization of the old cell poles of the pneumococcus by switching from a chemically defined medium with radioactive choline- 3 H to medium where choline was replaced by ethanolamine (Figure 2.C).

A new era of microbial cell biology

While bacteria were classified as Gram-positive versus negative as well as by shape, the vast diversity among bacteria only became clear decades later, with the entrance of whole genome sequencing. This revolution coincides with significant advances in microscopy and genetics. Without the discovery and development of fluorescent proteins as a tool to see proteins inside the cell in real-life and even follow them over time, no chapter of this thesis would exist.

While fluorescent proteins were used as a tool for fluorescence microscopy for a while in bacteria as *E. coli* and *B. subtilis*, this does not always easily translate to other bacteria. *S. pneumoniae* grows in microaerophilic conditions, while fluorescent proteins need oxygen to mature properly. When studying gene expression, fast maturation is important, but when studying localization, a bright signal and no protein degradation can be more important than maturation time. In our lab, different variants of green fluorescent proteins were tested to see which one to use for

which purpose (Overkamp *et al.* 2013). In the same spirit, in **chapter 2**, we tested different red fluorescent proteins in *Streptococcus pneumoniae* to give advice to the pneumococcal cell biology community on the use of red fluorescent proteins for both promoter and protein fusions. The protein fusions are for this study, to cell cycle protein FtsZ and nucleoid associated protein HU, are used in the next chapters to follow the bacterial cell and chromosome cycle.

1.

From images to insight

Along with the possibility to live-track single cells comes the next problem. How to transform the data of hundreds to thousands of single cells into useful biological insights? While in the beginning, many observations were done by eye and measurements were done by hand, soon different cell segmentation and fluorescence tracking programs were developed by different laboratories. This is an important development, not only to get a better understanding of the microscopy data, but also because automated segmentation can reduce bias. It is unavoidable to see patterns in images sometimes; measurements of these images and subsequent analysis can help to test whether these patterns are true.

Currently, many different cell segmentation programs exist for phase-contrast images of bacteria, all slightly different in their performance. However, after measuring the cells and localizing proteins inside the cell, it is still quite a challenge to analyze this data. To aid researchers in this process, we developed the R package BactMAP, a set of functions to gather cell segmentation data from different programs and experiments, analyze the data and visualize the cellular localization in different ways. The program is described in **chapter 3**.

Replication: a moving force for DNA?

One of the key questions in the seventies – and now, still – was how bacterial chromosomes manage to segregate properly after replication. As shown in **Figure 1.C**, bacterial chromosomes have to be compacted some orders of magnitude in size to be able to fit inside a small cell. This makes it even more astonishing that the chromosomes can still be accessed for replication, translation and segregate properly at the same time. If this does not happen in a coordinated way, it would probably be as hard as trying to disentangle the chord of your headphones while keeping the buds in your ears. One of the hypotheses was that bacterial chromosomes were membrane-bound, with conflicting evidence (Kleppe, Ovrebo, and Lossius 1979; Ryter 1968). In 1974, C. Wesley Dingman proposed his factory model for replication. Most bacteria have a circular chromosome with a single origin of replication from which replication occurs bidirectionally. When the replication machineries of both arms of the chromosome are fixed together at mid-cell, the chromosome arms have to move through – like products on an assembly line (Dingman 1974).

This is an attractive model, mostly because it is so simple. It requires no other regulation factors than something that pins down the replisomes (or replication forks). Over the years, reports supporting and rejecting this hypothesis have come out (Bates and Kleckner 2005; Japaridze *et al.* 2019; Lau *et al.* 2003; Mangiameli *et al.* 2018; Molina and Skarstad 2004; Reyes-Lamothe *et al.* 2008). Alternative hypotheses, like the ‘track’ model, where the replisomes move along the chromosome like a train on a track, and a combined model, were proposed (see Bates 2008 for a review). To this point, two careful conclusions could be made: firstly, there might be no general answer accounting for all bacteria. Secondly, in most cases, the replisomes are close together in the cell, but no compelling evidence for a physical linker between the two replisomes has been given so far. In many symmetrically dividing bacteria, such as *E. coli*, *B. subtilis* and *M. smegmatis*, the replisomes localize at mid-cell (Mangiameli *et al.* 2017; Santi and McKinney 2015).

Using time-lapse imaging of fluorescent protein-fusions of members of the replisome, the two replisomes are visible as one single spot at mid-cell. However, over time, the spot splits into two spots occasionally (Mangiameli *et al.* 2017; Reyes-Lamothe *et al.* 2008). In *Caulobacter crescentus*, the replisomes colocalize and move gradually from the pole to mid-cell (Jensen, Wang, and Shapiro 2001).

When the chromosome becomes less compacted due to the widening of *E. coli* cells (Japaridze *et al.* 2019), replisomes tracking along the chromosome can be observed. Similarly, deleting important chromosome organization genes *parB* or *smc* in *Mycobacterium smegmatis* separated and drastically changed the movement of the replisomes (Santi and McKinney 2015). In **chapter 3 and 4**, we will investigate the localization and dynamics of the pneumococcal replisome.

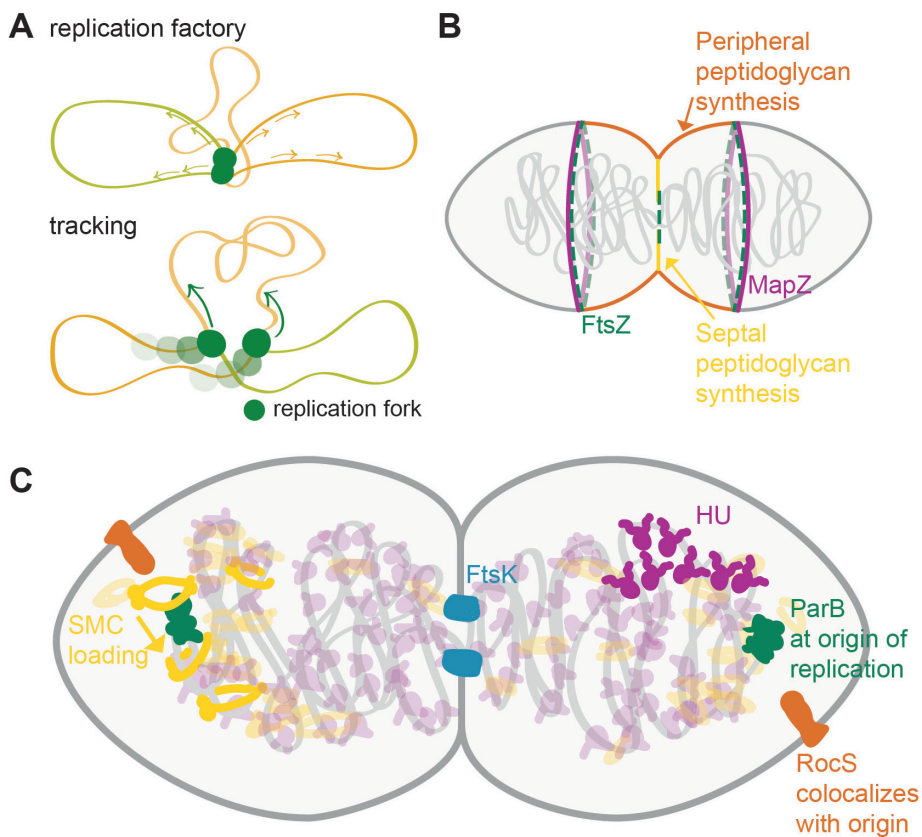


Figure 3. A. Two models for replication: the replication factory (top), where the two replisomes are physically bound and the DNA is spooling through. The tracking model (bottom), where the replication forks are moving along the chromosome while replicating the DNA. **B.** Simplified model for pneumococcal cell division. MapZ (pink) moves along with the newly synthesized peptidoglycan. FtsZ filaments are treadmilling around the septum, where it recruits the cell division machinery. The filaments move to the 1/4 positions of the new cell before cell division to start a new division round. **C.** Overview of the currently known proteins involved in chromosome structure. HU (pink) binds to the chromosome and is involved in DNA supercoiling. FtsK (blue) is an essential protein in *S. pneumoniae*, which localizes at the closing septum (see chapter 5). ParB (green) binds to the origin of replication, where it recruits SMC (yellow). RocS colocalizes with the chromosomal origin and interacts with ParB and the cell membrane.

Keeping it together (or apart)

There are other factors than replication managing the structure and shaping of the chromosome, so it can replicate and segregate while being available for transcription. Maintenance of the chromosome structure is necessary for the chromosome to be able to maintain compacted while being flexible. *In vitro*, long DNA molecules undergo positive supercoiling in high concentrations. *In vivo*, bacterial chromosomes are actively maintained in a negatively supercoiled state. This is especially important during replication: as the replication forks unwind the DNA duplex, bacteria maintain their DNA in a negatively supercoiled state with the help of several proteins: topoisomerases, DNA gyrase and nucleoid associated proteins (NAPs) (Agarwal *et al.* 2019). DNA gyrase induces negative supercoiling, while topoisomerases are important for DNA relaxation. In addition to that, topoisomerase IV (topoIV) is responsible for decatenation of the DNA close to replication termination (El Houdaigui *et al.*, 2019). DNA gyrase and topoIV are essential for cell viability and the target of antibiotics of the fluoroquinolones class.

Nucleoid associated proteins are generally small proteins with low DNA binding specificity. There are many NAPs known and their abundance and essentiality varies among bacterial species. One of the most abundant NAPs is HU (Heat Unstable protein, J Rouvière-Yaniv and Gros 1975). This small, dimer-forming protein was thought to be a bacterial version of histones because addition of (large amounts of) HU to DNA resulted in small round DNA knots *in vitro* (Josette Rouvière-Yaniv, Yaniv, and Germond 1979). Still, even then, this idea was coined with some caution, because the number of HU molecules inside the bacterial cell simply would not be high enough to have the same effect *in vivo*. Short after the discovery of HU in *E. coli*, HU homologs were reported in a wide range of prokaryotes and even in mitochondria (Drlica and Rouvière-Yaniv 1987; Haselkorn and Rouvière-Yaniv 1976).

Many functions are attributed over the years to this small, highly conserved protein. In different bacteria, HU affects gene expression in different ways. While it generally binds to all chromosome regions, in some bacteria it has a preference for AT-rich regions, or a higher binding profile closer to the origin of replication. In *Streptococcus pneumoniae*, HU (also known as HlpA for Histone-Like protein A) is the only known NAP. Pneumococcal HU facilitates negative supercoiling of DNA and is essential. In **chapter 5**, we investigate the role of HU in DNA organization in the pneumococcus.

Two other key players in chromosome organization and segregation are the Par(A)BS (Partitioning) system and the SMC (Structural Maintenance of Chromosomes) complex. ParB is a CTPase that binds to specific DNA sequences (*parS*) located close to the chromosomal origin (Osorio-Valeriano *et al.* 2019; Soh *et al.* 2019). ParA is a Walker-A-type ATPase whose activity can be stimulated by ParB and DNA. The interaction between ParB and ParA drive a directional movement of origin-bound ParB away from mid-cell. Several models exist explaining the mechanism by which ParA directs the movement of ParB (Hürtgen *et al.* 2019). Interestingly, *S. pneumoniae* lacks ParA (Minnen *et al.* 2011). Pneumococcal ParB binds to *parS* sites near the origin of replication, and GFP-bound ParB forms bright foci that segregate from mid-cell to the ¼ positions of the cell (Kjos and Veening 2014). How this movement is directed is unclear. Pneumococcal cells lacking ParB are impaired in chromosome segregation (Minnen *et al.* 2011; Kjos and Veening 2014), but the defect is not as severe as one might expect.

ParBS is however needed to properly load SMC onto the chromosome (Minnen *et al.* 2011). SMC is a member of the condensin family. Condensins are found in bacteria, archaea and eukaryotes alike. SMC forms a ring-like structure, which wraps around the chromosome at the origin of replication (Gruber 2018). From there, SMC migrates along the chromosome. SMC has an important role in the global organization of the chromosome, keeping the two chromosome arms together and/or forming large DNA loops (Ganji *et al.* 2018; Gruber 2018;

Wang, Montero Llopis, and Rudner 2013). In *S. pneumoniae*, deleting SMC leads to a slightly more severe phenotype than a ParB deletion (Kjos and Veening 2014; Minnen *et al.* 2011), but it is not essential under laboratory conditions.

1.

Two other proteins are known to impact chromosome segregation in the pneumococcus. The recently discovered membrane-bound protein RocS interacts and colocalizes with ParB. While the mechanism through which it impacts chromosome segregation is unknown, RocS deletion mutants show defects in segregation, and RocS is synthetic lethal with SMC and ParB (Mercy *et al.* 2019). The second protein is important for late chromosome segregation. FtsK is a motor protein that translocates DNA through the nearly-closing septum (Stouf, Meile, and Cornet 2013). FtsK is essential in *S. pneumoniae*, in contrast to the situation in *B. subtilis* (Marquis *et al.* 2008).

Apart from these chromosome shapers, there are more mechanisms influencing chromosome organization and segregation. In the last years, the role of phase separation in compacting and segregating the nucleoid has come to the surface. The size and compaction of the nucleoid seems to be correlated with ribosome concentration and mobility (Gray *et al.* 2019). Experiments with elongated *E. coli* as well as with L-form entrapped in microfluidic channels show that chromosomes segregate orderly as long as a rod shape is maintained (F. Wu, Swain, *et al.* 2019; L. J. Wu *et al.* 2019). Different models suggest that the combination of molecular crowding and a tubular confinement is enough to stimulate chromosome segregation (Dias, Dias and Rita 2019; Fisher *et al.* 2013; Jun and Wright 2010; De Vries 2010).

Following the chromosome

The bacterial chromosome is so condensed, that conventional fluorescence microscopy images using DNA staining generally reveal not much more than one or more intercellular ‘blobs’. Especially in small bacteria such as pneumococcus, the imaging resolution is not sufficient to reveal any more details. One way to get more information, is to move from snapshots to time-lapse microscopy. Fluorescent protein fusions to HU are popular for this, since HU is highly abundant and is generally distributed along the complete chromosome. In *S. pneumoniae*, a strain carrying HU-mKate2 and ParB-GFP was used to follow both the origin of replication and the nucleoid at the same time. This showed that the origin of replication splits early during the cell cycle (Kjos and Veening 2014).

A more detailed way to follow the chromosome is by marking specific loci on the chromosome with fluorescent DNA-binding proteins. There are several methods to achieve this. The FROS (Fluorescent Repressor-Operator) system uses promoter-repressor systems (for instance TetR/*tetO*): the operator sites are encoded on the location on the chromosome to be visualized, while the repressor is fused to a fluorescent protein (Belmont and Straight 1998). Another, similar method uses ParB/*parS* of another species than the host species, where ParB is bound to a fluorescent protein (Wang, Reyes-Lamothe, and Sherratt 2008). These methods have been used simultaneously to not only follow chromosomal locations, but also relate their localization to the localization of other parts of the chromosome. This led to the insight that different bacterial species segregate their chromosome in a different method: while the *E. coli* splits its chromosome from the middle to the $\frac{1}{4}$ positions from origin to terminus (Bates and Kleckner 2005), the chromosome of *B. subtilis* has a slightly more complex organization (Wang, Montero Llopis, and Rudner 2014). Cells dividing asymmetrically generally also segregate their chromosomes in an asymmetric manner (Viollier *et al.* 2004). In **chapter 4**, we elucidate the chromosomal organization of the pneumococcus by developing FROS systems for *S. pneumoniae* and following different chromosomal loci.

Since the development of superresolution microscopy a small decade ago, another way of unveiling the chromosomal shape has arrived. PALM (PhotoActivated Localization Microscopy) makes use of fluorescent proteins that, upon activation, can switch from one excitation/emission wavelength pair to another (photoswitchable) or where the fluorescence can be activated stochastically (photoactivatable). In both cases, not all fluorescent proteins are activated/switched at the same time. The blinking fluorescent proteins are recorded, after which the localization of each single protein is estimated. This is much more precise than normal fluorescence microscopy, because single molecules are localized without the noise of other molecules. Using PALM imaging, people have tried to determine chromosomal domains in *B. subtilis* and *E. coli* chromosomes (Marbouty *et al.* 2015; Spahn, Endesfelder, and Heilemann 2014).

SIM (Structured Illumination Microscopy) makes use of a patterned grid to enhance the resolution. It has the advantage over PALM that it does not require special fluorescent proteins and that less images are needed for the resulting image. However, the resolution limit of SIM is 50 nm (in x and y directions), while that of PALM is theoretically limitless. In the last years, SIM has become more and more common as a microscopy technique for bacterial cell biology. F. Wu, Japaridze, *et al.* 2019 used SIM on *E. coli* cells that are forced into a flat, round state to reveal a circular chromosome. They observed multiple chromosomal domains, but when removing HU, these clear domains turned into two large domains (left and right chromosome arm). In **chapter 5**, we used both PALM and SIM to image the dynamics and organization of the pneumococcal nucleoid.

Orchestrating the cell cycle

Even when we know how the bacterial chromosomes segregate after replication, the question is how chromosome segregation is coordinated together with cell division. How can the cell divide at the same time without ending up with guillotined nucleoids? How does the cell neatly keep an average of 1.5 chromosome per cell? In different bacteria, different players have been identified that can play a role in this process. The pneumococcus, however, lacks homologues of well-known systems such as Min or nucleoid occlusion.

What we do know, is that pneumococcal cell division revolves around FtsZ, which forms a ring (Z-ring) at mid-cell, where it recruits cell division proteins. FtsZ filaments are moving around in this ring (treadmilling). When the peptidoglycan synthesis machinery is assembled, the cell is growing (peripheral peptidoglycan synthesis, see **Figure 3.B**). The cell division protein MapZ is localized at the place where the new peptidoglycan is formed. Time-lapse microscopy of fluorescent fusions of MapZ show two rings, moving gradually from the old septum to the two new septa. There, FtsZ filaments will form two new Z-rings at the end of the cell cycle. Meanwhile, the septum closes by forming a cross-wall (septal peptidoglycan synthesis, see **Figure 3.B**).

SMC mutants are not only impaired in chromosome segregation, but are also larger, a phenotype more often found in cell division mutants. In **chapter 4**, we explore this further and propose a model where correct chromosome segregation is crucial for proper FtsZ placement. Recently, Gallay *et al.* 2019 discovered a novel protein CcrZ that is important for DNA replication initiation. CcrZ colocalizes and interacts with FtsZ and depletion mutants lead to large numbers of anucleate cells and chromosome guillotining. Interestingly, CcrZ mutants also have reduced DNA initiation. Gallay *et al.* 2019 propose a model where CcrZ is responsible for the firing of DnaA at the origin of replication when it is close to the Z-ring.

Disentangling the pneumococcal chromosome

1.

In this thesis, we aim to understand and visualize the organization of the pneumococcal chromosome. In order to do this, we developed several tools. In **chapter 2**, we investigate which red fluorescent proteins are the most suitable for fluorescent fusions. This is crucial for dual-color fluorescence microscopy, which we use frequently throughout the rest of the thesis. In addition, we show that transforming linear Gibson Assembly products directly into *S. pneumoniae* is a fast and efficient cloning technique. In **chapter 3**, we introduce BactMAP, an R package that can be used to analyze and visualize the data from different cell segmentation programs. We also visualize the origin of replication in three different Gram-positive bacteria, and follow the replisome of *S. pneumoniae*. BactMAP was used to analyze the data in **chapter 4**. In this chapter, we investigate the role of correct origin localization on cell division in *S. pneumoniae* and show that cells with delayed chromosome segregation also have mislocalized MapZ and – to a lower extent – FtsZ. We also show that MapZ is important for the correct placement of FtsZ on the width axis on the cell. Finally, in **chapter 5**, the role of nucleoid associated protein HU on chromosome segregation and structure is investigated. We show that cells with less HU have less condensed chromosomes and chromosome segregation defects. We also use SIM and PALM to investigate the shape and dynamics of the pneumococcal chromosome.

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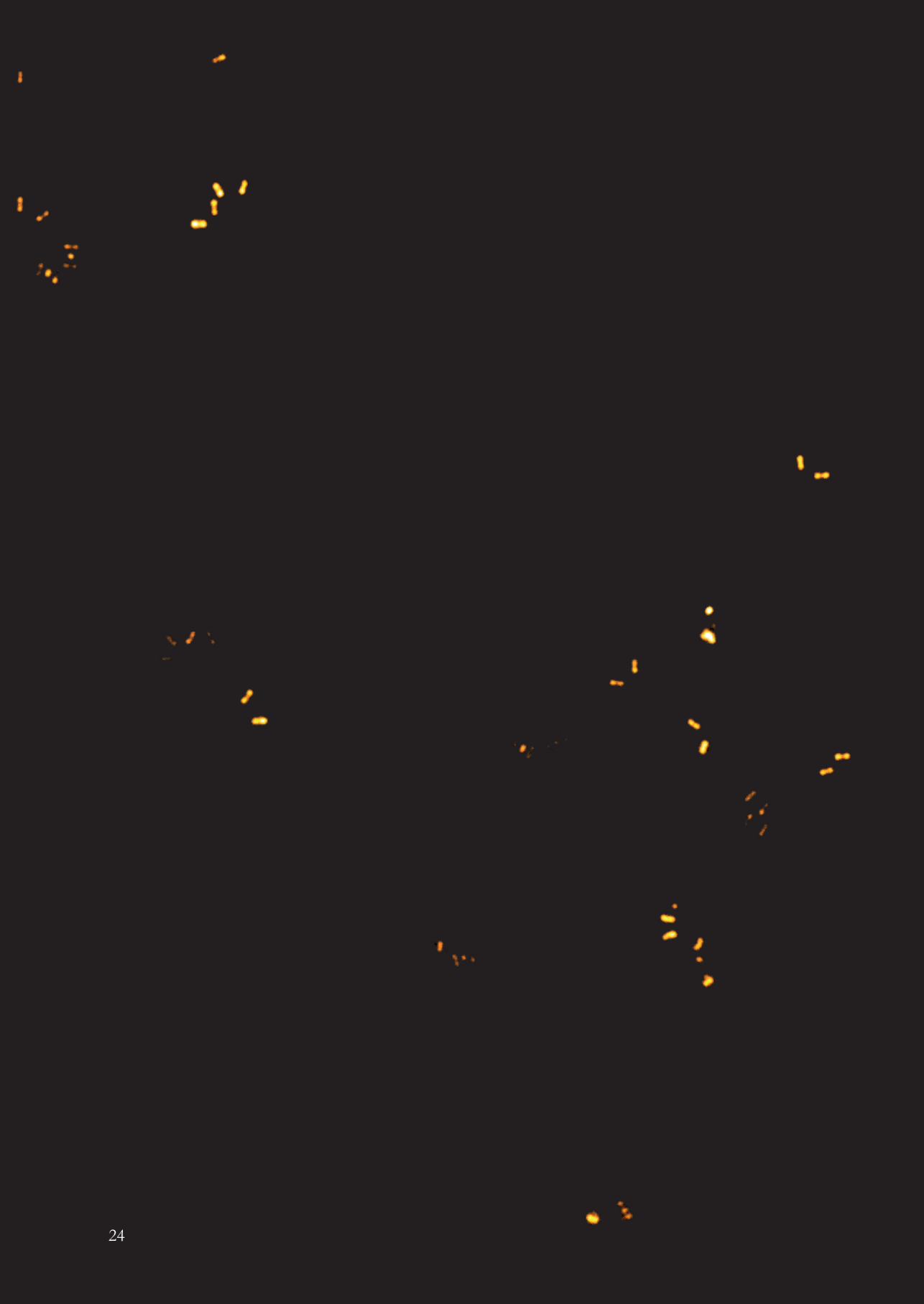
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1.



Chapter 2:

Red Fluorescent Proteins for gene expression and protein localization studies in *Streptococcus pneumoniae* and efficient transformation with DNA assembled via the Gibson Assembly method

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Abstract

2.

During the last decades, a wide range of fluorescent proteins (FPs) have been developed and improved. This has had a great impact on the possibilities in biological imaging and the investigation of cellular processes at the single-cell level. Recently, we have benchmarked a set of green fluorescent proteins (GFPs) and generated a codon-optimized superfolder GFP for efficient use in the important human pathogen *Streptococcus pneumoniae* and other low-GC Gram-positive bacteria. In the present work, we constructed and compared four red fluorescent proteins (RFPs) in *S. pneumoniae*. Two orange-red variants, mOrange2 and TagRFP, and two far-red FPs, mKate2 and mCherry, were codon optimized and examined by fluorescence microscopy and plate reader assays. Notably, protein fusions of the RFPs to FtsZ were constructed by direct transformation of linear Gibson assembly (isothermal assembly) products, a method that speeds up the strain construction process significantly. Our data show that mCherry is the fastest-maturing RFP in *S. pneumoniae* and is best suited for studying gene expression, while mKate2 and TagRFP are more stable and are the preferred choices for protein localization studies. The RFPs described here will be useful for cell biology studies that require multicolor labeling in *S. pneumoniae* and related organisms.

Introduction

Streptococcus pneumoniae is a human pathogen that annually kills about 1.5 million people worldwide (O'Brien *et al.*, 2009). It causes harmful infections such as pneumonia, meningitis, and sepsis, especially in elderly people, young children, and immunosuppressed patients. The increase of antibiotic resistance and the lack of long-lasting vaccines against this organism make it crucial to better understand the cell biology of *S. pneumoniae* and gain insights into essential processes such as cell division, chromosome segregation, and cell wall synthesis. Therefore, the development of tools for cell biological studies in this organism is important.

Studies of protein localization and gene expression at the single cell level have brought new insights into the cell biology of bacteria. Nevertheless, most of our knowledge on bacterial cell biology is based on experiments performed in rod-shaped model organisms such as *Escherichia coli* and *Bacillus subtilis*, and only recently have researchers been able to examine these processes in coccoid bacteria such as *Staphylococcus aureus* and *S. pneumoniae* (Pinho, Kjos and Veening, 2013).

S. pneumoniae is a microaerophilic organism that cannot tolerate high levels of oxygen. Folding and maturation of fluorescent proteins often require sufficient concentrations of oxygen, and microaerophilic conditions might have a negative effect on maturation (Tsien, 1998). More recently, molecular tools to study cell biology of living cells of *S. pneumoniae* have been published (Eberhardt *et al.*, 2009, Henriques *et al.*, 2013, Overkamp, Beilharz, Detert Oude Weme, Solopova *et al.*, 2013), and a set of green fluorescent proteins (GFPs) that can be used in *S. pneumoniae* with adequate efficiency has been described (Overkamp, Beilharz, Oude Weme, Solopova *et al.*, 2013).

The GFP from the jellyfish *Aequorea victoria* was the first FP to be described (Shimomura *et al.*, 1962) and cloned (Chalfie *et al.*, 1994, Prasher *et al.*, 1992). Other FP variants, such as blue, cyan, and yellow FPs, have been engineered from the jellyfish-derived GFP, whereas orange, red, and far-red variants were found in other sea animals (Shaner *et al.*, 2004, Shaner *et al.*, 2008, Shcherbo *et al.*, 2009). Most red fluorescent proteins (RFPs) naturally occur in tetrameric form, and monomerization mutants often resulted in a great loss of photostability and fluorescence intensities. However, in recent years, improved variants have been developed (Shaner *et al.*, 2008, Merzlyak *et al.*, 2009). The four different RFPs that we benchmarked in this study were codon optimized for low-GC Gram-positive bacteria. We chose four monomeric RFPs that can possibly be used in protein fusions. The orange-red fluorescent protein we tested, mOrange2, is a monomeric derivative of DsRed from the coral *Dictyosoma sp.* with improved photostability (Shaner *et al.*, 2008). TagRFP, a monomeric derivative of eqFP578 from the sea anemone *Entacmaea quadricolor* (Merzlyak *et al.*, 2007), is an orange-red FP for which high brightness but low photostability has been reported (Merzlyak *et al.*, 2007). For far-red variants, we tested mCherry (Shaner *et al.*, 2004), another derivative of DsRed and one of the most widely used RFPs in bacterial research, and mKate2, derived from *E. quadricolor* and with excellent pH resistance and photostability (Shcherbo *et al.*, 2009). While the four selected RFPs share a high degree of sequence and structural similarity, mKate2 has the highest reported quantum yield when examined *in vitro* (Shcherbo *et al.*, 2009).

To identify which of the four RFPs is the brightest and most rapidly maturing variant *in vivo* in *S. pneumoniae*, we placed the respective genes under control of inducible promoters P_{Zn} and P_{sigB} and integrated them stably as a single copy within the *S. pneumoniae* genome. The speed of FP maturation is not always important, for instance, when the question is simply where the protein localizes. In such cases, brightness or functionality of the FP fusion might be more crucial. Therefore, we tested the properties of TagRFP, mCherry, and mKate2 when used as C-terminal protein fusions to the tubulin-like protein FtsZ and the histone-like protein HlpA

(also known as HU). We successfully used Gibson's one-step isothermal (ISO) DNA assembly method (Gibson, 2011) to generate several of our constructs. Importantly, using *S. pneumoniae*'s natural competence, we show that pneumococcus can take up and integrate the linear assembly product even directly from the reaction mixture. The RFPs and the use of a fast DNA assembly method (Gibson assembly) for direct transformation presented in this study further expand the cell biological toolbox available for *S. pneumoniae* and related organisms.

2. Materials and Methods

Bacterial strains, plasmids, media, and growth conditions

All strains and plasmids used in the study are listed in Table 1. *Streptococcus pneumoniae* was grown in liquid C+Y medium at 37°C (Martin *et al.*, 1995). Blood agar plates were prepared from Columbia agar with addition of 3% defibrinated sheep blood (Johnny Rottier, Kloosterzande, The Netherlands). The P_{Zn} promoter was induced with addition of $ZnCl_2$ to a final concentration of 0.1 mM or 0.15 mM, and P_{ssbB} was induced by adding 100 ng/ml of competence-stimulating peptide (CSP). *Escherichia coli* DH5 α was used for cloning and grown in LB medium at 37°C with shaking or on LB medium solidified with 1.5% (wt/vol) agar. When required, 100 μ g/mL ampicillin (Amp) for *E. coli* or 1 μ g/mL tetracycline (Tet) and 4.5 μ g/mL chloramphenicol (Cm) for *S. pneumoniae* was used for selection.

Recombinant DNA techniques and oligonucleotides

Procedures for DNA isolation, restriction, ligation, agarose gel electrophoresis, and transformation of *E. coli* were performed as described by Sambrook and Russell (2001). Plasmid DNA and PCR products were isolated and purified using the High Pure plasmid isolation kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Enzymes were purchased from Fermentas (Vilnius, Lithuania) and used as described by the manufacturer. For PCR amplification, Phusion and Taq DNA polymerases (Fermentas) were used. *S. pneumoniae* was transformed as described by Martin *et al.*, 1995. The oligonucleotides used in this study are listed in Table 2 and were purchased from Biolegio (Nijmegen, The Netherlands). Constructs were verified by sequencing.

Codon optimization

mKate2, *tagRFP*, and *mOrange2* were codon optimized for *S. pneumoniae* using OptimumGene (GenScript Inc., Piscataway, NJ, USA), resulting in genes *mKate2(Sp)*, *tagRFP(Sp)*, and *mOrange2(Sp)*, while the sequence for a codon-harmonized variant of *mCherry* (based on the *Bacillus subtilis* codon usage table) was obtained from DSM Biotechnology Center (Delft, The Netherlands), resulting in gene *mCherry(Bs)*. Genes were synthesized by GenScript [*mKate2(Sp)*, *tagRFP(Sp)*, and *mOrange2(Sp)*] or by GeneArt (Regensburg, Germany) [*mCherry(Bs)*]. Note that throughout this paper we refer to these codon-optimized genes as *mKate2*, *tagRFP*, *mOrange2*, and *mCherry*.

Construction of promoter fusion plasmids

Different RFP genes were first fused to the Zn^{2+} -inducible promoter P_{Zn} , resulting in construct *pKB01-PZn-RFP*. *mKate2* and *tagRFP* were amplified from the synthetic genes using primer pairs *mKate-F+XbaI/mKate-R-SpeI* and *tagRFP-F+XbaI/tagRFP-R-SpeI*, respectively, and cut using *XbaI* and *SpeI*. *mCherry* was cut from the GeneArt plasmid pMA-mCherry using *XbaI* and *SpeI* and purified. Digested *mKate2*, *tagRFP*, and *mCherry* were ligated in *XbaI*- and *SpeI*-cut plas-

mid pJWV102 (Overkamp, Beilharz, Oude Weme, Solopova et. al, 2013), resulting in plasmids pKB01-mKate2, pKB01-tagRFP, and pKB01-mCherry, respectively. *mOrange2* was amplified using *mOrange-F*+*NotI*/*mOrange-R*+*SpeI*, cut using *NotI* and *SpeI*, and ligated in similarly digested pJWV102. Fragments were ligated accordingly, resulting in plasmid pKB01-mOrange2.

Subsequently, the zinc-inducible promoter was replaced by the competence-induced promoter P_{ssbB} (Prudhomme *et al.*, 2006), resulting in plasmid pKB01-PssbB- RFP, which was excised from pLA18 using *SphI* and *EcoRI* and introduced into pKB01-RFP plasmids cut with the same restriction enzymes.

Construction of strains with P_{Zn} - and P_{ssbB} -controlled RFP gene expression

S. pneumoniae strains KB1-69, KB1-70, KB1-71, and KB1-72, expressing different RFP variants under control of the Zn^{2+} -inducible promoter (P_{Zn}), were obtained by transformation of wild-type strain D39 with pKB01 derivatives. Correct integration by double crossover in the *bgaA* locus was tested by colony PCR using primer pairs integration 1/in- tegration 2 and integration 5/integration 6.

Similarly, strains KB04-03, KB04-04, KB04-05, KB04-06, expressing RFPs from the competence-activated promoter P_{ssbB} , were obtained by transforming the corresponding plasmids into D39. Correct integration in the *bgaA* locus was checked as described above.

Construction of strains expressing translational HlpA-RFP fusions

The construction of strain MK119 (*hlpA hlpA-mKate2; Camr*), containing an *hlpA-mKate2* fusion (in which *hlpA* and *mKate2* is separated by a flexible linker) and a chloramphenicol resistance gene on the same transcriptional unit as *hlpA*, is described elsewhere (Kjos and Veening, 2014).

To construct strain KB1-64 (D39 *hlpA-mCherry; Camr*), the *hlpA-up* fragment (containing the *hlpA* gene and its promoter) was amplified from D39 chromosomal DNA using *hlpA-up-F*/*hlpA-up-R*+*BamHI*, and the *hlpA-down* fragment (containing ~1,000 bp immediately downstream of the *hlpA* stop codon, including a chloramphenicol resistance marker) was amplified from strain MK119 (D39 *hlpA hlpA-mKate2; Camr*) using primer pair *camR-up-F*+*EcoRI*/*hlpA-down-R*. *mCherry* including a flexible linker was amplified from pMA-mCherry using *linker-mCherry-F*+*BamHI*/*mCherry-R*+*EcoRI*. Fragments were purified and cut using *BamHI* (fragment), *hlpA-up* *BamHI* and *EcoRI* (*linker-mCherry*), or *EcoRI* (*hlpA-down*). Cut and purified fragments were ligated. *S. pneumoniae* D39 was transformed with the ligation mix, and transformants were selected on plates containing chloramphenicol. A single transformant was sequence verified, and this strain was named KB1-64.

To construct strain MK218 (D39 *hlpA hlpA-mCherry; Camr*), an *hlpA-up* fragment (containing *hlpA* and the upstream sequence) was amplified from genomic DNA of D39 using primers *hlpA-up-F* and *hlpA-RSphI*. The *hlpA-mCherry; Camr* fragment (containing the *hlpA-mCherry* fusion with a downstream; *Camr* resistance gene and the *hlpA* downstream sequence) was amplified from strain KB1-64 using primers *hlpAF-rbs-SphI* and *hlpA-down-R*. The two fragments were digested with *SphI* and ligated. The ligation product was transformed into D39, and transformants were selected on plates containing chloramphenicol. Transformants were verified by PCR and sequencing.

To construct strain KB1-65 (D39 *hlpA hlpA-tagRFP; Camr*), the *hlpA-linker-up* fragment, containing an extra copy of *hlpA* including a flexible linker downstream of *hlpA*, was amplified

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Relevant properties	Source or reference
<i>E. coli</i> DH5α	F ⁻ <i>araD139</i> 11(<i>ara-leu</i>)7696 11(<i>lac</i>)X74 <i>galU galK hsdR2 mcrA mcrB1 rpsL</i>	Invitrogen, CA
<i>S. pneumoniae</i>		
D39	Encapsulated strain	Avery, MacLeod and McCarty, 1944
KB1-69	D39 Tet <i>bgaA</i> ::PZn- <i>mOrange2</i>	This study
KB1-70	D39 Tet <i>bgaA</i> ::PZn- <i>tagRFP</i>	This study
KB1-71	D39 Tet <i>bgaA</i> ::PZn- <i>mCherry</i>	This study
KB1-72	D39 Tet <i>bgaA</i> ::PZn- <i>mKate2</i>	This study
MK119	D39 Cam <i>hlpA hlpA-mKate2</i>	Kjos and Veening, 2014
KB1-64	D39 Cam <i>hlpA-mCherry</i>	This study
MK218	D39 Cam <i>hlpA hlpA-mCherry</i>	This study
KB1-65	D39 Cam <i>hlpA hlpA-tagRFP</i>	This study
KB04-03	D39 Tet <i>bgaA</i> ::PssbB- <i>mOrange2</i>	This study
KB04-04	D39 Tet <i>bgaA</i> ::PssbB- <i>tagRFP</i>	This study
KB04-05	D39 Tet <i>bgaA</i> ::PssbB- <i>mCherry</i>	This study
KB04-06	D39 Tet <i>bgaA</i> ::PssbB- <i>mKate2</i>	This study
DRR27	D39 Ery <i>ftsZ-mCherry</i>	This study
DRR28	D39 Ery <i>ftsZ-mKate2</i>	This study
DRR54	D39 Ery <i>ftsZ-tagRFP</i>	This study
MK125	D39 Cam <i>hlpA hlpA-mKate2</i> Tet <i>bgaA</i> ::PZn- <i>gfp-stkP</i>	This study
MK289	D39 Cam <i>hlpA hlpA-mCherry</i> Tet <i>bgaA</i> ::PZn- <i>gfp-stkP</i>	This study
MK290	D39 Cam <i>hlpA, hlpA-tagRFP</i> Tet <i>bgaA</i> ::PZn- <i>gfp-stkP</i>	This study
MK292	D39 Ery <i>ftsZ-mCherry</i> Tet <i>bgaA</i> ::PZn- <i>gfp-stkP</i>	This study
MK293	D39 Ery <i>ftsZ-mKate2</i> Tet <i>bgaA</i> ::PZn- <i>gfp-stkP</i>	This study
MK294	D39 Ery <i>ftsZ-tagRFP</i> Tet <i>bgaA</i> ::PZn- <i>gfp-stkP</i>	This study
Plasmids		
pKB01_sfgfp(<i>Sp</i>)	<i>bla tet bgaA</i> ::PZn- <i>sfgfp</i> (<i>Sp</i>)	Overkamp <i>et al.</i> , 2013
pKB01_mOrange2	<i>bla tet bgaA</i> ::PZn- <i>mOrange2</i> (<i>Sp</i>)	This study
pKB01_tagRFP	<i>bla tet bgaA</i> ::PZn- <i>tagRFP</i> (<i>Sp</i>)	This study
pKB01_mCherry	<i>bla tet bgaA</i> ::PZn- <i>mCherry</i> (<i>Bs</i>)	This study
pKB01_mKate2	<i>bla tet bgaA</i> ::PZn- <i>mKate2</i> (<i>Sp</i>)	This study
pKB01-PssbB-mOrange2	<i>bla tet bgaA PssbB-mOrange2</i> (<i>Sp</i>)	This study
pKB01-PssbB-tagRFP	<i>bla tet bgaA PssbB-tagRFP</i> (<i>Sp</i>)	This study
pKB01-PssbB-mCherry	<i>bla tet bgaA PssbB-mCherry</i> (<i>Sp</i>)	This study
pKB01-PssbB-mKate2	<i>bla tet bgaA PssbB-mKate2</i> (<i>Sp</i>)	This study
pJWV25-stkP	<i>bla tet bgaA PZn-gfp-stkP</i>	Beilharz <i>et al.</i> , 2012
pLA18	<i>bla tet bgaA::PssbB-luc-gfp</i>	Slager <i>et al.</i> , 2014

Table 2 Oligonucleotides used in this study

Oligonucleotide	sequence (5' to 3') ^a
mOrange-F+NotI	GCAGGCGGCCGCGAGGAAAATTAATGTCTAAGGGAG
mOrange-R+SpeI	GCAGACTAGTCTCATGAATCTTTCTCGAGTTAGG
mKate2-F+XbaI	GCAGTCTAGAGGAGGAAAATTAATGTCAGAACTTATCAAGG
mKate2-R+SpeI	GCAGACTAGTGCGAGATCTCATGAATCTTTCTCG
tagRFP-F+XbaI	GCAGTCTAGAGGAGGAAAATTAATGTCAGAAC
tagRFP-R+SpeI	GCAGACTAGTCTCATGAATCTTTCTCGAGTTAGGATCC
hlpA-up-F	AACAAGTCAGCCACCTGTAG
hlpA-up-R+BamHI	CTGCGGATCCTTTAACAGCGTCTTTAAGAGCTTTACCAGC
camR-up-F+EcoRI	ACTCGGAATCTTAATGAGCACTAGTAGGAGGCATATC
hlpA-down-R	CGTGGCTGACGATAATGAGG
Linker-mCherry-F+BamHI	CGATGGATCCGGATCTGGTGGAGAAGCTGCAGCTAAAGGAAGCAAAGGAG-AAGAAGATAACATGGCAATCATC
mCherry-R+EcoRI	GCGCGAATCTTATTATTTGTAAAGCTCATCCATTCCGCC
hlpA-F-rbs-SphI	CGTGCATGCTGGAGGAATCATTAACATGGCA
hlpA-R-SphI	CGCGCATGCAGACTGATTATTAACAGCGTC
hlpA-linker-up-R+BglII	GCGCAGATCTTCTTTAGCTGCAGCTTCTCC
mKate2-tagRFP-F+BamHI	CGTGGATCCTCAGAACTTATCAAGG
tagRFP-R+EcoRI	GCATGAATCTTATTAACGGTGTCCCAATTTACTAG
integration 1	CTTGATGAAACCTACATTTG
integration 2	GCTTCCATTAAGGATAGTTC
integration 5	GCTATCGCTGAGCGCCGG
integration 6	AGCTAGAGTTCGCAATTGG
FtsK-down-RV	ATCAAAACCGAACTCACCTGTTGAT
ftsZ-down_FW_eryOE	GGGAGGAAATAAGCGGCCGATGAATGTAAAAGAAAATACAGAACTTG
ftsZ-up-FW	CCTGTTATTGCTCGTATCGCCAAA
ftsZ-up-RV-mCherryOE	GCTTCTCCACCAGATCCGGAACGATTTTGTAAAAATGGAGGTG
ery-FW-mcherryOE	GAATGGATGAGCTTTACAAATAATAAGCCCGGAGGAATTTTCATATGAAC
ery-RV-ftsZOE	CAAGTTCTGTATTTCTTTTACATTCATCGGCCGCTTATTTCTCTCC
ery_F_mKateOE	GTAAATTGGGACACCGTTAATAACCCGGAGGAATTTTCATATGAAC
mKate_RV_eryOE	GTTTCATATGAAAATTCCTCCGGGTATTAACGGTGTCCCAATTTAC
tagRFP_RV_eryOE	GTTTCATATGAAAATTCCTCCGGGCTAGTTTATTTATGTCCCAATTTAC
ery_F_tagRFP OE	GTAAATTGGGACATAAAATAAAGTCCCGGAGGAATTTTCATATGAAC
FtsZ_checkRV	AGTGGTGCCGATATGG
FtsZ_checkFW	TGACAGCGAAGGCTAC
mCherry-RV-eryOE	GTTTCATATGAAAATTCCTCCGGGCTTATTATTTGTAAAGCTCATCCATTC
mCherry-FW-ftsZOE	CACCTCCATTTTCAAAAATCGTTCCGGATCTGGTGGAGAAGC

^a Underlined sequences represent the endonuclease restriction sites; italic sequences represent the overlapping sequences for Gibson assembly.

from MK119 using primer pair *hlpA-up-F/hlpA-linker-R*+*BglIII*, and the *hlpA-down* fragment was amplified as described for KB1-64. *tagRFP* was amplified from pUC57-*tagRFP* using primers *mKate2-tagRFP-F+BamHI/tagRFP-R+EcoRI*. Fragments were purified and cut with *BglIII* (*hlpA-linker-up*), *EcoRI* (*hlpA-down*), and *BamHI* and *EcoRI* (*tagRFP*). The fragments were ligated and directly transformed into *S. pneumoniae* D39. Positive transformants were selected on plates containing chloramphenicol. A single transformant was sequence verified, and the strain was named KB1-65.

2.

Gibson assembly for construction of FtsZ-RFP strains

Strains expressing the different *ftsZ*-RFP fusions were constructed by direct transformation of linear Gibson assembly products. The parts for strain DRR27 were assembled using Gibson's one-step ISO assembly of overlapping double-stranded DNA (dsDNA) (Gibson, 2011). The *ftsZ-up* and *ftsZ-down* fragments were amplified from chromosomal DNA using *ftsZ-up-FW* and *ftsZ-up-RV-mCherryOE* for *ftsZ-up* and *ftsZ-down-RV* and *ftsZ-down_FW_eryOE* for *ftsZ-down*. The fragment encoding erythromycin resistance was amplified from plasmid JWV502 using *ery-FW-mCherryOE* and *ery-RV-ftsZdOE*. *mCherry* including a flexible linker was amplified from strain KB1-64 using primers *mCherry-RV-eryOE* and *mCherry-FW-ftsZuOE*. All four parts included a sequence of around 20 bp (Table 2) that overlapped with the next part. Gibson one-step ISO assembly was done as described by Gibson (2011), and 10 μ l of the reaction mixture was directly transformed into *Streptococcus pneumoniae* D39.

For DRR28 and DRR54, *ery-ftsZdown* was amplified from strain DRR27 using *ery-F-mKateOE* and *ftsZ-down-RV* for DRR28 and *ery-F-tagRFP*OE and *ftsZ-down-RV* for DRR54. *mKate2* was amplified from strain MK119 using *mCherry-FW-ftsZuOE* and *mKate_RV_eryOE*, and *tagRFP* was amplified from strain KB1-65 using *mCherry-FW-ftsZuOE* and *tagRFP_RV_eryOE*. *ftsZ-up* was amplified the same way as for DRR27. For DRR28, *ery-ftsZdown* (with overlap with *mKate2*), *ftsZ-down*, and *mKate2* and for DRR54, *ery-ftsZdown* (with overlap with *tagRFP*), *ftsZ-down*, and *tagRFP* were combined in equimolar amounts for Gibson assembly and directly transformed into *S. pneumoniae* D39. Integration via double crossover was checked in all three strains using primers *FtsZ-checkRV* and *FtsZ-checkFW*.

Construction of RFP/GFP-double-labeled strains

Double-labeled strains with *GFP-stkP* and *ftsZ*-RFP were made by transformation of plasmid pJWV25-*stkP* (19) into the FtsZ-RFP fusion strains DRR27, DRR28, and DRR54, to generate strains MK292 (*ftsZ-mCherry*, *GFP-stkP*), MK293 (*ftsZ-mKate2*, *GFP-stkP*), and MK293 (*ftsZ-TagRFP*, *GFP-stkP*).

Double-labeled strains with *GFP-stkP* and *hlpA*-RFP were made in a similar manner by transformation of plasmid pJWV25-*stkP* into *hlpA*-RFP fusion strains to generate MK125 (*hlpA-mKate2*, *GFP-stkP*), MK289 (*hlpA-mCherry*, *GFP-stkP*), and MK290 (*hlpA-TagRFP*, *GFP-stkP*).

Fluorescence measurements using a plate reader

S. pneumoniae RFP gene-expressing strains and the D39 wild-type strain were grown at 37°C in liquid C+Y medium to an optical density at 600 nm (OD₆₀₀) of 0.1 and diluted 1:100 with fresh C+Y containing 0.15 mM ZnCl₂ for induction of P_{Zn}. Cells were then further grown and harvested at an OD₆₀₀ of 0.15. For plate reader assays, cells were first collected by centrifugation and washed with phosphate-buffered saline (PBS). Fluorescence was measured with the following equipment and settings: Infinite 200 Pro plate reader (Tecan Group Ltd.) with I-control 1.7.1.12 software (Tecan Group Ltd.), RFP filter set (excitation at 590 nm and emission at 635 nm with a 50% dichroic mirror), and RFP signals collected as top readings with a gain setting of 100. RFP

fluorescence values were corrected for background fluorescence, OD₆₀₀, and negative controls (values for the wild-type strains). The OD₆₀₀ levels used were corrected for the background value of the corresponding buffer.

Fluorescence microscopy

Cells of *S. pneumoniae* were grown at 37°C in liquid C+Y medium to an OD₆₀₀ of 0.1, diluted 1:100 with fresh C+Y, and further grown to an OD₆₀₀ of 0.05 before 0.15 mM ZnCl₂ was added to the cultures for induction of P_{Zn}. Cells were then further grown and collected at different time points. For microscopy, 0.4 µl of the cell suspension was directly spotted onto a microscope slide carrying a thin layer of 1.2% agarose in PBS covered with a coverslip. Images were taken with an Olympus IX71 microscope (Personal DV, Applied Precision; assembled by Imsol [Preston, United Kingdom] and Chromaphor [Münster, Germany]) using a CoolSNAP HQ2 camera (Princeton Instruments, Trenton, NJ, USA) with a 100X phase-contrast objective and Trulight (Deltavision, GE Healthcare, USA) illumination. The following standard fluorescence filter sets from Chroma Technology Corporation (Bellows Falls, VT, USA) were used to visualize RFPs: for mCherry, excitation at 575/25 nm, emission at 632/60 nm, and polychroic mirror QUAD2 (580 to 630 nm); for tetramethyl rhodamine isocyanate (TRITC), excitation at 542/27 nm, emission at 594/45 nm, and polychroic QUAD1 (550 to 630 nm). (Note that the second number indicates the bandwidth around the wavelength; e.g., 575/25 indicates excitation with light from 562.5 to 587.5 nm). For the Zn²⁺-inducible constructs, the exposure time was 1.4 s with 100% excitation for all RFPs. To visualize the RFP fusions to HlpA, an exposure time of 0.9 s was used. Softworx 3.6.0 (Applied Precision, Issaquah, WA, USA) software was used for image capturing. Phase-contrast images were segmented automatically, and fluorescence signal intensities were extracted using Microbetracker (Sliusarenko *et al.*, 2011). Fluorescence levels were corrected for background fluorescence of the medium.

Western blot analysis and immunodetection

Cells were grown in C+Y liquid medium at 37°C to an OD₆₀₀ of 0.1, diluted 1:100 in C+Y containing 0.15 mM ZnCl₂ for induction, and harvested at an OD₆₀₀ of 0.15. Cells were harvested by centrifugation at 8,000 rpm for 10 min. For lysis, the pellet was resuspended in 100 µl of SEDS lysis buffer (150 mM NaCl, 15 mM EDTA, 0.02% SDS, 0.01% deoxycholate) and was incubated for 5 min at 37°C. Lysates were diluted in 100 µl 2X SDS loading buffer and boiled for 5 min. After separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to a polyvinylidene di-fluoride (PVDF) membrane by Western blotting. Red fluorescent proteins were detected with polyclonal anti-RFP antibody (Molecular Probes) and rabbit anti-IgG-horseradish peroxidase (HRP) antibody (GE Healthcare) according to the manufacturer's instructions. Note that the polyclonal anti-RFP antibodies recognize all four RFPs due to the high structural homology.

Nucleotide sequence accession numbers

The sequences of the *mKate2*, *tagRFP*, *mOrange2*, and *mCherry* genes have been deposited in GenBank under accession numbers KJ908191, KJ908188, KJ908189, and KJ908190, respectively.

Results and Discussion

Expression of codon-optimized RFPs in *S. pneumoniae*

2. New variants of monomeric red fluorescent protein have been discovered and developed in recent years. They are important tools for use in protein fusions to study protein localization and dynamics and for multicolor labeling, for instance, in combination with GFP, cyan fluorescent protein (CFP), or yellow fluorescent protein (YFP). In the past, the brightness of monomeric RFPs has been problematic, but more recently, improved variants have been described. We selected four of these “next-generation” variants of red fluorescent proteins and codon optimized the genes for low-GC Gram-positive bacteria (Table 3), including those for mOrange2 and mCherry from the “mFruits” palette (Shaner *et al.*, 2008), as well as TagRFP (Merzlyak *et al.*, 2007) and mKate2 (Shcherbo *et al.*, 2009). mCherry is one of the most commonly used RFPs, with a reported short maturation time (varying between 15 min (Shaner *et al.*, 2004) and 40 min (Merzlyak *et al.*, 2007)) but a relatively poor photostability (Shaner *et al.*, 2008). mOrange2 is an orange-red-emitting FP variant and was reported to have a high photostability and high intrinsic brightness but a relatively long maturation time (~ 4.5 h) compared to the doubling time of *S. pneumoniae* (~ 30 min under our experimental conditions) (Shaner *et al.*, 2008). TagRFP is a bright orange-red fluorescent protein with a reported maturation time of 100 min and high brightness (Table 3) (Shaner *et al.*, 2008, Merzlyak *et al.*, 2009). mKate2 was also reported to have a short maturation time (<20 min) and good photostability (Table 3) (Shcherbo *et al.*, 2009). mKate2 and mCherry are far-red variants and therefore highly suitable for the use in combination with green and yellow fluorescent proteins. All variants are reported to be monomeric which makes them suitable for use in protein fusions. In order to directly compare the four RFPs (mOrange2, TagRFP, mCherry, and mKate2), we used plasmid pJWV102 (Overkamp, Beilharz, Oude Weme, Solopova *et al.*, 2013), a pPP2 derivative (Halfmann *et al.*, 2007) containing the Zn^{2+} -inducible promoter P_{Zn} (Eberhardt *et al.*, 2009) or competence-inducible promoter P_{ssbB} (Prudhomme *et al.*, 2006), that integrates in the chromosome by double crossover at the nonessential *bgaA* locus. To exclude translational differences, the same ribosome binding site (RBS) was used for all RFPs. This allows for evaluation and unbiased comparison of the fluorescence intensities and folding times of the individual RFPs. A schematic presentation of chromosomally recombined RFP gene-carrying vectors is shown in Figure 1.

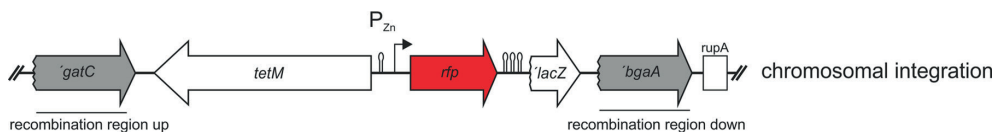


Figure 1. Inducible RFP expression in *Streptococcus pneumoniae*. The scheme for RFP gene expression strains after integration of the P_{Zn} -*rfp* promoter fusion at the nonessential *bgaA* locus by double crossover is shown. Strains were constructed by transformation with plasmids pKB01- P_{Zn} -*rfp* that carry P_{Zn} -*rfp* (*mCherry*, *mOrange2*, *tagRFP*, or *mKate2*) and a tetracycline resistance marker between the homologous recombination sites (shown in gray; *gatC* and *bgaA*). Lollipops depict transcriptional terminators. Similar constructs where the P_{Zn} promoter was replaced with the P_{ssbB} promoter were also made (see Figure S1 in the supplemental material).

Table 3 Red fluorescent proteins

Fluorescent protein	Maximum λ_{ex} (nm)	Maximum λ_{em} (nm)	Color	Brightness ^a	Reference
mOrange2	549	565	Orange	35	Shaner <i>et al.</i> , 2008
TagRFP	555	584	Red	40	Merzlyak <i>et al.</i> , 2007
mCherry	587	610	Far red	16	Shaner <i>et al.</i> , 2008
mKate2	588	633	Far red	25	Shcherbo <i>et al.</i> , 2009

^a Brightness was determined as follows: (extinction coefficient X quantum yield)/1,000.

Comparison of RFP intensities at the population and single-cell levels

The intensities of the different RFPs were analyzed at the population level using a plate reader and at the single-cell level using epifluorescence microscopy. First, to check whether all RFPs were expressed under our experimental conditions, we performed immunodetection on whole-cell lysates using an anti-RFP polyclonal antibody (Molecular Probes, Life Technologies). Cells were grown to exponential phase in the presence of Zn^{2+} for induction, and immunodetection was performed on whole-cell extracts, which allows for only relative quantification of protein levels. All fluorescent proteins were efficiently produced in *S. pneumoniae*, and only mCherry showed signs of protein degradation (Figure 2A). Fluorescence measurements with the plate reader were also performed on Zn^{2+} -induced cells in exponential phase which were resuspended in PBS. The plate reader we used contains a standard RFP filter set (excitation [λ_{ex}], 590 nm; emission [λ_{em}], 635 nm) which is best suitable for the far-red FPs. Not surprisingly, the signals for cells expressing TagRFP and mOrange2 hardly appeared above background. Interestingly, mCherry had the highest signal-to-noise ratio, with approximately 6-fold higher fluorescence than mKate2 (Figure 2B), while mKate2 was reported to be brighter in vitro (Shcherbo *et al.*, 2009).

For fluorescence microscopy, cells were grown to an OD_{600} of 0.05 before expression of RFPs was induced with 0.15 mM ZnCl_2 . Cells were then harvested for microscopy at different time points. We used filter sets that are standard in most fluorescence microscopes: mCherry (Aex, 575/25 nm; Aem, 632/60 nm; QUAD 2 polychroic [580 to 630 nm]) and TRITC (Aex, 542/27 nm; Aem, 594/45 nm; QUAD 1 polychroic [550 to 630 nm]). The same light output and exposure times were used for all samples. Using the mCherry filter set, signals from mCherry and mKate2 were already visible after 30 min of induction (Figure 2C). After 120 min, TagRFP also showed good fluorescence, similar in strength to that of mKate2 but still approximately 6 times less bright than mCherry (Figure 2C). With these imaging conditions, signals for mOrange2 could not be detected. When the TRITC filter set was applied, which should be better suited for mOrange2 and TagRFP than for the far-red RFPs, mCherry still outperformed the other RFPs (Figure 2D). With this filter set, TagRFP gave a good signal above background, with approximately half of that of mCherry for the different time points, while the signal from mKate2 was consistently very low, and mOrange2 still gave no signals above background levels after 3.5 h.

To rule out that fluorescence intensity differences depended specifically on the Zn^{2+} regulation, we swapped the zinc-inducible promoter P_{Zn} with the competence-induced promoter P_{ssbB} (Prudhomme *et al.*, 2006) and measured fluorescence intensities after 1.5 h of induction with the competence-stimulating peptide (CSP) using the same filter sets (see Figure S1 in the supplemental material). The results are consistent with the ones we obtained for the zinc induction after 1.5 h. It should be noted that the TRITC filter set and the QUAD1 polychroic mirror might block a large amount of the emitted photons from mOrange2. Also, the slow fluorophore maturation

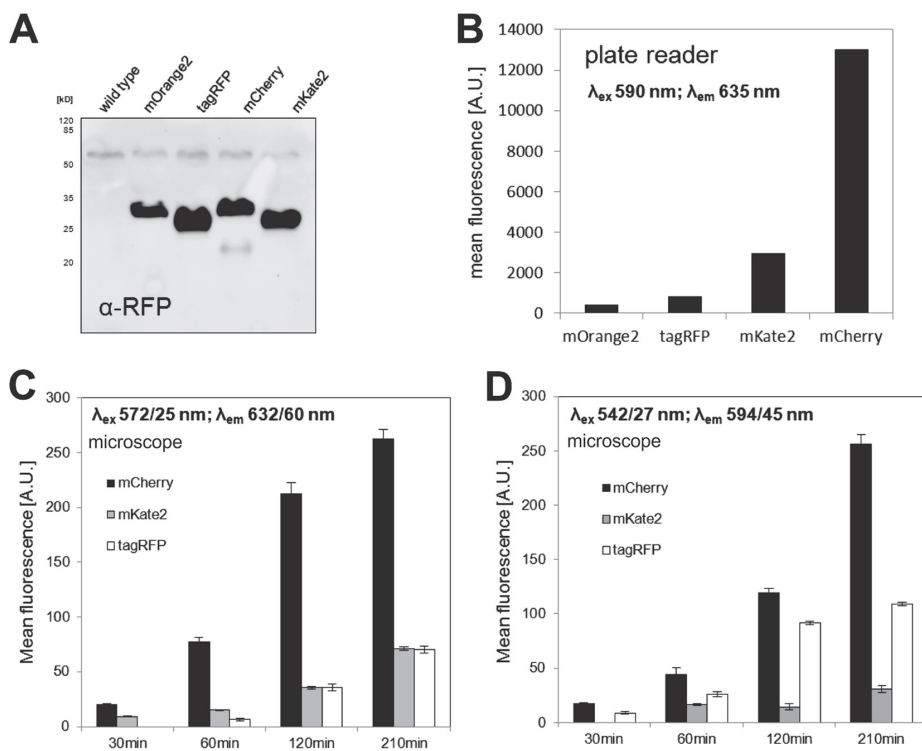


Figure 2. Fluorescence intensities of the different red fluorescent proteins. (A) Western blot analysis of expression levels of RFPs in whole-cell extracts detected using anti-RFP antibody. (B) Mean fluorescence on the population level determined using a plate reader equipped with a standard RFP filter set. See Materials and Methods for details of the filters used. (C and D) Average fluorescence intensities on the single-cell level measured by fluorescence microscopy using an mCherry filter set and polychroic mirror QUAD2 (C) or a TRITC filter set and polychroic mirror QUAD (D). Values are in arbitrary units (A.U.). Single-cell fluorescence levels were determined using MicrobeTracker (Slusarenko *et al.*, 2011). Error bars depict the standard error of the mean. mOrange2 values are not shown in the plots since signals above background level could not be detected.

time of mOrange2 (Shaner *et al.*, 2008) might be an explanation for the absence of signal and thus makes this FP unsuitable for imaging in *S. pneumoniae*. TagRFP gives appreciable signals with a standard TRITC filter set and is a good orange-red RFP for use in *S. pneumoniae* (Figure 2D), but it might be less suitable in multicolor experiments that include yellow fluorescent proteins due to possible spectral overlap. In total, these results show that mCherry is the fastest maturing RFP in *S. pneumoniae* with the highest fluorescence signal when used in promoter fusions. This is probably not simply because of more efficient protein production, since all four RFPs seem to be produced to approximate similar levels using qualitative Western blotting (Figure 2A).

Fast assembly of protein fusion constructs by Gibson assembly

In previous studies, mCherry has been used to tag *S. pneumoniae* FtsZ (Sham *et al.*, 2011) and the capsule protein Wze (CpsD) (Henriques *et al.*, 2013), while mKate2 has been used to tag the eukaryotic-type serine/threonine kinase StkP (Beilharz *et al.*, 2012) and also FtsZ (Fleuriot *et al.*, 2014). However, a systematic comparison of how different RFPs perform when fused to proteins for localization studies has not been made.

In order to compare the performances of different RFPs in protein fusions, the far-red and

fast-folding mCherry and mKate2, as well as the red-orange TagRFP, were all fused to the C-termini of FtsZ and HlpA (also called HU or Hup, SPD_0997). TagRFP has been suggested as a suitable FP for protein fusions but, unlike mCherry and mKate2, has not yet been used for this purpose in *S. pneumoniae*. mOrange was not included in this part of the study, since we did not observe any signal when this FP was expressed alone (Figure 2).

While fusions to *hlpA* were constructed by conventional methods (see Materials and Methods), the FtsZ-RFP constructs were all made using Gibson's one-step ISO assembly of overlapping ds-DNA (Gibson assembly) (Gibson, 2011) (Figure 3A). With this method, the four different parts of the construct were amplified with 40-bp overlaps, and the different parts could then be fused without any restriction or ligation steps. Since no restriction sites are needed, it is possible to choose the optimal sequence for protein linkage. Importantly, as *S. pneumoniae* readily takes up linear DNA fragments via the competence pathway, we wondered whether we could directly use the Gibson assembly product, which is assembled in 1 h, to transform *S. pneumoniae* without any intermediate cleanup or amplification steps. Indeed, transformation with unpurified Gibson mixtures readily yielded correct transformants. Thus, this direct transformation of assembly products is a fast and efficient method for strain construction and is now the preferred construction method for linear fragments in our laboratory.

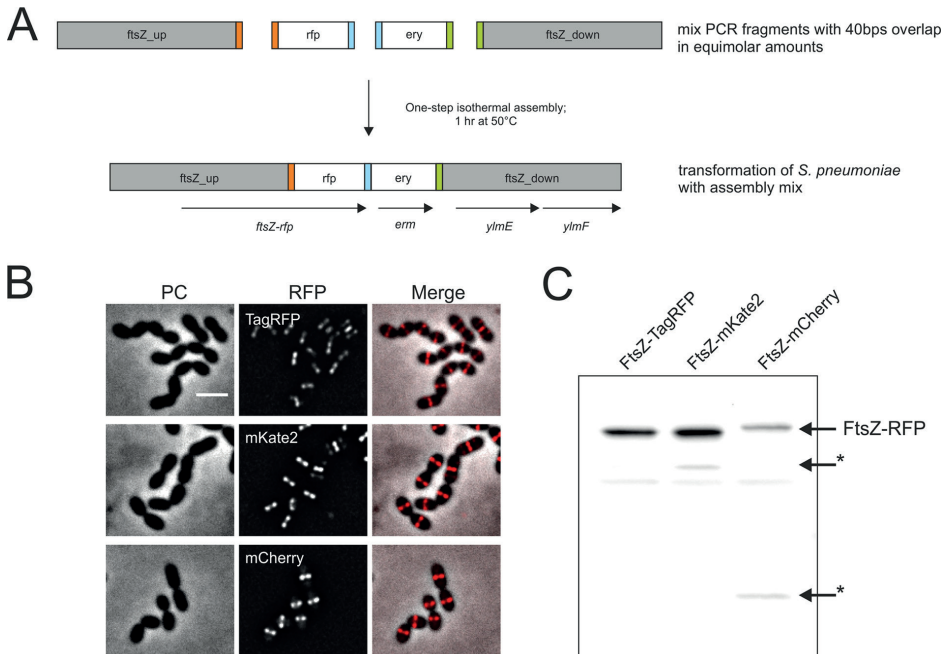


Figure 3. Fast generation of RFP fusions to FtsZ in *S. pneumoniae*. **A.** Gibson assembly procedure (Gibson, 2011) for construction of FtsZ-RFP-expressing strains. **B.** Images show localization of FtsZ fused to TagRFP, mKate2, and mCherry. Phase-contrast (PC), RFP, and overlay (merge) images are shown. Scale bar, 2 μ m. **C.** Immunodetection of FtsZ-RFP fusion proteins in whole-cell extract by anti-RFP antibody. Asterisks indicate degradation products.

Characterization of RFPs in a fusion to FtsZ and HlpA

The highly conserved tubulin-like protein FtsZ polymerizes at the septum during early cell division. The localization pattern of the FtsZ ring is well studied in bacteria, making it an excellent candidate for comparing the performances of the three red fluorescent proteins. HlpA is the only known histone-like protein encoded in the *S. pneumoniae* D39 genome (Lanie *et al.*, 2007) and binds unspecifically to DNA and thus localizes to the nucleoid in a fashion similar to that for the intercalating DNA dye DAPI (4',6'-diamidino-2-phenylindole) (Kjos and Veening, 2014). C-terminal fusions of *ftsZ* to *tagRFP*, *mCherry*, and *mKate2* were obtained by Gibson assembly replacing wild-type *ftsZ*. We also attempted to construct C-terminal fusions of these fluorescent proteins to HlpA by integrating the fusion gene into the native locus. Interestingly, we obtained the *hlpA-mCherry* fusion strain but were unable to make strains expressing the HlpA-mKate2 and HlpA-TagRFP fusions. In our attempts to make these strains, we always observed mutations in the linker sequence generating premature stop codons or deletion of (parts of) the RFP genes. Therefore, we instead constructed merodiploid strains where the *hlpA* fusions are present as an extra copy immediately downstream of the native *hlpA* gene and thus also under the control of the endogenous *hlpA* promoter. These strains could be readily made (Table 1) (see Materials and Methods).

Fluorescence microscopy of cells growing in mid-exponential phase showed that the total amount of fluorescence of both FtsZ and HlpA fused to TagRFP was comparable to that of the fusions with mKate2, whereas signals of the mCherry fusion proteins were lower (data not shown). Signals for the FtsZ-mKate2 and FtsZ-TagRFP strains appeared as sharp defined Z rings at midcell even before deconvolution (Figure 3B and 4A), whereas FtsZ-mCherry demonstrated a slightly more diffuse and heterogeneous signal (Figure 3B and 4A). The same was the case for the HlpA-fusions; the merodiploid TagRFP and mKate2 fusions appeared as sharp defined nucleoid-localized spots in the cytoplasm (Figure 4B; see Figure S2 in the supplemental material), whereas both HlpA-mCherry strains frequently demonstrated a more diffuse and heterogeneous signal (Figure 4B; see Figure S2 in the supplemental material).

Western blot analysis of both proteins showed the presence of degradation products in the FtsZ-mCherry strain and in both HlpA-mCherry strains, whereas for fusions with mKate2 and TagRFP, no or only marginal degradation was observed (Figure 3C; see Figure S2B in the supplemental material). Degradation of mCherry might lead to a pool of untagged HlpA, which might be the reason why the single copy HlpA-mCherry could be made whereas this was not possible with mKate2 and TagRFP. In total, these results show that mCherry originating from our codon-optimized gene is more readily degraded than mKate2 and TagRFP in *S. pneumoniae* (Figure 2A and 3C), making the latter RFPs more suitable for protein fusions. Note that protein functionality always needs to be tested, since FP fusions may not always be fully functional, as probably is the case for the HlpA-mKate2 and HlpA-TagRFP fusions.

Far-red fluorescent proteins are particularly suitable for double-labeling experiments in combination with GFP or YFP due to nonoverlapping excitation and emission spectra and thus limited chance for cross talk. We show that all three RFPs can be successfully used in combination with GFP using our standard filter sets, as exemplified by double-labeled strains showing the localization of two cell division proteins, FtsZ and StkP (Figure 4A), or localization of the division site relative to the nucleoid (Figure 4B).

Final remarks

Here we benchmarked four commonly used RFPs (mOrange2, TagRFP, mCherry, and mKate2) in the important human pathogen *S. pneumoniae*. The genes encoding these RFPs were codon optimized, and Western blotting showed that all proteins are efficiently produced in *S. pneumoniae*.

Under our experimental conditions, no fluorescent signals were detected with mOrange2, likely because of its long maturation time. The three other RFPs were all reported to be fast folding. However, mCherry clearly was the fastest-folding variant in our assays and generated the highest fluorescence signals, and it is thus the best RFP to use in promoter fusions. On the other hand, mCherry is also partially degraded, making it less suitable in protein fusions. It would be interesting to identify the degradation site in mCherry and mutate it to generate a superior mCherry. However, for the time being, we recommend the use of mKate2 or TagRFP as a red fluorescent localization marker protein in *S. pneumoniae*. Further, we showed that mKate2, TagRFP, and mCherry are suitable for dual-color imaging using standard filter sets. Finally, we demonstrated that direct transformation of Gibson assembly products could be used as a fast and straightforward cloning method in *S. pneumoniae*.

2.

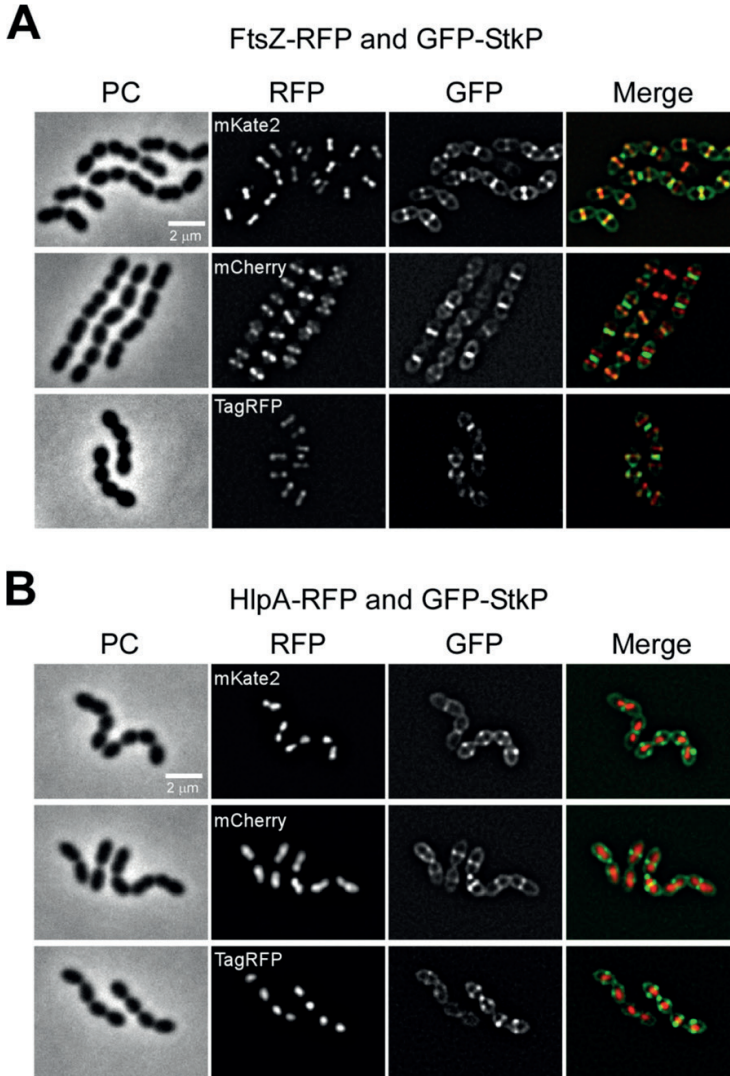


Figure 4. Double labeling with RFP and GFP in *S. pneumoniae*. **A.** Localization of FtsZ-RFP (different RFP variants in the different panels) and GFP-StkP. **B.** Localization of HlpA-RFP (different RFP variants in the different panels) and GFP-StkP. Phase-contrast (PC), RFP, and GFP images are shown, in addition to an overlay of GFP and RFP (merge). Scale bars, 2 μ m.

Acknowledgements

We thank Amaya Aramendía de Goñi for initial characterization of FPs in *S. pneumoniae*. We thank Hans Roubos (DSM) for kindly providing the mCherry sequence.

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2.

Author Contributions

KB, MK, RR and JWV designed and KB, MK and RR performed the experiments. KB, MK, RR and JWV wrote the manuscript.

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Supplemental Figures

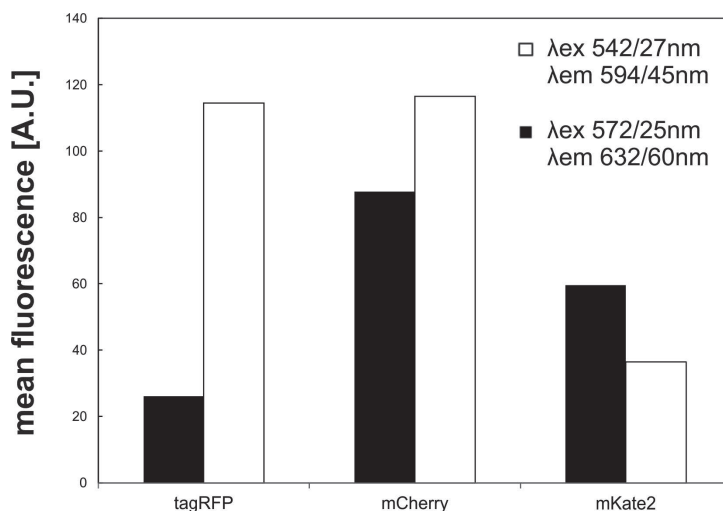


Figure S1. Comparison of competence-regulated RFP fluorescence intensities.

Cells of strain carrying *PssbB*-RFP were grown in C+Y medium, and induced at OD₆₀₀ 0.05 with CSP (1 µg/ml) and analyzed after 1.5h. Average fluorescence intensities at the single cell level measured by fluorescence microscopy using an mCherry filterset, polychroic mirror QUAD2 (*) or a tritc filter set; polychroic mirror QUAD1 (°). Values are in arbitrary units [A.U.]. Single cell fluorescence levels were determined using MicrobeTracker (Sliusarenko *et al.* 2011).

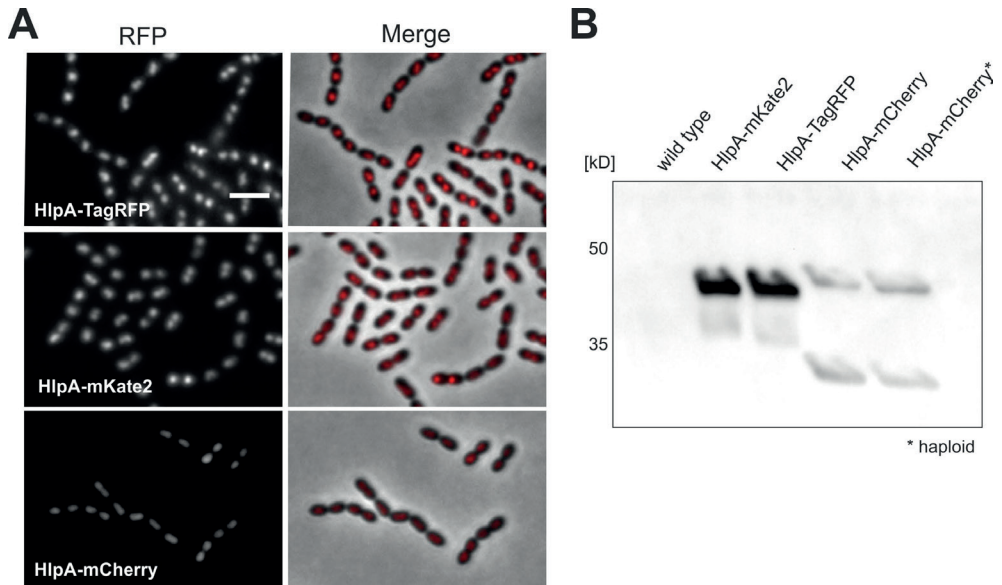
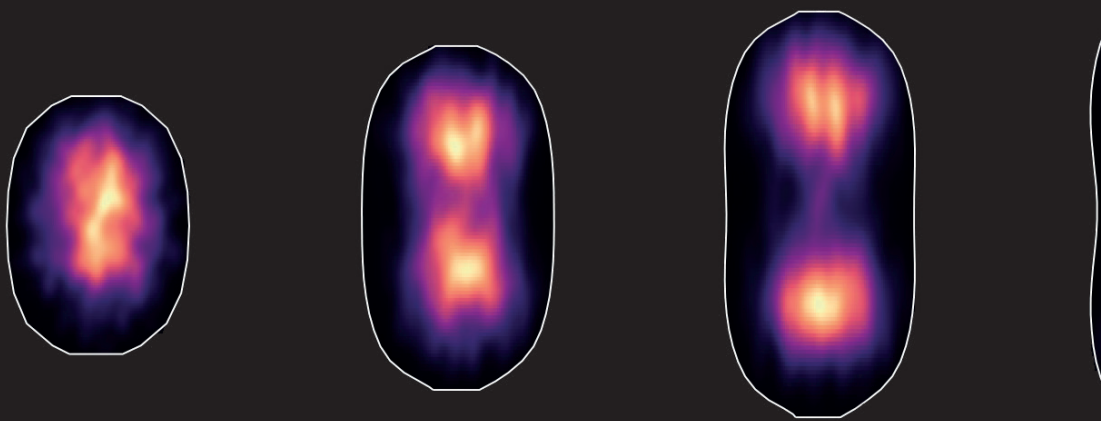


Figure S2. Comparison of tagRFP, mCherry and mKate2 in a C-terminal protein fusion to the histone-like protein HlpA.

A. Non-deconvolved, unprocessed micrographs of merodiploid D39 strains expressing HlpA-TagRFP (KB1-65), HlpA-mKate2 (MK119) and HlpA-mCherry (MK218), respectively. Scale bar equals 2 μ m. The filter sets TRITC for HlpA-TagRFP or mCherry for HlpA-mKate2 and HlpA-mCherry were used. **B.** Immunodetection of fusion proteins in whole cell extracts of strains KB1-65 (*hlpA*, *hlpA-tagRFP*), MK119 (*hlpA*, *hlpA-mKate2*), MK218 (*hlpA*, *hlpA-mCherry*) and KB1-64 (*hlpA-mCherry*) using anti-RFP antibodies.

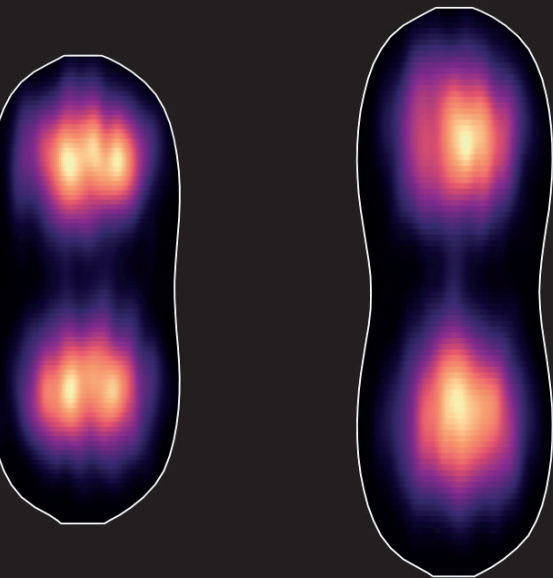


Chapter 3:

BactMAP: an R package for integrating, analyzing and visualizing bacterial microscopy data

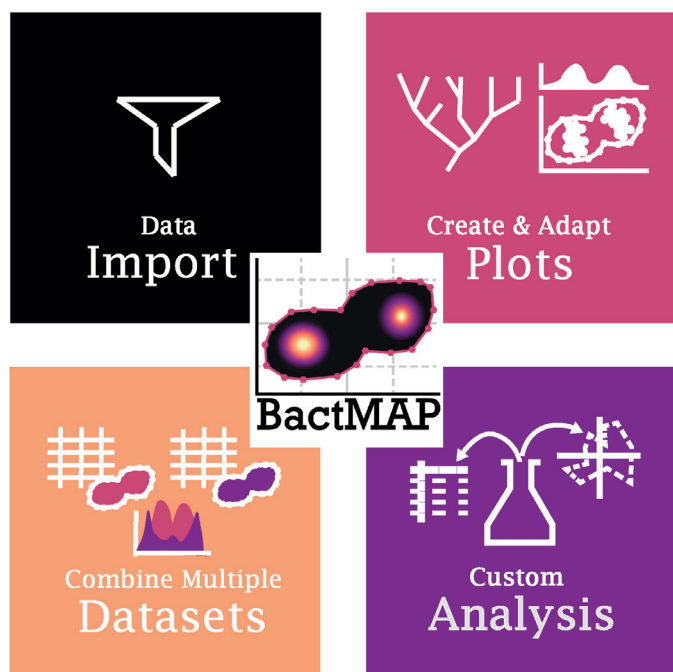
Renske van Raaphorst, Morten Kjos & Jan-Willem Veening

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Abstract

High-throughput analysis of single-cell microscopy data is a critical tool within the field of bacterial cell biology. Several programs have been developed to specifically segment bacterial cells from phase-contrast images. Together with spot and object detection algorithms, these programs offer powerful approaches to quantify observations from microscopy data, ranging from cell-to-cell genealogy to localization and movement of proteins. Most segmentation programs contain specific post-processing and plotting options, but these options vary between programs and possibilities to optimize or alter the outputs are often limited. Therefore, we developed BactMAP (Bacterial toolbox for Microscopy Analysis & Plotting), a command-line based R package that allows researchers to transform cell segmentation and spot detection data generated by different programs into various plots. Furthermore, BactMAP makes it possible to perform custom analyses and change the layout of the output. Because BactMAP works independently of segmentation and detection programs, inputs from different sources can be compared within the same analysis pipeline. BactMAP complies with standard practice in R which enables the use of advanced statistical analysis tools, and its graphic output is compatible with ggplot2, enabling adjustable plot graphics in every operating system. User feedback will be used to create a fully automated Graphical User Interface version of BactMAP in the future. Using BactMAP, we visualize key cell cycle parameters in *Bacillus subtilis* and *Staphylococcus aureus*, and demonstrate that the DNA replication forks in *Streptococcus pneumoniae* dissociate and associate before splitting of the cell, after the Z-ring is formed at the new quarter positions. BactMAP is available from <https://veeninglab.com/bactmap>.



Graphical abstract

Introduction

Segmentation tools for analyses of phase contrast microscopy images of bacterial cells are improving rapidly. Over a decade ago the first software packages became available, making it possible to track cells (semi-) automatically using programs like Bacterial Home Vision (BHV), Cell-Profiler, Schnitzcells and MicrobeTracker (Stewart *et al.*, 2005; Lamprecht *et al.*, 2007; Sliusarenko *et al.*, 2011; Young *et al.*, 2011). However, using these tools and transforming the data could be time-consuming and still heavily relied on manual adjustments. In recent years, more user-friendly and quicker tools have been developed to segment phase-contrast images or fluorescence images, making cell segmentation a standard tool for bacterial microscopy analysis. With the creation of datasets of cell shape, size, growth and internal fluorescent signal information so readily at hand, the next challenge is to gain biologically useful insights.

Most of the current popular cell segmentation programs include basic tools for exploring the data in the form of histograms or fluorescence profiles. Oufiti (Paintdakhi *et al.*, 2016), ObjectJ (Vischer *et al.*, 2015) and MicrobeJ (Ducret *et al.*, 2016) have excellent tools for exploring several characteristics of the dataset. Oufiti has a few fast time-lapse plotting and exploring tools, while MicrobeJ gives the user the possibility to plot characteristics of sub-groups of cells in many different ways. Within ObjectJ it is relatively easy to add custom measurements to the analysis and make quick visualizations. Supersegger (Stylianiidou *et al.*, 2016) includes a set of plotting and filtering tools inspired by flow cytometry analysis (Cass *et al.*, 2017).

While the options for analysis within these programs are improving and expanding, there are limitations to having the analysis toolbox and the detection tool within the same program. After exploring the quality of the detection and the general distribution of the data by for instance fluorescence histograms and dot plots of the cell size, more in-depth custom analysis will often be necessary. Apart from that, multiple datasets containing data from different conditions or replicates will often need to be combined before analyzing them altogether. This is not always straightforward as the different software packages are mostly built to directly proceed from segmentation and detection. Finally, with so many new and excellent tools at hand for single molecule tracking, cell segmentation and cell shape identification, it can be very useful to combine datasets generated from different programs or to compare the output of similar programs to see which one is optimal for different purposes.

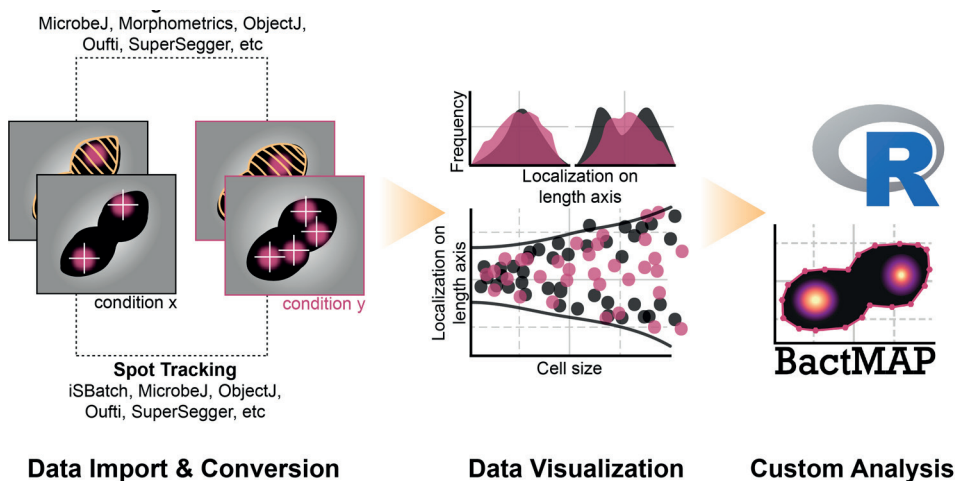


Figure 1. Visualization and analysis of microscopy data using BactMAP.

Therefore we created BactMAP (Bacterial toolbox for Microscopy Analysis & Plotting), an R package (<https://www.r-project.org/>), which acts as a funnel for segmentation and fluorescence detection data (Figure 1). BactMAP orders the input data in a standard way, filtering out the most-used information, but keeping all original data attached. It is possible to gather data from different sources and combine them, visualize the data and perform custom analysis using the BactMAP toolbox together with the vast number of tools already available in R. BactMAP, its code and a user guide is available at <https://veeninglab.com/bactmap> and <https://github.com/veeninglab/bactmap>.

We benchmarked the various options of BactMAP by analyzing the localization of the replication fork and origin of replication in three different Gram-positive organisms: *Bacillus subtilis*, *Streptococcus pneumoniae* and *Staphylococcus aureus*. These organisms all have a different cell shape, leading to different challenges in segmentation. Indeed, we show that certain programs perform segmentation better on specific cell types than others (Figure S1 and S2). All data generated with these programs was transformed using BactMAP. To obtain useful biological insights from the data, the BactMAP toolbox was used to perform exploratory localization plotting, time-lapse analyses and finally we show how BactMAP can be used as a starting point for custom analysis with R.

Results

Visualization and analysis of microscopy data using BactMAP

BactMAP was built for automated import of segmentation data and for visualization of cells and their internal fluorescence signals (Figure 1). BactMAP is built as an R package: a set of command-line based functions which can be used within the R framework. This allows for more flexibility in the analysis and visualization and a seamless connection with other available functions in R. This functionality, however, comes with the drawback of a steep learning curve. The most-used functionality of BactMAP will be further developed into a graphical user interface (GUI), to make it more user-friendly for those unfamiliar with command lines. To communicate with users and gain insights into which parts of BactMAP should be changed and updated, users can follow progress and make suggestions in the “Issues” section of our GitHub page (<https://github.com/veeninglab/bactmap/issues>).

To make BactMAP as useful as possible for the bacterial cell biology community, we standardized the import of datasets from five popular segmentation programs and one single-molecule tracking program: SuperSegger (Stylianidou *et al.*, 2016), MicrobeJ (Ducret *et al.*, 2016), Oufi (Paintdakhi *et al.*, 2016), Morphometrics (Ursell *et al.*, 2017), ObjectJ/ChainTracer (Vischer *et al.*, 2015; Syvertsson *et al.*, 2016) and iSBatch (Caldas *et al.*, 2015). Furthermore, new programs with improved or specialized cell segmentation capabilities are being developed constantly, as recently for instance BacStalk (Hartmann *et al.*, 2018), a specialized tool for segmentation of cells with complex morphologies and the deep learning and machine-learningbased segmentation programs DeepCell (Bannon *et al.*, 2018), DeLTA (Lugagne *et al.* 2019) and Ilastik (Berg *et al.*, 2019). Therefore, to make BactMAP compatible with a wide range of current and future segmentation software packages, we also implemented a generic import function for segmentation input.

The visualization options of BactMAP are summarized in Figure 2. In short, there are three main categories of plotting functions in BactMAP: 1) the visualization of intracellular fluorescence using information from the image itself, 2) visualization of subcellular localization of spots and objects as identified by any of the software packages and 3) plots which can be used in the analysis of time-lapse movies.

Exploratory localization plotting using BactMAP of the origin of replication in three differently shaped bacteria.

To demonstrate the functionality of exploratory localization plotting using BactMAP, we analyzed the subcellular localization of the origin of replication in three Gram-positive bacteria: *Bacillus subtilis*, *Streptococcus pneumoniae* and *Staphylococcus aureus*. In *B. subtilis*, the origin of replication was visualized using a TetR/*tetO* system (*tetR*-RFP, *ori::tetO*, Veening *et al.*, 2009), while in *S. pneumoniae* the origin was marked with a ParBp/*parSp*-marker (*ParBp*-GFP, *ori:parSp*, (van Raaphorst *et al.*, 2017)). In *S. aureus*, a protein-fusion of the native, origin-binding ParB to GFP was used as a marker for the origin of replication (Pinho and Errington, 2004). These three bacteria all have their own challenges in segmentation analysis due to differences in cell shape and mechanisms of growth and division.

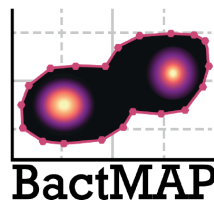
B. subtilis divides by forming a cross-wall septum, which cannot be visualized by phase-contrast imaging. In this case, segmentation based on fluorescence images (e.g. cells stained with a fluorescent cell wall or membrane dye) is therefore necessary. The only currently available program which combines phase-contrast segmentation with semi-automated septum detection based on fluorescence is ChainTracer (Syvertsson *et al.*, 2016). Other programs segment the *B. subtilis* chains well, but cannot locate septa. Morphometrics (Ursell *et al.*, 2017) has an option for segmentation of peripheral fluorescence, which can be used for *B. subtilis* segmentation.

Segmentation Software	Software Needed	Cell Outline	Edit Outlines	Edit Septa	Cell Tracking	Set Cell Polarity	Longitudinal Axis	Record Chain / Clump	Membrane-based Segmentation	Cell Shape
SuperSegger	Matlab	Pixel Mask	Draw / remove	Add/ remove pre-defined septa	✓					Multi-directional division, cocci e.g. <i>S. aureus</i>
MicrobeJ	ImageJ	Subpixel	Draw / remove	Split cells manually	✓	✓	✓	✓		Dividing longitudinal, ovococci or rods e.g. <i>S. pneumoniae</i> , <i>E. coli</i>
Oufti	Standalone	Subpixel	Draw / refine / remove	Split & join cells manually	✓	✓	✓			Longitudinal division, curved cells e.g. <i>C. crescentus</i>
Morphometrics	Matlab	Subpixel	Draw / remove		✓	✓	✓		✓	Branching e.g. <i>S. coelicolor</i>
ObjectJ/ Chaintracer	ImageJ	Bounding Box	Remove	Add/ remove septa		✓	✓	✓	✓	Rod-shaped, septum invisible in phase-contrast e.g. <i>B. subtilis</i>

Figure 3. Overview of the functionality of five programs compatible with BactMAP. Of the five tested programs, three are matlab-based (SuperSegger, Morphometrics and Oufti) and two are ImageJ Plugins (MicrobeJ, ObjectJ). While Oufti is Matlab-based, it comes as a standalone program for 64x operating systems. In addition to measuring the outlines, Oufti, MicrobeJ, Morphometrics and SuperSegger can track cells over time and provide information on growth speed and cell genealogy. Oufti, MicrobeJ, Morphometrics and ObjectJ estimate the cell length and curvature over the longitudinal axis. MicrobeJ offers a range of options for detection and counting of cell chains and clumps, while both MicrobeJ and ObjectJ offer options to detect cell features such as curvatures or invaginations as specified by the user. Finally, both SuperSegger and MicrobeJ give users the option to group cells based on user-specified cell features. All programs offer some options for manual editing of the results. In Oufti, a user can split or join cells, delete cells and draw new cell outlines. In Morphometrics, MicrobeJ and ObjectJ it is also possible to delete or add cells. For both Morphometrics and Oufti, it is not possible to move septa to a manually chosen subcellular location. In MicrobeJ this is possible, just as ObjectJ's ChainTracer allows users to check, add and delete detected septa manually. Also in SuperSegger, it is possible to delete cells, but it is only possible to delete or add septa on pre-calculated positions. The right panel shows which program performs cell segmentation best on which kind of shaped cells in our experience.

3.

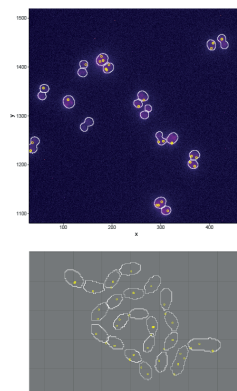
Visualization of Microscopy Data with



Intracellular (raw) fluorescence

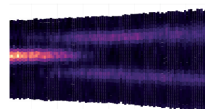
plotRaw()

Visualize a raw TIFF image, project cell outlines and/or localized spots

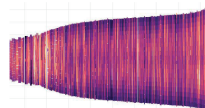


bactKymo()

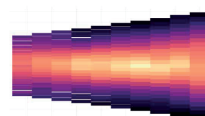
Make kymographs and demographs of:



Length/width axis profiles of a single cell over time



Length/width axis profiles of a group of cells over cell size



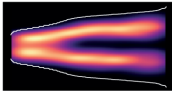
Length/width axis profiles of cells binned by their point in cell division

Figure 2. Overview of BactMAP's plotting functions. Intracellular (raw) fluorescence. *plotRaw()* and *bactKymo()* are both useful visualization tools that use cell outlines and the original image in TIFF format. *plotRaw()* shows the original microscopy pictures with the cellular outlines and/or the localization data. *bactKymo()* makes kymographs and demographs of single cells and cell groups. **Subcellular localization.** For plotting of subcellular fluorescent spot localizations *createPlotList()* is used. This function returns a list of demographs, histograms and projections. For larger fluorescent objects, *plotObjects()* plots intracellular object shapes and localization through cell projections. When MicrobeJ or ISBatch are used to track fluorescent spots over time, *plotTrack()* can be used to visualize them. Moreover, *plotOverlay()* can be used to plot cell towers and localization over time of different fluorescent channels and/or experimental conditions. **Time-lapse analysis.** *percDivision()* will categorize each cell based on growth speed and determine when a cell underwent a full division. *plotTreeBasic()* uses the package ggtree (Yu *et al.* 2017) to plot Oufiti's or SuperSegger's genealogy information as a tree plot. To visualize single-cell growth and fluorescence, *plotCellsTime()* uses cell outlines and raw microscopy images to create single-cell towers or movies.

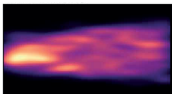
Subcellular localization

createPlotList()

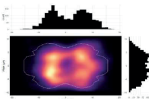
Summary function for intracellular localization of fluorescent spots containing:



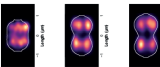
localization of spots on length axis of cell plotted over the order of the cells (by cell length)



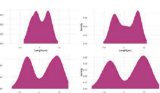
localization of spots on width axis of cell plotted over the order of the cells (by cell length)



projection of x/y-localization of all spots on the average cell



a list of n x/y-localization projections of cells grouped by their cell length



localization of spots on the length axis of the cell displayed as a density plot

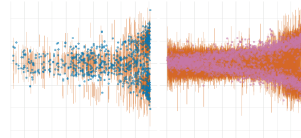
plotObjects()

Plot cell towers of intracellular localization of fluorescent objects



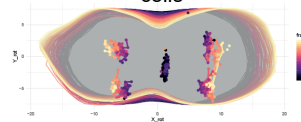
plotOverlay()

Combine & plot data from different conditions



plotTrack()

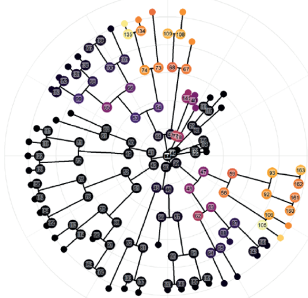
Plot localizations and trajectories in single cells



Time-Lapse Analysis

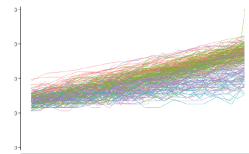
plotTreeBasic()

ggtree-based plotting of cell genealogy and attributes, for instance mean fluorescence



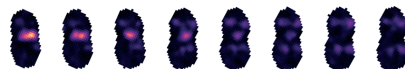
percDivision()

Show and categorize the individual cell



plotCellsTime()

Plot single cell towers or movies based on raw fluorescence intensity per pixel



Encapsulated, oval-shaped *S. pneumoniae* cells can be difficult to segment, because they are small and form chains of cells under certain conditions (Domenech *et al.*, 2018). As part of a BactMAP test session, we asked three of our lab members to use their program of choice to segment the same image of *S. pneumoniae* cells. The number of cells detected by each program was comparable, however, measured cell widths and lengths were strikingly different between programs as well as between parameter settings (see Figure S1). This underlines the importance of keeping the same global segmentation parameters within experiments, as well as of visual inspection of the segmentation results to make sure these are consistent. It is important to note and be aware that the preferences and interpretations of the user have a major influence on segmentation results. Since all programs are in principle capable of segmentation of phase contrast images of *S. pneumoniae* cells, the program choice largely depends on the goal of the experiment (e.g. comparing cell sizes, measuring internal fluorescence or tracking cells). To aid in this decision, we summarized the options and strengths of the five segmentation programs we tested in Figure 3.

S. aureus detection and segmentation is notoriously difficult because the cells are small cocci, not completely homogeneous in phase-contrast and they divide in multiple perpendicular planes (Pinho *et al.*, 2013). For the analysis of *S. aureus* it was thus challenging to find a segmentation algorithm which created an outline of the whole cell based on phase contrast images. Both Oufi and ObjectJ are capable of creating a mask around *S. aureus* cells taken with phase-contrast microscopy, but both software packages have trouble attributing cell parameters to the mask because the algorithms are assuming a rod-shaped cell with a medial axis. Morphometrics and MicrobeJ both have the option of medial-axis based segmentation, and they also offer other options for subpixel segmentation, while SuperSegger only creates a pixel-resolution cell mask. We tested the last three programs and used BactMAP to display and compare the optimal segmentation result of the three programs (Figure S2). All three programs performed well at segmenting the cells. Both MicrobeJ and Morphometrics, using a variety of different settings, however, still missed detection of many cells, while SuperSegger detected almost twice as many (Figure S2). SuperSegger is therefore our program of choice for segmentation of *S. aureus* cells based on phase contrast images (see section below). Another option is to use fluorescence-based segmentation using a membrane dye or protein-fusion. While most segmentation programs have options for fluorescence-based segmentation, of the programs tested here, Morphometrics is the only program which combines segmentation options specifically developed for membrane-bound fluorescence with segmentation options for cells that are not rod-shaped.

While different software packages were used for detection of cell outlines in the different species (as explained above), the single-molecule fluorescence detection program iSBatch (Caldas *et al.*, 2015) was used to detect fluorescent foci for all three bacterial species (Figure 4A). After imaging, fluorescence spot detection and cell detection, all datasets were imported using BactMAP.

Using BactMAPs *createPlotList*-function we plotted a projection of the subcellular localization of the origin of replication in five cell-size groups (Figure 4B). *B. subtilis* was grown in lysogeny broth at 30°C. Due to multifork replication under these conditions, a median of 4 ± 2 origins/cell were detected ($\pm$ indicating the standard deviation). The variable number of spots lead to a diffuse signal in the density plots of the different cell size groups (Figure 4B), but when only the coordinates of the origins on the length axis are plotted (Figure 4C), a splitting of the origins between the two cell halves is visible. For *S. pneumoniae*, the origin of replication segregates early in the cell cycle, after which it moves to the future septa of the cell. In the dataset presented here, we detected 2 ± 2 spots/cell.

Similar to *S. pneumoniae*, *S. aureus* only initiates DNA replication one time per cell division. However, contrary to both *B. subtilis* and *S. pneumoniae*, *S. aureus* divides in consecutive perpendicular planes, making it especially hard to extract cell cycle information from microscopy snap shots. *S. aureus* cells only elongate slightly when the septal cross wall is being formed (Monteiro *et al.*,

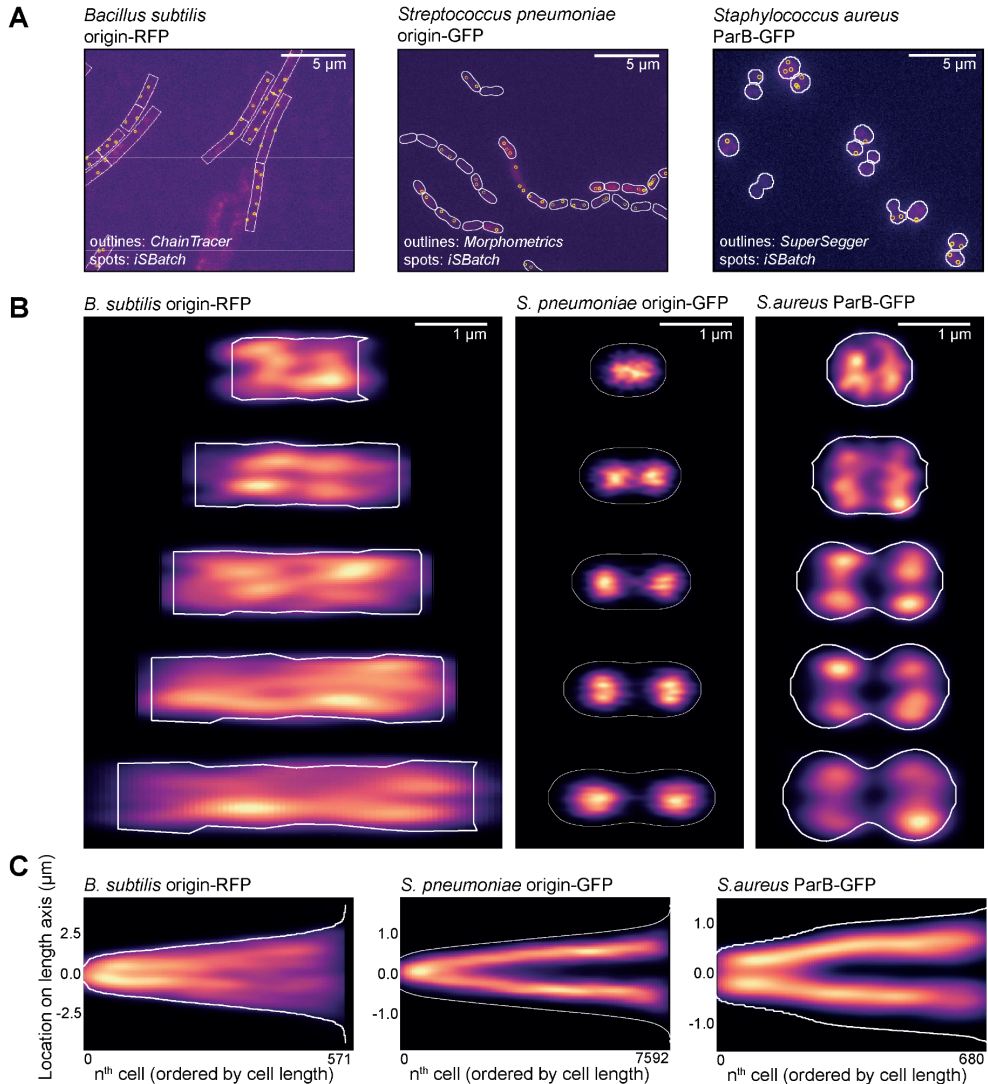


Figure 4. BactMAP output of the segmentation and origin localization of three differently shaped bacteria. A. For all three bacteria; a cutout of the raw image file with an overlay of the cell segmentation and detected fluorescent spots is shown. The raw images, segmentation data and spot detection data was loaded into R using BactMAP's *extr*-functions, and the overlay images were created using *plotRaw()*. **B.** Cell towers showing the x,y-projection of the origin/ParB inside the cell. The five groups are divided by cell length and contain an equal number of cells. Localizations are displayed as a heatmap; brighter color indicates more localization in this x,y position. **C.** Projection of the localization of the origin/ParB on the longest axis of the cell, where all cells are ordered by cell length.

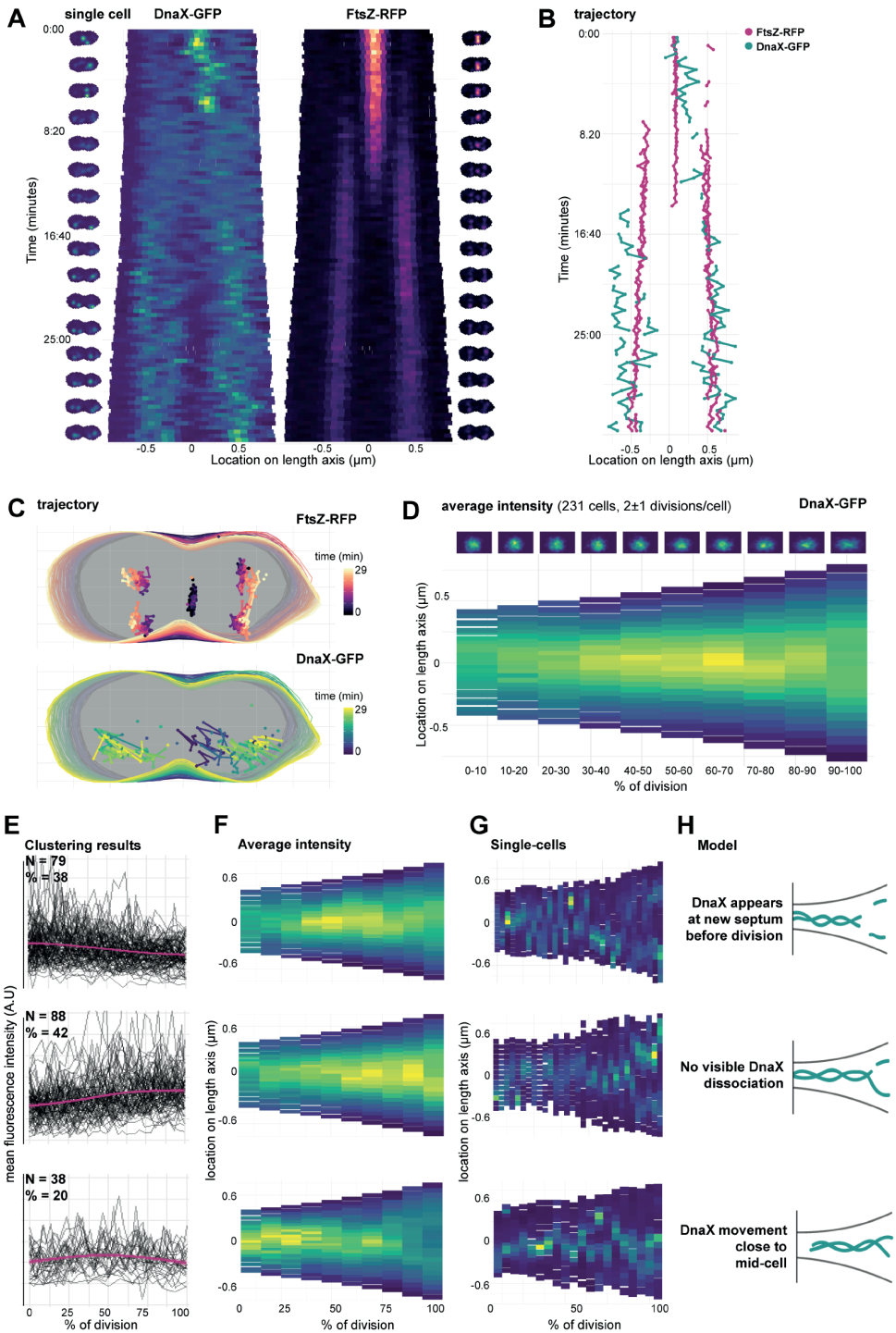
2015), after which they quickly split into two new daughter cells. To facilitate cell size grouping, we chose to record connected daughter cells as one cell (Figure 4A and B, right panel). In this way, we could not only observe cells dividing in one plane, but also connected daughter cells that started to grow and divide in the perpendicular direction. Thus, in Figure 4B, the two first cell groups represent cells before splitting, while the three last cell groups are cells already undergoing splitting. As observed in Figure 4B, the average grouped cells indeed get mainly larger by length first, then by cell width. The origin is segregated in small cells, prior to splitting, without preference for location on the width axis (Figure 4B). However, as observed in the third cell group, the second segregation takes place over the width axis (perpendicular to the initial segregation) and four spots are visible in the last three cell groups (Figure 4B). This corresponds to the average number of detected ParB-GFP spots in the *S. aureus* dataset (4 ± 3 spots/cell). In total, this analysis shows that BactMAP is capable of handling different input data and converting it into intuitive and qualitative as well as quantitative insightful reproductions of key cell cycle parameters.

Single-cell time-lapse analysis of the replication fork of *S. pneumoniae* using BactMAP

In *S. pneumoniae*, the origin of replication moves to the new division sites very early in the cell cycle (Figure 4B and C). The movement of the origin of replication is restricted, while the replication fork of *S. pneumoniae* is very mobile (van Raaphorst *et al.*, 2017), in contrast to the replication fork of *E. coli* (Wallden *et al.*, 2016) or *B. subtilis* (Liao *et al.*, 2015, Mangiameli *et al.*, 2018). Snap shots of fast-moving DnaX (the clamp loader of the replication fork) fused to GFP lead to blurred demographs, making it hard to interpret the localization of the replisome. Previously, we saw in single-cell movies using epifluorescence microscopy that the bulk of FtsZ and DnaX often localize on the same plane on the length axis of the cell. A recent study employing total internal reflection fluorescence (TIRF) microscopy (Perez *et al.*, 2019) showed that FtsZ is not moving *en masse* from the division plane to the $\frac{1}{4}$ -positions of the cell, but few treadmilling FtsZ clusters move along with MapZ, after which the concentration of FtsZ polymers colocalizing with MapZ gradually increases into the ring as seen by epifluorescence microscopy.

For our previous work (van Raaphorst *et al.*, 2017), we imaged cells with DnaX-GFP and FtsZ-RFP (strain MK396) every 20 seconds for one hour. For this study, we re-analyzed these movies. We first used Oufiti to track the cells and iSBatch to track internal FtsZ-RFP filaments and DnaX-GFP foci. After tracking of cells, filaments and foci, we used BactMAP to combine the information. Figure 5 displays some examples on how such data can be plotted with BactMAP.

Figure 5 (right). Single-cell time-lapse analysis of the replication fork and FtsZ in *Streptococcus pneumoniae*. **A.** Kymographs of a single cell (strain MK396, dnaX::dnaX-GFP-eryR, ftsZ::ftsZ-RFP-kanR) imaged every 20 seconds for one hour. Cell outlines recorded with Oufiti and combined with the raw image data using BactMAP. Left/right of the kymographs are movie strips of the single cell, created with BactMAP. **B.** Trajectory over the length-axis of the cell over time of FtsZ-RFP bundles and DnaX-GFP foci in the same cell as shown in A. Foci/bundles were tracked with iSBatch. **C.** X/Y trajectory over time of the cell shown in A and B. Outlines recorded with Oufiti, tracks recorded with iSBatch. **D.** The growth curves of all cells were determined and curves of non-growing cells and cells with incomplete cell cycles were discarded. Cell parameters were binned in ten groups by % of division. Bottom: average intensity of GFP signal over the length axis of the cell per % of division. Top: density plots of X/Y coordinates of recorded GFP foci per binned % of division. X/Y coordinates were recorded with iSBatch, cell outlines with Oufiti and the raw image files were used by BactMAP to determine the average intensity per bin. **E.** Clustering results: average cellular intensity over division percentage. Mean cellular fluorescence intensity (arbitrary units) over percentage of division (pink, standard deviation in shade), with single-cell fluorescence intensity paths shown in grey. Top-bottom shows each cluster, the number of clusters (N) and the percentage of the total number of cells (%). **F.** Average intensity profile. Average intensity of the length axis of the cell binned in 10 groups based on percentage of division for each of the three clusters (top-bottom). **G.** Single cells. Example kymographs of single cell members of each cluster. **H.** Schematic models of the various dynamics that were observed for pneumococcal DnaX.



3.

Figure 5A shows kymographs of the fluorescence intensity across the length-axis in a single cell over time. While the bulk of FtsZ has arrived at the $\frac{1}{4}$ -positions of the cell around 10 minutes from the start of the movie, there is already an increase in fluorescence visible next to mid-cell from 4 minutes on. This is confirmed by the trajectories recorded by iSBatch (Figure 5B). Interestingly, the timing of movement from mid-cell to the quarter positions of the cell is not completely the same in the left and the right side of the cell, as was also shown by (Perez *et al.*, 2019). As shown in Figure 5C, the FtsZ-RFP tracks are short and sometimes even static. It is not surprising that we do not observe treadmilling in these conditions, since we imaged the cells in HiLo-mode (highly inclined and laminated optical sheet mode) where we focused on the middle of the cell and not in TIRF mode as performed by (Perez *et al.*, 2019). While these conditions are good to follow DnaX and to see the general localization of FtsZ, we miss the focus on the edge of the cell and thereby the movement of FtsZ along the Z-ring.

While FtsZ moves to the new septa early during the cell cycle, DnaX seems to disassociate at mid-cell and assemble at the new quarter positions within a timeframe of 8 minutes. To see if we can observe association and dissociation in more cells, we re-analyzed a time-lapse movie of pneumococcus with DnaX-GFP imaged every 2 minutes for 4 hours (van Raaphorst *et al.*, 2017). The lower time resolution allowed us to record multiple divisions while keeping the time resolution high enough to be able to see DnaX appear and disappear in single cells. BactMAPs function `perc_Division()` uses cell length as a proxy to determine when a cell in a time-lapse has divided, if this is not yet determined by the segmentation software used as input. After this, it uses the division time to determine how far into the cell cycle every single cell is at a given point in time. In this way, BactMAP can order and compare cells. Figure 5D shows a demograph of the fluorescence intensity over the length axis of the cell averaged by the percentage of division. Notably, no drop in fluorescence intensity is visible during cell division in this demograph, meaning that at the population level, DnaX association and dissociation is not measurable. This can be due to the lower time-resolution, difference in fluorescence intensity per cell or a high variability in association/dissociation timing between cells. This exploratory analysis shows that to follow the timing and localization of DnaX accurately, one needs to examine individual cells in time.

Timing and localization of DnaX association/dissociation at the replication fork: custom analysis

Visual inspection of kymographs of individual cells over time showed that there is indeed a high variability in the localization of DnaX between cells. Using the R package TSclust (Montero and Vilar, 2014), we created a dissimilarity matrix and performed hierarchical clustering based on the fluorescence profile over the course of division of each cell (see Figure S3). Before clustering, 10% of the cells were discarded because they showed no growth or too little fluorescence. After the clustering analysis, we grouped the cells into three main groups (Figure 5E-H). In 38% of the remaining cells, the mean fluorescence drops just before division, after which it slightly increases again. This corresponds to cells where DnaX moves as in Figure 5A-C: after moving around mid-cell, DnaX disappears for a few minutes, after which it reappears at the two new septa. However, in 42% of the cells, we observed no drop in fluorescence: DnaX moves gradually to the two cell poles. It could be that for this cell group, dissociation and association happened within our 2 minute interval. In 20% of the cells, DnaX remains at the old septum until division (Figure 5E-H). It might be possible that the segmentation software split these cells earlier in their division cycle than the cells of the largest group, leading to an early drop in fluorescence instead of a late one. Segmenting cells based on membrane or cell wall markers could give more insight into whether this is the case. Nevertheless, this analysis using BactMAP provides new insights into DnaX dynamics during the pneumococcal cell cycle. We now show that the replication forks dissociate and associate before the cell splits and after the Z-ring is formed at the new $\frac{1}{4}$ positions.

Discussion

In this work, we have developed BactMAP, a tool for visualization of microscopy data obtained from different image analysis programs. Using BactMAP, cell segmentation, fluorescence intensity and fluorescence spot detection results obtained from different software packages are all converted to the same format and can be plotted the same way. This allows for easy comparison of results by eye or by quantitative measures. BactMAP thus enables users to not only compare outputs from different software packages, but also combine them in one analysis. Finally, data from different experiments and different color channels can be easily combined in a uniform manner.

BactMAP is currently available as an R package, which is a set of functions to be executed in scripts or command-line. With R being one of the most-used languages in biological analysis, this has the advantage that functions from BactMAP can be added to existing workflows. Another advantage of using the functions within R is that plots generated with BactMAP can be adapted using standard functions from the R package *ggplot2*. To make it easier for users unfamiliar with R code to use BactMAP, we are currently working on a graphical user interface (GUI). To keep users informed on the progress and get feedback on the functionality of BactMAP, we have opened an issues page on our GitHub page (<https://github.com/veeninglab/bactmap/issues>).

When creating BactMAP, our focus was to make a tool for plotting of intracellular fluorescence and automated import of different datasets. However, there are more types of analyses possible, some of which we slightly touched; for instance single molecule tracking, cell shape classification, analysis of cell lineages or visualization of localization microscopy. For advanced single molecule tracking, there are already software packages available, which can combine tracking analysis with single cell outlines from other programs (e.g. ISBatch (Caldas *et al.*, 2015) and SM-Tracker (Rösch *et al.*, 2018)).

One of the more alarming outcomes of our analysis of segmentation outputs is that both the software and the user influence the outcome of the analysis to a great extent. Only keeping the same software parameters between experiments are not enough to keep consistent segmentation, because background noise and other day-to-day differences influence the parameters needed to segment the cells. This is why all segmentation programs rely on the user to check whether the segmentation reflects the biology (e.g. when a cell divides) of the cell. Especially when comparing cell sizes from different experiments or when switching software within a lab, it is important to keep these limitations in mind and document the differences. As exemplified in Figure S1 and S2, BactMAP can be used to investigate these differences.

Using our newly developed R package BactMAP, we could revisit our previously analyzed microscopy images and time-lapse movies and gather new information. Most notably, using averaged datasets and observations of single-cell movies of DnaX-GFP movement during cell division, we concluded previously that the formation of the new replisome happens simultaneously with Z-ring formation. Using single-cell analysis instead of looking at the cells at the population level, we see that the situation is more nuanced. In 38% of the cases, the replisome is formed after Z-ring formation, but in the remaining 62% of the cells we see DnaX foci moving gradually to the new septum or not moving away from the septum at all. In many cells, association and dissociation of the replication forks probably happens within a very short time interval. We also could get a more detailed view of global FtsZ movement during cell division.

Recently, more R packages and Shiny apps have been developed to enable researchers with little experience in R to use the plotting tools available with little effort, for example PlotsOfData (Postma and Goedhart, 2019). With BactMAP, researchers can use advanced plotting of microscopy data in R and have autonomy over both the way of plotting as well as the layout presented. After plotting, the data output can be used further for custom analysis. All in all, BactMAP is

a tool for both initial data investigation and publication-ready plotting, and as a starting point for more in-depth data analysis. A user guide and the source code of BactMAP is available at <https://veeninglab.com/bactmap>.

Experimental procedures

Conversion to standard data structure

3.

BactMAP converts data from segmentation and localization analysis tools into R dataframes with a standard structure, depending on the information enclosed in the input data: 1) meshframe, containing cell contours, 2) spotframe, containing spot x/y coordinates, 3) objectframe, with x/y coordinates of the outline of fluorescent objects, 4) spots_relative and object_relative, with the x/y coordinates of the spots or object translated to intercellular coordinates, 5) timelapsedata, containing information on cell genealogy, and 6) cellList, where the original input data is saved with as little change as possible.

BactMAP's import functions are at least compatible with the current versions of segmentation and fluorescence detection software: Oufi (2015-current), Morphometrics (>version 0.1, Oct 25 2016), ObjectJ (version 1.04), MicrobeJ (version >5.13), SuperSegger (version 1.0.1) and isBatch (version >0.3.5).

Segmentation data standardly creates a meshframe, containing x/y-coordinates of the contours of the cells, the length and width of the minimal bounding box, cell and frame ID and the coordinates of the cell when the cells are turned on their long axis with their mid-point at [0,0]. The R package shotGroups (<https://CRAN.R-project.org/package=shotGroups>) was used to create the function of finding the minimal bounding box and the cell angle needed to turn every cell on their length axis. For importing MATLAB (.mat) files directly into R, the R package R.matlab (<https://CRAN.R-project.org/package=R.matlab>) was used.

Similarly, when localization information is transformed by BactMAP, a spotframe is created which contains the coordinates of the spots and the frame ID. When segmentation and localization data are imported together, the data is combined to finally create the dataframe spots_relative. The R package SMDTools (VanDerWal *et al.*, 2014) was used to build the function to connect the spot data to the cell outline data.

While the spotframe, meshframe and spots_relative dataframes are the most common output formats of transformation of BactMAP data, BactMAP also has standard functions to transform fluorescent object coordinates, cell genealogy information and raw TIFF microscopy images. A summary of transformation functions and their output can be found in the documentation of the BactMAP package available at <https://veeninglab.com/bactmap>.

Visualization

The BactMAP plotting toolbox can be divided in three groups (see Figure 2): (1) visualization of cells based on the raw image data and unprocessed cell outlines, (2) plots used to visualize, combine and categorize information on subcellular localization and (3) plots intended for time-lapse analysis (e.g. genealogy trees and extraction of single-cell movies).

One of the primary goals of this project was to make it easy to generate and edit publication-ready plots. For this reason, all plots generated using the BactMAP package are made using ggplot2 (Wickham, 2016). Since ggplots are easy to edit and build upon by adding layers of layouts, it is possible to change the color schemes and fonts or add new layers of data. All plot-

ting functions also return the data used to make the plot, so a user can also decide to build the plot from scratch. Plots generated in R can be saved in vector-format PDFs. The standard color schemes used in BactMAP are colorblind-friendly (the viridis diverging heatmap color schemes or the palette designed by (Ichihara *et al.*, 2010)). Using the R package gganimate (<https://gganimate.com/>) both time-lapse and localization plots faceted by cell size can easily be converted into movies. When indicated, BactMAP plot outputs are compatible with the interactive plotting package plotly (<https://plot.ly>), which enables a user to change the axes manually, zoom by dragging/dropping or make interactive movies.

Tutorials & Documentation

The source code and full documentation of all functions included in BactMAP can be found on <https://veeninglab.com/bactmap>, on the GitHub page of the Veening Lab (<https://github.com/veeninglab>), and are included in the BactMAP package.

The datasets used in this study can be downloaded as example datasets from <https://veeninglab.com/bactmap>. Consequently, in a set of tutorials we explain how to recreate these plots using the example data on <https://veeninglab.com/bactmap>.

Strains

Strain	Genotype	Reference
<i>Bacillus subtilis</i>		
VL429/JW147	sp168; <i>hutM::tetO</i> , <i>Km^R</i> , <i>amyE::Pxyl-tetR-mCherry</i> , <i>Spec^R</i> , <i>PdnaN-gfp-dnaN</i> , <i>Cm^R</i>	Veening <i>et al.</i> , 2009
<i>Staphylococcus aureus</i>		
IM67	SH1000, pLOW- <i>parB-m(sf)gfp</i>	Laboratory collection
<i>Streptococcus pneumoniae</i>		
VL369/RR23	D39V; <i>dnaX::dnaX-m(sf)GFP</i> , <i>ery^R</i>	van Raaphorst <i>et al.</i> , 2017
VL451/MK359	D39V, $\Delta bgaA::P_{zn}$ - <i>tetR-mKate2_p</i> , <i>parB^{mut}-gfp</i> ; <i>tet^R</i> , <i>comCDE</i> , <i>parS_p</i> , <i>kan^R</i> , <i>pulA</i> , <i>tetO48</i> , <i>spc^R</i>	van Raaphorst <i>et al.</i> , 2017
VL469/MK396	D39V, <i>ftsZ::ftsZ-mKate2</i> , <i>kan^R</i> , <i>dnaX::dnaX-m(sf)GFP</i> , <i>ery^R</i>	van Raaphorst <i>et al.</i> , 2017

Table 1. Strains used in this study

Growth Conditions

Bacillus subtilis was incubated in Lysogeny Broth (LB, Miller) and grown O/N shaking (220 rpm) at 37°C. The culture was diluted 200 times in fresh LB and allowed to grow for 2 hours at 30°C. The culture was induced with D-Xylose (1% w/v, Sigma-Aldrich) for 30 minutes before the cells were washed in PBS containing 0.5 ug/mL Mitotracker Far Red (Invitrogen). The cells were washed again in PBS and were immobilized on 1% agarose in PBS as described before (de Jong *et al.* 2011) for microscopy.

S. aureus SH1000 carrying pLOW-*parB-m(sf)gfp* was grown in BHI broth (Oxoid) containing 5 µg/ml erythromycin overnight. The culture was diluted 100 times in fresh medium containing 100 µM IPTG and incubated for 2.5 hours. Cells were then immobilized on 1% agarose in PBS

for microscopy.

Streptococcus pneumoniae with DnaX-GFP (strain VL369/RR23) and DnaX-GFP/FtsZ-RFP (strain VL469/MK396) were grown as described before (van Raaphorst *et al.*, 2017). *Streptococcus pneumoniae* strain VL451/MK359 was grown in C+Y medium recipe 2018 (Domenech *et al.*, 2018) until an OD of 0.1 and diluted 100 times in fresh medium containing 0.1 μ M ZnCl₂. The culture was grown up to OD 0.1 again and immobilized on 1% agarose in PBS as described before for microscopy (de Jong *et al.*, 2011).

Microscopy

3. The images of *B. subtilis* and *S. pneumoniae* VL451/MK359 were acquired on a Leica DMI8 microscope with a DFC9000 GT camera, a 100x/1.40 NA phase-contrast objective (Leica) and a Lumencor SpectraX light engine with the following filter settings: GFP: SpectraX-Quad cube (Chroma #89000) 470/24 excitation & 515/40 emission; RFP: SpectraX-Quad cube (Chroma #89000) 575/35 excitation & 595/40 emission; far-red (MitoTracker Red): Alexa 633 cube (Leica #11103136): 610/655 excitation & 620/720 emission.

S. aureus was imaged using a Zeiss AxioObserver with ZEN Blue software. Images were captured using an ORCA-Flash4.0 V2 Digital CMOS camera (Hamamatsu Photonics), a 100x phase contrast objective. HPX 120 Illuminator (Zeiss) was used as a fluorescence light source. The following filter settings were used for GFP: filter set 38 HE (Zeiss) 470/40 excitation and 525/50 emission.

Streptococcus pneumoniae with DnaX-GFP (strain VL369/RR23) and DnaX-GFP/FtsZ-RFP (strain VL469/MK396) were imaged as described before (van Raaphorst *et al.*, 2017). In short, the cells were imaged on a Deltavision Elite microscope in HiLo mode using a 488 nm (GFP) and 568 (RFP) laser through a Quad cube (Chroma #89000), either every 20 seconds in 30°C for one hour or every two minutes in 37°C for three hours an 48 minutes.

Image Analysis

Images were converted to TIFF files using FIJI (Schindelin *et al.*, 2012). Cell segmentation and fluorescence detection/segmentation was done with isBatch (Caldas *et al.*, 2015), MicrobeJ (Ducrot *et al.*, 2016), SuperSegger (Stylianidou *et al.*, 2016), Morphometrics (Ursell *et al.* 2017), ObjectJ (Vischer *et al.*, 2015), ChainTracer (Syvertsson *et al.*, 2016) and Oufi (Paintdakhi *et al.*, 2016) as indicated in the results section. The parameters used for each experiment are documented in the tutorials and documentation on <https://veeninglab.com/bactmap>.

For testing the segmentation of *Streptococcus pneumoniae*, we asked members of the Veening lab to segment one single microscopy image with their segmentation program of choice. Four lab members including one of the authors participated, of which one lab member performed segmentation twice, using two different programs, resulting in five datasets. These datasets were analyzed as described in the results section. A full transcript of the analysis including the datasets can be found as a tutorial on <https://veeninglab.com/bactmap>.

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Supplementary Figures

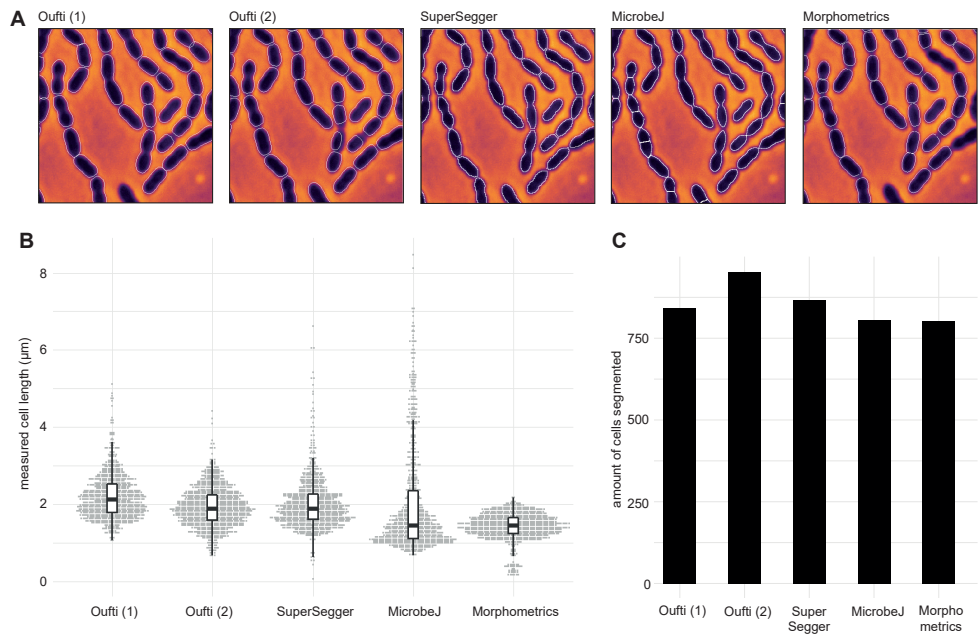


Figure S1. Comparison of phase-contrast segmentation of *Streptococcus pneumoniae* cells by Oufiti, SuperSegger, MicrobeJ & Morphometrics. Four lab members carried out cell segmentation of the same phase-contrast picture using the program they regularly use. One lab member decided to segment the image twice, one time using Oufiti (Oufiti (2) in the Figure above) and one time using SuperSegger. **A.** Cell outlines of a cutout of a microscopy image of chained *S. pneumoniae* D39 cells (400*400 pixels or 20*20 μm) as segmented using the different programs. **B.** The measured cell sizes of all cells measured by each program. **C.** The amount of cells detected by each program.

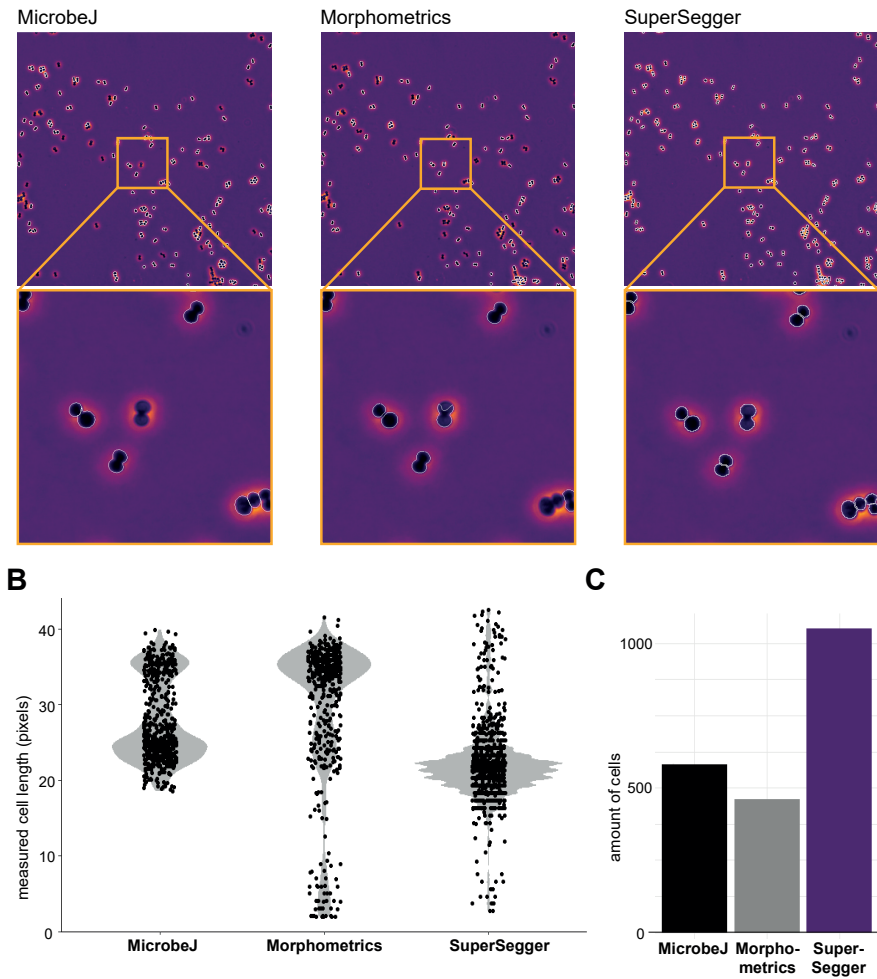


Figure S2. Comparison of *Staphylococcus aureus* segmentation using MicrobeJ, Morphometrics and SuperSegger. **A.** Plot of full phase-contrast image of *S. aureus* (2048*2048 px² or 113*113 μm²) with cell outlines obtained with either MicrobeJ, Morphometrics or SuperSegger (left-right) with zoomed-in cut-outs below (300*300 px² or 19.5*19.5 μm²). **B.** Cell length distribution (in pixels) as measured by MicrobeJ, Morphometrics and SuperSegger, respectively. **C.** The total amount of cells found by MicrobeJ, Morphometrics and SuperSegger.

3.

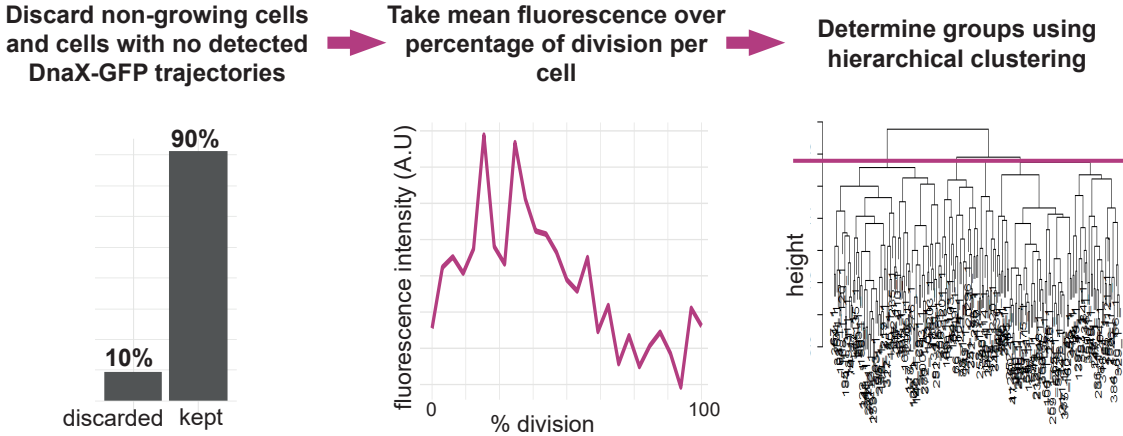
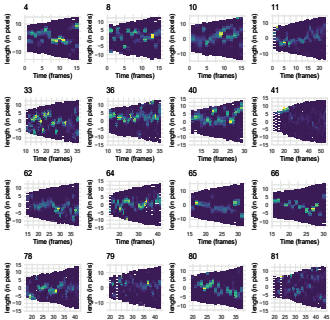
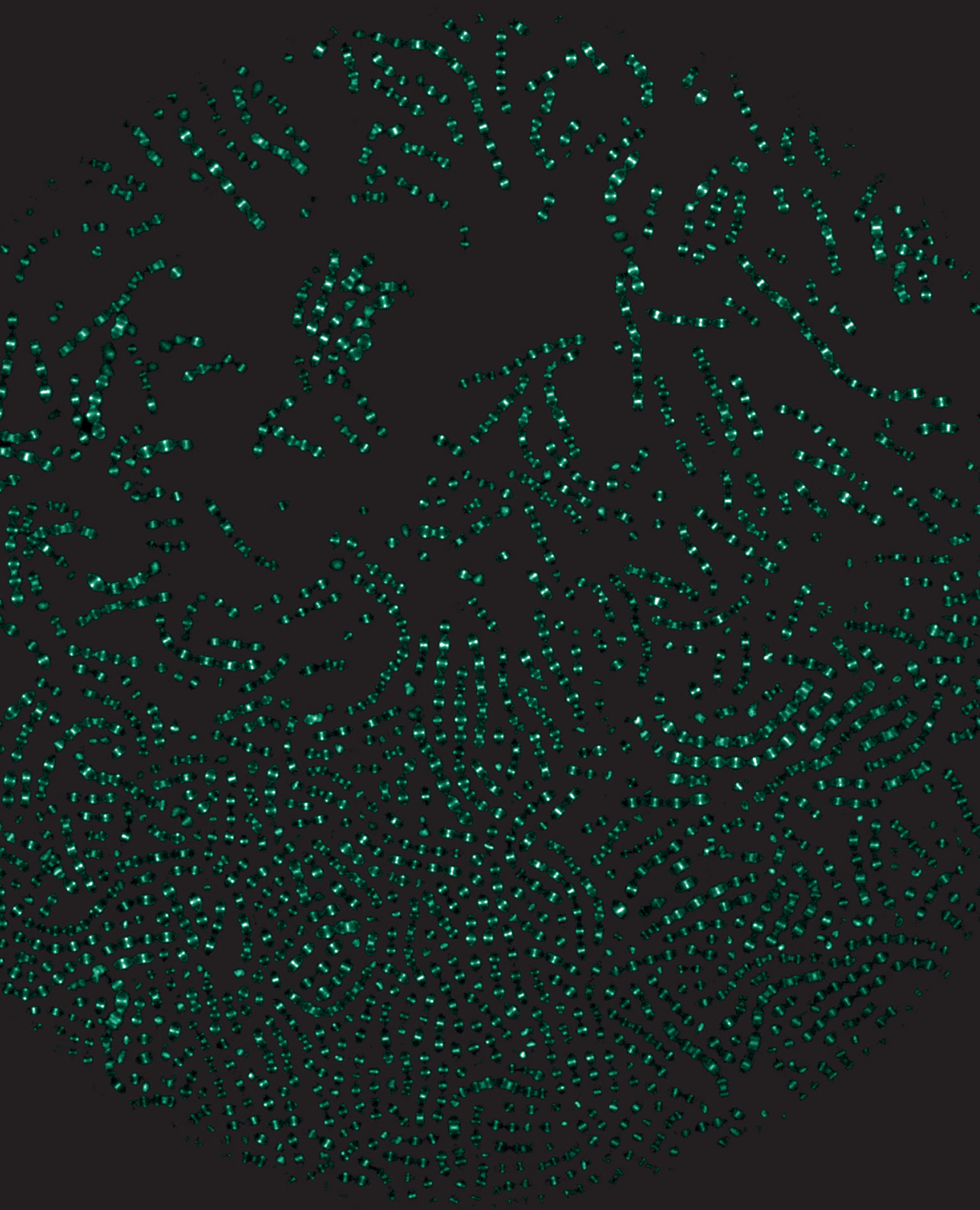


Figure S3. Clustering strategy used for Figure 5.

3.

➡ Plot & investigate groups





Chapter 4:

Chromosome segregation drives division site selection in *Streptococcus pneumoniae*

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Abstract

Accurate spatial and temporal positioning of the tubulin-like protein FtsZ is key for proper bacterial cell division. *Streptococcus pneumoniae* (pneumococcus) is an oval-shaped, symmetrically dividing opportunistic human pathogen lacking the canonical systems for division site control (nucleoid occlusion and the Min-system). Recently, the early division protein MapZ was identified and implicated in pneumococcal division site selection. We show that MapZ is important for proper division plane selection; thus the question remains what drives pneumococcal division site selection. By mapping the cell cycle in detail, we show that directly after replication both chromosomal origin regions localize to the future cell division sites, prior to FtsZ. Interestingly, Z-ring formation occurs coincidentally with initiation of DNA replication. Perturbing the longitudinal chromosomal organization by mutating the condensin SMC, by CRISPR/Cas9-mediated chromosome cutting or by poisoning DNA decatenation resulted in mistiming of MapZ and FtsZ positioning and subsequent cell elongation. Together, we demonstrate an intimate relationship between DNA replication, chromosome segregation and division site selection in the pneumococcus, providing a simple way to ensure equally sized daughter cells.

4.

Introduction

In eukaryotic cells, DNA replication, chromosome segregation and cell division are tightly coordinated and separated in time (Alberts *et al.*, 2014; Fededa & Gerlich, 2012; Willet, McDonald & Gould, 2015). In most bacteria this is less obvious as these processes occur simultaneously. However, in the last decade, it has become evident that the bacterial cell cycle is a highly regulated process, in which both cell cycle proteins as well as the chromosome have defined spatial and temporal localization patterns (Bouet, Stouf, Lebailly & Cornet, 2014; Xindan Wang & Rudner, 2014). The tubulin-like protein FtsZ (forming the Z-ring) is key for initiating divisome assembly in virtually all bacteria (Margolin, 2005). Accurate cell division is mostly exerted through regulation of FtsZ positioning in the cell. However, the mechanisms that control FtsZ positioning can be highly diverse among bacterial species. In well-studied rod-shaped model organisms, such as *Bacillus subtilis* and *Escherichia coli*, precise formation of the Z-ring at midcell is regulated by the so-called Min-system and nucleoid occlusion (Adams, Wu & Errington, 2014; Rowlett & Margolin, 2015). These are both negative regulators of FtsZ polymerization, which prevent premature Z-ring formation and cell division near cell poles and across unsegregated chromosomes, respectively (de Boer, Crossley & Rothfield, 1989; Wu & Errington, 2004). These two regulatory mechanisms have been found in many bacteria. However, in some species other dedicated proteins are used for this purpose, including MipZ in *Caulobacter crescentus* (Thanbichler & Shapiro, 2006), SsgB in *Streptomyces coelicolor* (Willemse, Borst, De Waal, Bisseling & Van Wezel, 2011) and PomZ in *Mycococcus xanthus* (Treuner-Lange *et al.*, 2013). It is important to note that none of these FtsZ regulation mechanisms are essential for bacterial growth, and other mechanisms of cell cycle control must therefore also exist (Männik & Bailey, 2015; Monahan, Liew, Bottomley & Harry, 2014; Zaritsky & Woldringh, 2015). In this context, it has been suggested that there are important links between different cell cycle processes, such as DNA replication and Z-ring assembly (Donovan, Schauss, Krämer & Bramkamp, 2013; Hajduk, Rodrigues & Harry, 2016; Monahan *et al.*, 2014; Wallden *et al.*, 2016; Zaritsky & Woldringh, 2015).

As for the opportunistic pathogen *S. pneumoniae*, the orchestration of replication and chromosome dynamics remains largely unknown. Ovococcal *S. pneumoniae* lacks a nucleoid occlusion system and has no Min-system (Fadda *et al.*, 2007; Pinho, Kjos & Veening, 2013). Recently, MapZ (or LocZ) was proposed to be a division site selector in *S. pneumoniae* (Fleurie, Lesterlin, *et al.*, 2014; Holečková *et al.*, 2014). This protein localizes early at new cell division sites and positions FtsZ by a direct protein-protein interaction (Fleurie, Lesterlin, *et al.*, 2014). MapZ is binding peptidoglycan (PG) via an extracellular domain, and is also a protein substrate of the master regulator of pneumococcal cell shape, the Ser/Thr kinase StkP (Beilharz *et al.*, 2012; Fleurie, Lesterlin, *et al.*, 2014; Holečková *et al.*, 2014). Together, this suggests that for division site selection in *S. pneumoniae*, FtsZ is controlled via the MapZ beacon at midcell (Bramkamp, 2015; Grangeasse, 2016; Monahan *et al.*, 2014).

Furthermore, the mechanisms of chromosome segregation in pneumococci also seem to be different from rod-shaped model bacteria; *S. pneumoniae* harbors a single circular chromosome with a partial partitioning system that only contains the DNA-binding protein ParB with *parS* binding sites, but lacks the ATPase ParA. Furthermore, the ubiquitous condensin protein SMC is not essential (Minnen, Attaiech, Thon, Gruber & Veening, 2011). Although both ParB and SMC are involved in chromosome segregation in pneumococci, *parB* and *smc* mutants have minor growth defects and a low percentage of anucleate cells (1-4 %) (Kjos & Veening, 2014; Minnen *et al.*, 2011). In contrast, in *B. subtilis* deletion of *smc* is lethal at normal growth conditions (Britton, Lin & Grossman, 1998). To gain more understanding of the progression of the pneumococcal cell cycle, we therefore investigated the relationship between DNA replication, chromosome segregation and division site selection in this pathogen. We show that MapZ is not involved in division site selection as suggested before, but is crucial for correctly placing the Z-ring perpendicularly to the length axis of the cell. By establishing new tools to

visualize the replisome and different genetic loci, we show that there is an intimate relationship between DNA replication, chromosome segregation and division. Importantly, we demonstrate that correct chromosomal organization acts as a roadmap for accurate division site selection in pneumococcus and possibly other bacteria.

Results

MapZ identifies the division plane but does not select the division site

4.

MapZ has been proposed as the division site selector in *S. pneumoniae*, and Z-ring positioning and angle were shown to be perturbed in *mapZ* null-mutants (Fleurie, Lesterlin, *et al.*, 2014; Holečková *et al.*, 2014). In contrast to what can be expected for a protein involved in division site selection, $\Delta mapZ$ mutants are not elongated but on average shorter than wild type cells with relatively minor distortions in cell morphology (Fleurie, Lesterlin, *et al.*, 2014; Holečková *et al.*, 2014), raising questions on what the actual biological function of MapZ is (Boersma *et al.*, 2015). To reassess the $\Delta mapZ$ phenotype, we fused MapZ at its N-terminus to a monomeric superfolder green fluorescent protein (GFP). Using the cell-segmentation software Oufi (Paintdakhi *et al.*, 2016), to detect cell outlines and fluorescent signals, in combination with the newly developed R-package SpotprocessR to analyze the microscopy data (see Methods), GFP-MapZ localization was determined in exponentially growing cells (balanced growth). Note that by balancing growth by re-diluting exponentially growing cells several times, pneumococcal cell length becomes an accurate proxy for the cell cycle state (Beilharz *et al.*, 2012; Fadda *et al.*, 2007). Cells were ordered by length, and this order was plotted against the position of GFP-MapZ on the long axis of the cells as a density histogram (Figure 1a). In line with previous reports (Fleurie, Lesterlin, *et al.*, 2014; Holečková *et al.*, 2014), GFP-MapZ localized to the division site (Figure 1a). As new cell wall is synthesized at midcell (Pinho *et al.*, 2013), MapZ seems to move from the current division site, probably via attachment to PG, and ends up at the interface between the new and old cell halves. This position will eventually become the future division site where the Z-ring assembles. It should be noted that we do not observe three MapZ rings, which is in line with previous results from *S. pneumoniae* D39 (Holečková *et al.*, 2014), but different from what has been found for *S. pneumoniae* R800 (Fleurie, Lesterlin, *et al.*, 2014). Deleting *mapZ* in the encapsulated D39 genetic background led to irregularly shaped and shorter, sometimes branched or clustered cells (Figure 1b). Similar observations were made in serotype 4 strain TIGR4 and in the unencapsulated R6 laboratory strain (Figure S1a and b).

To examine FtsZ localization, we constructed a C-terminal monomeric red fluorescent protein (mCherry) fusion to FtsZ expressed from its own locus as the only copy (Beilharz, van Raaphorst, Kjos & Veening, 2015). While the FtsZ-mCherry strain showed a normal cell size distribution in a wild type background, when combined with the $\Delta mapZ$ mutant, a clear synthetic phenotype arose and cells were misformed (Figure S2a and b), suggesting that the previously described *mapZ* phenotype in the presence of FtsZ-fusions should be interpreted with caution (Boersma *et al.*, 2015). Therefore, we replaced FtsZ-mCherry by a more functional FtsZ-CFP or FtsZ-mKate2 (FtsZ-RFP) fusion (Figure S2c and d), and reassessed FtsZ localization in $\Delta mapZ$ cells. As reported before (Fleurie, Lesterlin, *et al.*, 2014; Holečková *et al.*, 2014), localization of FtsZ to future division sites occurs when MapZ is already localized at this position (Figure 1a and c). Note, however, that in stark contrast to MapZ, which gradually moves as new cell wall is synthesized, FtsZ is highly dynamic and remodels quickly from the previous to the future division site. Thus, there is only a short period of the cell cycle where MapZ and FtsZ colocalize (compare Figure 1a and c) (Fleurie, Lesterlin, *et al.*, 2014; Holečková *et al.*, 2014). To compare the localization of FtsZ in the wild type and mutant cells, we first determined the relative cell size

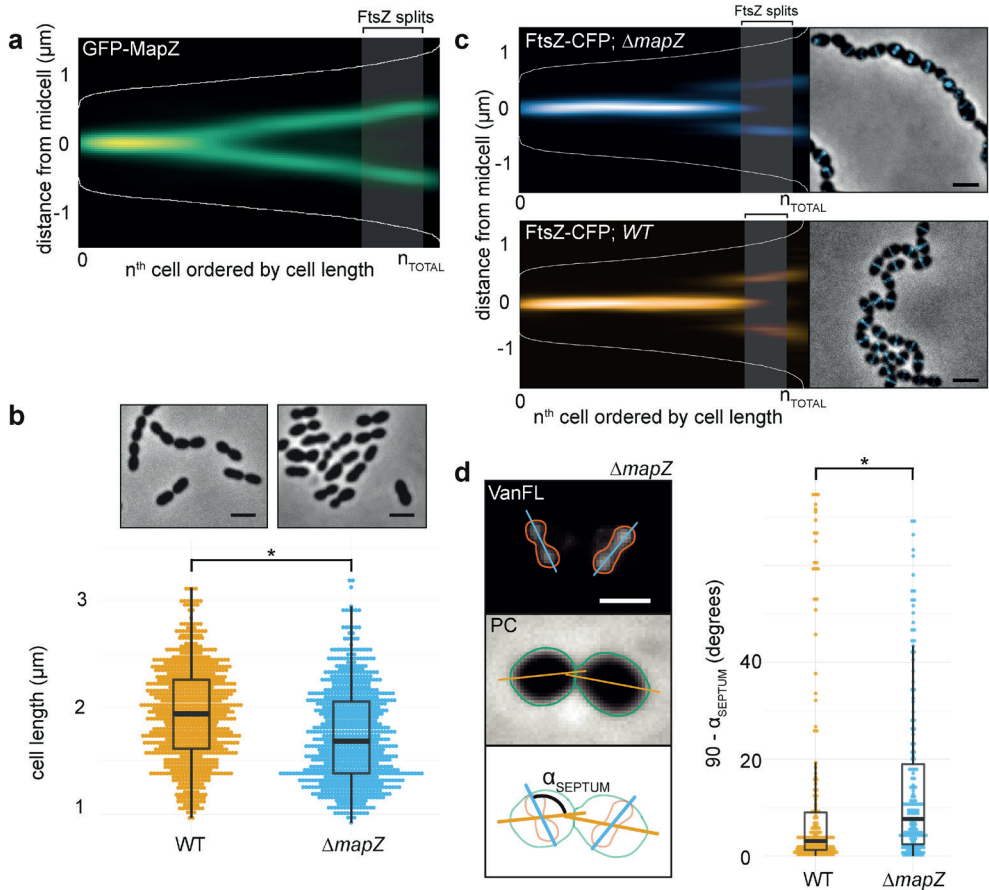


Figure 1. MapZ sets the pneumococcal division plane but is not involved in division site selection. a. Fluorescence microscopy of 2976 cells, 7020 spot localizations were quantified and analyzed using Ufti & SpotprocessR (see Methods). The distance of GFP-MapZ (strain RR101) from midcell was plotted in a heatmap where all localizations are ordered by cell length, and the color saturation represents the density of the localizations. The shaded area represents the point in the cell cycle where 50% of the FtsZ proteins move to the $\frac{1}{4}$ positions of the cell (124 ± 9.04 % of the mean cell length, see Methods). **b.** Cell size distribution of wild type D39 and ΔmapZ cells (strain RR93), representing measurements of 1692 and 705 cells, respectively. Top: phase contrast microscopy images. Scale bar is 2 μm . **c.** The localization of FtsZ-CFP in wild type (strain RR23) and ΔmapZ (strain RR105) cells as shown by histograms and micrographs from overlays of phase contrast images with CFP signal. Scale bar is 2 μm . The plots are based on data from 617 cells/957 localizations for ΔmapZ and 1717 cells/2328 localizations for wild type. The shaded area represents the point where 50% of FtsZ moves to the $\frac{1}{4}$ positions in wild type cells. **d.** The angle of the septum relative to the length axis of the cells is larger and more variable in ΔmapZ cells. Left: wild type D39 and ΔmapZ cells (strain RR93) were stained with fluorescently labeled vancomycin (VanFL). Fluorescence image (top), phase contrast image (middle) and a schematic drawing of the analysis (bottom) are shown. The angle, α_{SEPTUM} was measured automatically by measuring the angle between the long axis of the bounding boxes of the cell outlines and the long axis of the bounding box of the VanFL signal. The angles of both wild type and ΔmapZ cells are significantly different from 90° (sign test, $p \leq 2.2 \times 10^{-16}$), however, the septum angle is significantly more skewed in ΔmapZ cells ($p < 0.05$, Kolmogorov-Smirnov test). Scale bar is 1 μm . Right: the α_{SEPTUM} plotted in wild type cells and ΔmapZ cells. 177 and 181 cells were measured for wild type and ΔmapZ , respectively.

where FtsZ localization is at the $\frac{1}{4}$ positions in 50% of the cells in the wild type background using eight datasets of both FtsZ-CFP and FtsZ-RFP localizations (totaling 26 986 cells). The cell size where 50% of FtsZ splits was strikingly consistent between the datasets ($1.97 \pm 0.137 \mu\text{m}$). Since the cell sizes of mutant cells differ from wild type cell sizes, we converted the splitting point to a percentage of the mean cell length ($P_{\text{SPLIT}_N} = 124 \pm 9.04 \%$) and visualized this as a shading area in both the wild type and ΔmapZ density histograms (Figs. 1a and c). Importantly, FtsZ localization over the length axis of the cell was not affected in ΔmapZ cells, suggesting that MapZ is not essential for accurate timing of Z-ring assembly. To gain more insights into the role of MapZ during septum formation, we stained cells with fluorescently-labeled vancomycin (Van-FL) to image sites of cell wall synthesis (Daniel & Errington, 2003), and measured the angle of the areas in the cell enriched with Van-FL relative to the long axis. Interestingly, the angle of the septum was not perfectly perpendicular to the cell length in wild type cells (median difference from 90° $\alpha_{\text{SEPTUM}} = 3.08 \pm 1.47^\circ$), and this difference from 90° was found to be significant (one sided sign test, $p \leq 2.2 \cdot 10^{-16}$). Notably, however, in ΔmapZ cells, this angle was significantly more skewed than wild-type (Figure 1d, median difference from 90° $\alpha_{\text{SEPTUM}} = 7.65 \pm 1.27^\circ$, $p = 0.014$, Kolmogorov-Smirnov test). Measuring the angle of FtsZ-CFP in the same manner as with Van-FL confirmed that the angle of the Z-ring was significantly more skewed in ΔmapZ cells (Figure S2e).

These results are in line with previous observations (Fleurie, Lesterlin, *et al.*, 2014; Holečková *et al.*, 2014) and could explain the variability in cell shapes observed in ΔmapZ mutants. The observed cell shape alterations are reminiscent of *E. coli* mutants lacking certain low molecular weight penicillin binding proteins (LMW-PBPs) such as PBP5 that have defected division plane selection and mislocalized Z-rings (Potluri, de Pedro & Young, 2012). LMW-PBPs modify PG by trimming amino acid linkages from the glycan side chains (Popham & Young, 2003). Since MapZ has a large extracellular PG binding domain and is controlled by the Ser/Thr kinase StkP (Fleurie, Lesterlin, *et al.*, 2014; Manuse *et al.*, 2016), which is proposed to be a key player in tuning peripheral and septal peptidoglycan synthesis (Beilharz *et al.*, 2012; Fleurie, Manuse, *et al.*, 2014), it is tempting to speculate that MapZ has a function in cell wall remodeling and subsequently maintaining the perpendicular Z-ring plane. Although this remains to be verified experimentally, it has been shown that mutations in peptidoglycan hydrolases as well as depletion of Pbp2b involved in cell elongation, can lead to non-parallel division and cell shape alterations in *S. pneumoniae* (Berg, Stamsas, Straume & Havarstein, 2013; Boersma *et al.*, 2015; Tsui *et al.*, 2014), similar to the phenotype of ΔmapZ cells.

The replisome of *S. pneumoniae* is dynamic around midcell

Since the ΔmapZ mutant has moderate effects on division site selection under our experimental conditions, another system must be in place. Since *S. pneumoniae* lacks the canonical systems, we hypothesized that ovococci might coordinate division via chromosome replication and segregation (Hajduk *et al.*, 2016). To test this, we first aimed at imaging the DNA replication machine (replisome) and constructed inducible, ectopic fusions of the single-strand binding protein (SSB), the β sliding clamp (DnaN) and the clamp loader (DnaX) with GFP or RFP (mKate2). Fluorescence microscopy showed enriched signals as bright diffraction limited spots for all fusions, mainly localized in the middle of the cells, similar to what has been observed for *B. subtilis* and *E. coli* (Lemon & Grossman, 2000; Reyes-Lamothe, Possoz, Danilova & Sherratt, 2008) (Figure 2a). Notably, the background signal of SSB-RFP was higher than the background of the other fusions, as also reported for *E. coli* (Reyes-Lamothe *et al.*, 2008). Chromosomal replacements of the fusion constructs with the original gene could only be obtained for *dnaX*, but not for *ssb* and *dnaN*, suggesting that the fusion tags of these two latter genes are not fully functional. To validate that the localizations of the fusions represent biologically active replisomes, we examined their colocalization patterns. As expected, the ectopically expressed fluorescent fusions of DnaX, DnaN and SSB to RFP colocalize with the functional DnaX-GFP

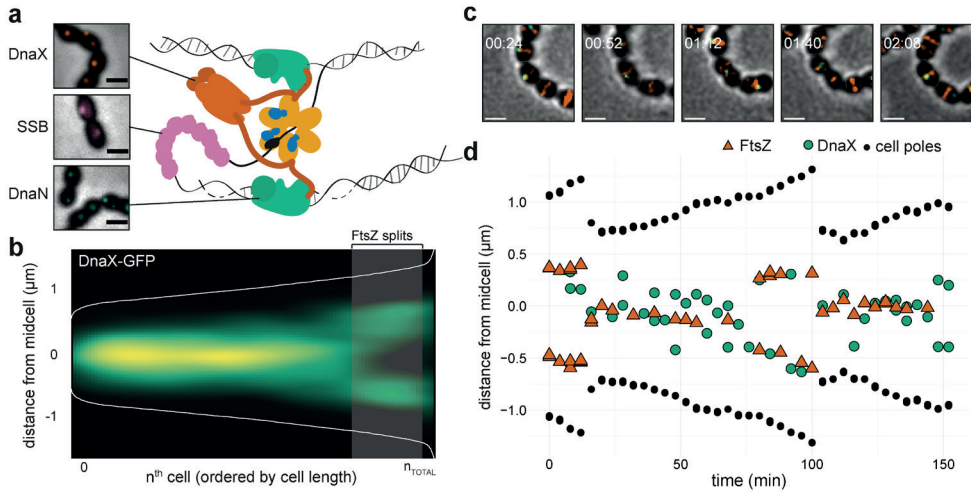


Figure 2. Localization of the pneumococcal replisome. **a.** Localization of DnaX-GFP (RR31), GFP-DnaN (DJS02) and SSB-GFP (RR33). A cartoon of the bacterial replication fork at initiation shows the role of DnaX (clamp loader), SSB (single-strand binding protein) and DnaN (β sliding clamp). DNA Polymerase is replicating the leading strand (top) and Okazaki fragments at the lagging strand (bottom). The cartoon is based on what is known about the replication fork in *Escherichia coli*. **b.** Plotting the localization of DnaX-GFP (RR22) shows that the replisome is localized at midcell. Data from a total of 3574 cells, 3214 unique localizations. The shaded area represents the point in the cell cycle where 50% of FtsZ has localized to the 1/4 positions of the cell. **c.** Snap shots from a representative time-lapse movie of strain MK396 (*dnaX-GFP, ftsZ-mKate2*). Overlays of GFP, RFP and phase contrast are shown. Scale bar is 1 μm **d.** Transcript of the cell shown in Figure 2C. The distance of FtsZ (red), DnaX (green) and the cell poles (black) to midcell is plotted against time. The data is also shown in Movie S1. Transcripts of more single cells are shown in Figure S3c.

fusion in live cells (91 % colocalization or more, Figure S3a).

DnaX-GFP is positioned close to midcell with a similar pattern as FtsZ-RFP, although the DnaX-GFP localization is less precise than FtsZ-RFP (Figure 2b). To analyze the correlation between DnaX appearance at the new division site and FtsZ splitting, a DnaX-GFP/FtsZ-RFP double-labeled strain was made. A cluster analysis on both DnaX and FtsZ localizations was performed to determine the “fork point” of the cell length where 50% of the proteins are localized at the new division sites. This point was at 1.856 μm for DnaX and 1.998 μm for FtsZ, respectively (1234 cells analyzed), which is a difference within the resolution limit. Furthermore, plotting the localization densities of both the mid-cell clusters and 1/4-position clusters against each other, show that there is no significant difference in the fork points between DnaX and FtsZ (Figure S3b). To validate these results, we tracked single cells during growth using time-lapse fluorescence microscopy (Figure 2c and d, Figure S3c, Movie S1 and S2). This showed that although the replisome(s) is dynamic, it stays in near proximity to the Z-ring. Together, these data demonstrate that the replisome moves to the future cell division sites coincidentally with FtsZ and the Z-ring does not linger for cell division to finish (Figure 2d).

To gain more insight into the nature of the movement of the replisome, we imaged DnaX-GFP in short-time interval movies (1 sec, Movie S3) using total internal reflection fluorescence (TIRF) microscopy. We tracked DnaX-GFP using the single molecule tracking software U-track (Jaqaman *et al.*, 2008) and analyzed mobility using SpotprocessR (Figure S3d). As expected, replisome mobility was significantly lower than that of free diffusing GFP (Elowitz, Surette, Wolf, Stock & Leibler, 1999). However, compared to ParB-GFP, which binds to the origin of replication (*oriC*) (Minnen *et al.*, 2011), DnaX-GFP showed a nearly two-fold higher mobility

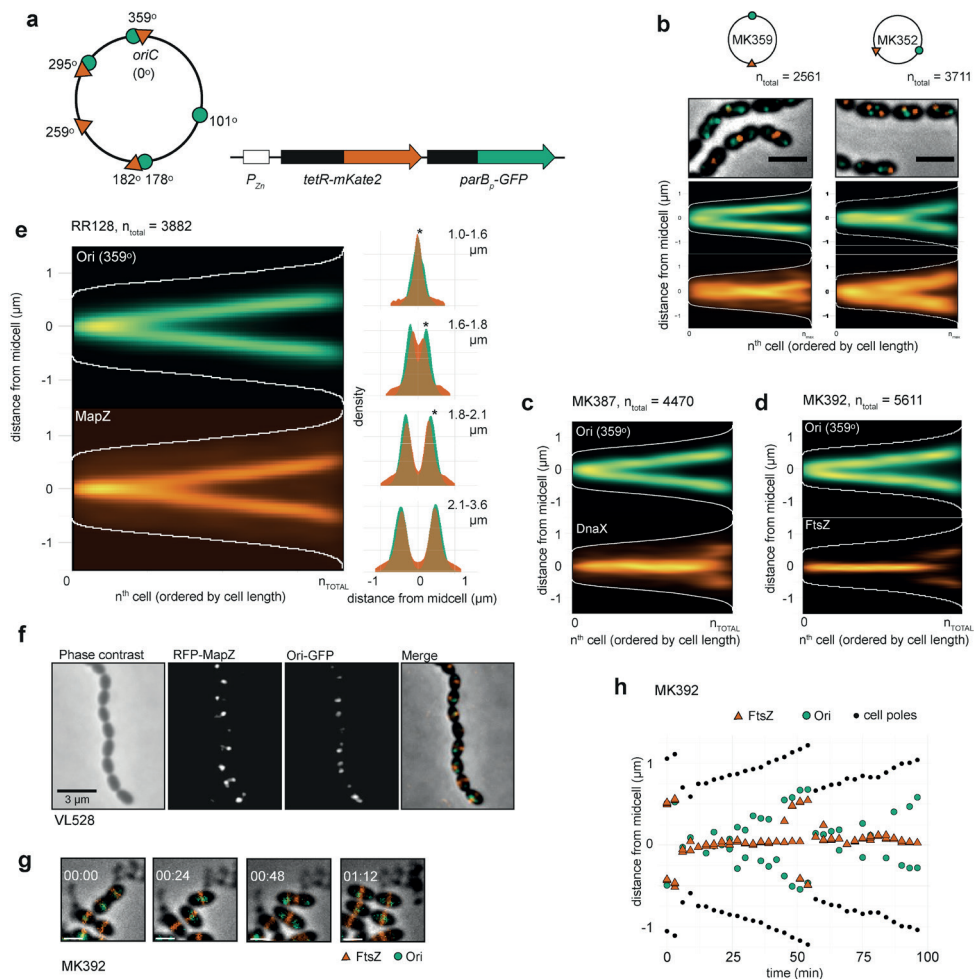


Figure 3. Chromosomal organization in *S. pneumoniae*. **a.** Visualizing specific genetic loci in live cells by fluorescence microscopy was done by developing two independent chromosomal markers systems; TetR-mKate2/*tetO* (*tetO* integration sites indicated by red triangles on the chromosome map) and ParB_p-GFP/*parS_p* (*parS_p* integration sites indicated by green circles). *tetR-mKate2* and *parB_p-GFP* are ectopically expressed from the non-essential *bgaA* locus under control of the Zn²⁺-inducible promoter P_{Zn} . **b.** Localization of the origin and terminus (MK359, left panel) and left and right arm (MK352, right panel) in exponentially growing cells. Overlays of GFP signal, RFP (mKate2) signals and phase contrast images are shown. Scale bars are 2 μm. The data represents 2561 cells/3815 GFP-localizations/2793 RFP-localizations from MK359 and 3711 cells/5288 GFP-localizations/4372 RFP-localizations from MK352. **c.** Localization of the origin (ParB_p-GFP/*parS_p* at 359°) and DnaX-RFP (MK387). The data represents 4470 cells/5877 GFP-localizations/4967 RFP-localizations. **d.** Localization of the origin (ParB_p-GFP/*parS_p* at 359°) and FtsZ-RFP (MK392). The data represents a total of 5611 cells/6628 GFP-localizations/26674 RFP-localizations. **e.** Localization of the origin (ParB_p-GFP/*parS_p* at 359°) and RFP-MapZ (RR128) on the length-axis of the cell shown as heatmaps (left) and overlay of both localization density plots when the cells are grouped in four quartiles by cell length (right). Stars indicate a significant difference between GFP and RFP localization (Kolmogorov-Smirnov test, $p < 0.05$). The data represents 3882 cells/1785 GFP-localizations/8984 RFP localizations. **f.** Microscopy images of strain VL528 showing subsequent origin and MapZ splitting. Phase contrast, RFP, GFP and overlay images are shown. Scale bar = 3 μm. **g** and **h.** Time-lapse microscopy shows that the origins move to the next cell halves before FtsZ. Snap shots from a representative time-lapse experiment (**g**) and plotting of the distances of FtsZ, the origins and the cell poles relative to midcell in a single cell (**h**) are shown. More examples of origin and FtsZ localizations in single cells are shown in Figure S7.

(MSD = $2.66 \times 10^{-2} \mu\text{m}^2$, $D_{app} = 2.44 \times 10^{-3} \mu\text{m}^2/\text{s}^{-1}$ compared to MSD = $8.8 \times 10^{-3} \mu\text{m}^2$, $D_{app} = 3.19 \times 10^{-4} \mu\text{m}^2/\text{s}^{-1}$; Figure S3d and e), indicating that DnaX movement is not strictly confined by the chromosome.

The pneumococcal chromosome segregates in a longitudinal fashion

The on average midcell localization of the replication machineries in *S. pneumoniae* suggests that DNA replication at midcell might determine an ordered chromosomal organization. To examine this, methods for tagging chromosome positions in this bacterium were established (Figs. S4 and S5). We first constructed a novel chromosome marker system based on fluorescent protein fusions to ParB of plasmid pLP712 (Wegmann, Overweg, Jeanson, Gasson & Shearman, 2012) from *Lactococcus lactis* (hereafter named ParB_p), which was found to require insertion of only a 18 bp *parS* binding site (hereafter named *parS*_p) in the pneumococcal genome for visualizing genetic loci by microscopy (Figs. S4a and c). The *parS*_p sequence is simpler compared to existing ParB/*parS* systems and does not require additional host factors (Funnell & Gagnier, 1994; Li, Sergueev & Austin, 2002). Importantly, this system does not perturb DNA replication and is completely orthogonal to *S. pneumoniae* chromosomal ParB (Figs. S4b and S5a-d). Secondly, we adapted the TetR/*tetO* fluorescence repressor-operator system (FROS) (X. Wang, Montero Llopis & Rudner, 2014) for *S. pneumoniae* and validated that it does not interfere with DNA replication (Figs. S4d and S5a-c). To verify the localization patterns and compatibility of both systems, we constructed a strain containing both *parS*_p and *tetO* near *oriC* and showed that *parB*_p-*gfp* and *tetR*-*rfp* foci colocalize (Figs. S5e and f).

In total, five chromosomal locations were marked using ParB_p/*parS*_p and/or TetR/*tetO*; the origin-region (359° degrees), right arm (101°), *ter*-region (178° and 182°) and two positions on the left arm (259° and 295°) (Figure 3a). Using double-labeled strains under balanced growth, the localizations of loci were compared revealing that the pneumococcal genome is organized in a longitudinal fashion (Figure 3b, Figure S6). The left and right arms move at the same time to the new daughter cells (Figure 3b). The terminus region seemed less confined in space during the cell cycle (Figure 3b). Strikingly, the origins never localized near the cell poles as is common in other bacteria (Viollier *et al.*, 2004; X. Wang *et al.*, 2014; Xindan Wang & Rudner, 2014), and arrived to future division sites at a very early stage, before DnaX and FtsZ, with a similar timing as MapZ (Fig 3c-f). The early segregation of *oriC* was also observed when single cells were tracked over time (Figure 3g and h, Figure S7 and Movie S4). Note that this organization is strikingly different from organisms with nucleoid occlusion where FtsZ preferably polymerizes across DNA-empty regions or regions near the terminus that lack Noc protein.

SMC is required for correct segregation of *oriC* and cell shape

The observation that *oriC* arrives at future cell division sites prior to FtsZ opens the question whether MapZ has a role in directing the chromosome. However, the origin still localized to the future division site in ΔmapZ (Figure 4a). The localizations of MapZ and *oriC* were further analyzed in a wild type background by sorting the cells into four subgroups according to cell size and plotting the localizations as histograms over the cell lengths (Figure 3e, right panel). This shows that MapZ is localized slightly closer to the old midcell in smaller, newborn cells (Figure 3e, right panel, stars indicate a significant difference in the first three groups of cells, Kolmogorov-Smirnov test, $p < 0.05$), and indicates that *oriC* localizes to the new midcell before MapZ. To study this in more detail, we constructed a strain with GFP labelled *oriC* and two copies of *rfp-mapZ* (strain VL528) to improve RFP-MapZ signal (Figure 3e). For this strain, we determined, per single cell, whether the origin or MapZ localizes furthest away from mid-cell. In 71% of the cells, the origin and MapZ localized together (distance <200 nm). Of the rest

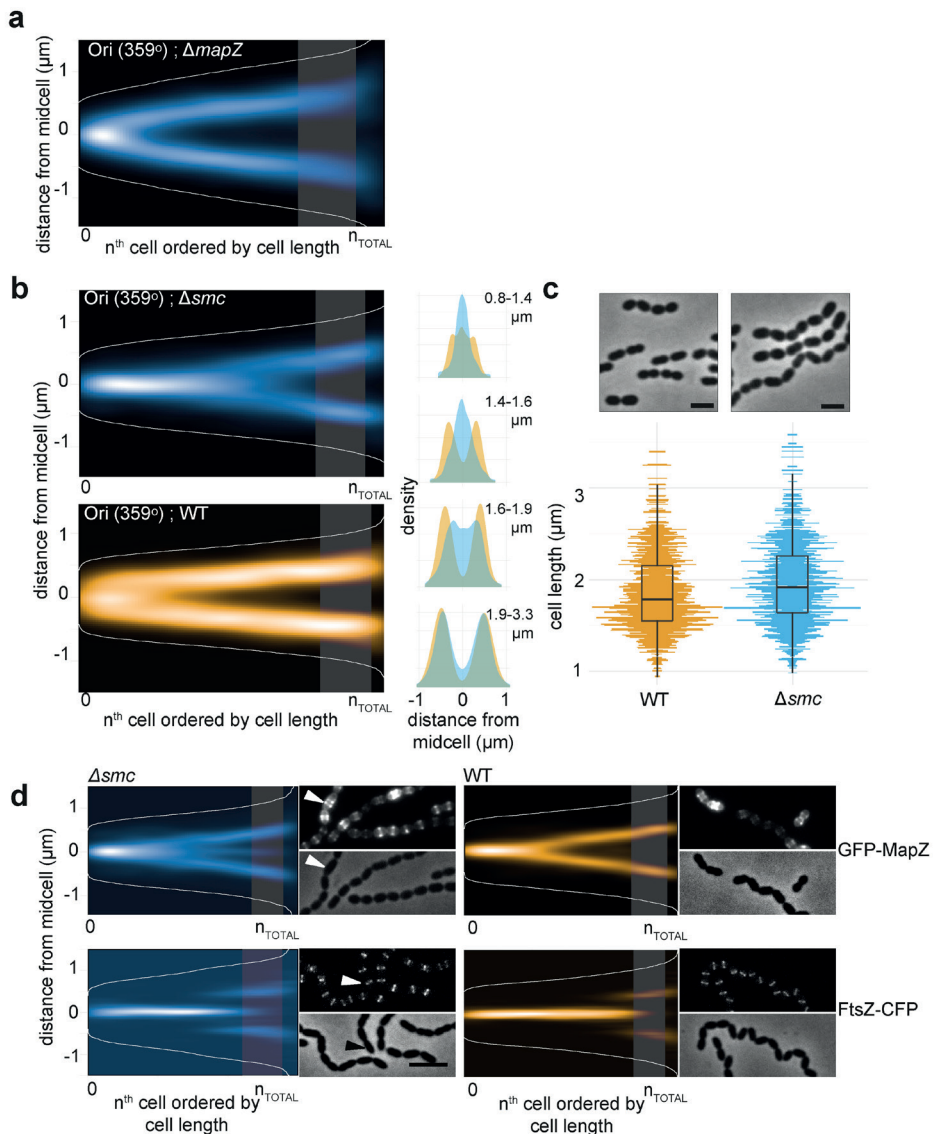


Figure 4. SMC is required for origin segregation and accurate division site selection. A. Localization of the chromosomal origin (ParB_o-GFP/*parS*_o at 359°) in a ΔmapZ background shows that MapZ does not affect origin segregation (RR99). The data represents 2976 cells/7020 localizations. The shaded areas in all density histograms in this Figure represent the point in wild type cells where 50% of FtsZ localizes to the 1/4 positions of the cell. **B.** The origins (ParB_o-GFP/*parS*_o at 359°) are segregated at a later stage in the cell cycle in Δsmc compared to wild type. The localizations are shown as heatmaps when cells are sorted according to length (left) and as overlay of both localization density plots when the cells are grouped in four quartiles by cell length (right). The data represents 2012 cells/3815 localizations for wild type (MK359) and 3908 cells/5192 localizations for the Δsmc mutant (MK368). **C.** Phase contrast images of wild type D39 and Δsmc cells (AM39). The scale bar is 2 μm (top). Comparison of cell lengths between the wild type (1407 cell analyzed) and Δsmc (1035 cells analyzed, bottom). **D.** Localization of GFP-MapZ and FtsZ-CFP in wild type versus Δsmc . Fluorescence and phase contrast micrographs are shown along with heatmaps. The arrowhead in the micrograph points to a cell with clearly mislocalized MapZ. Data represents 2560 cells/5314 localizations (Δsmc , GFP-MapZ, RR110), 1300 cells/2257 localizations (Δsmc , FtsZ-CFP, RR84), 3908 cells/3128 localizations (wild type, GFP-MapZ, RR101) and 3422 cells/29464 localizations (wild type, FtsZ-CFP, RR70).

29%, the origin localized furthest away from mid-cell than MapZ in 68% of the cells, (2240 cells analyzed). Although the difference is small, this confirms that the origin, on average, localizes to the new midcell slightly before MapZ.

Given the early movement of the origin to the future cell division sites, we wondered whether instead the chromosome or nucleoid associated proteins could play a role in guiding Z-ring positioning. In many prokaryotes, condensin-like proteins called Structural Maintenance of Chromosomes (SMC) play a role in the organization and compaction of the chromosome (Gruber, 2014). In *Streptococcus pneumoniae*, deletion of *smc* leads to approximately 2% anucleate cells and problems in chromosome segregation (Kjos & Veening, 2014; Minnen *et al.*, 2011). To specifically investigate how the absence of SMC affected chromosome organization, the origin, terminus and left/right arm chromosome positions were determined in Δsmc cells. In line with what has been found using temperature-sensitive or degradable alleles in *B. subtilis* (Gruber *et al.*, 2014; Xindan Wang *et al.*, 2014), the origin region arrived to the new midcell at a considerable delay when SMC was absent (Figure 4b). Quantitative analysis of the origin localization showed a significant different localization of *oriC* in Δsmc vs wild type ($p < 1.5 \times 10^{-3}$, Kolmogorov-Smirnov test, Figure 4b). Eventually, however, the origins still segregated to their correct localization in subsequent larger cells. Also, segregation of the left arm, right arm and terminus did not differ significantly from wild type (Figure S8b-d). Thus, *S. pneumoniae* SMC is specifically important for the early segregation of *oriC*. SMC-GFP showed, in line with previous observations (Minnen *et al.*, 2011), a nucleoid-like pattern with foci in a subset of cells with poorly defined localizations (Figure S8a).

In the current data, we also found that Δsmc cells are longer, more irregularly shaped and form long chains (Figure 4c). The same observation was also made upon deletion of *smc* in strains TIGR4 and R6 (Figure S1a and b). This suggests that *smc* mutants are somehow defective, not only in chromosome segregation, but also in cell division. We therefore compared the localization of MapZ and FtsZ in wild type and Δsmc cells (Figure 4d). MapZ showed an obvious mislocalization; part of the MapZ-rings arrived at the new septa at a later stage, while a large fraction stayed at midcell in larger cells (11% of the MapZ-localizations in the largest quartile of the cell population stayed at midcell in Δsmc cells vs. 4% in wild type cells, Figure 4d). For FtsZ, the effect is less pronounced, but a small difference in localization accuracy is observed at the time when new Z-rings were formed (Figure 4d). This subtler difference in FtsZ localization between wild type and Δsmc cells is not surprising, though, given that in the Δsmc cells, the origins have started segregating at the time of FtsZ assembly (Figure 3b). Note that the angle of the division ring is not affected in Δsmc cells (Figure S1c). Together, these results suggest that SMC and/or origin localization is important for timely and precise positioning of the cell division machinery in *S. pneumoniae*.

Knowing that both origins and MapZ localize very early in the pneumococcal cell cycle to the future division sites and that individual perturbation of origin localization or MapZ cause division problems, we deleted both *smc* and *mapZ* to understand more about the link between them. The double mutant strain was readily obtained, although the strain had severe defects in growth and cell shape (Figure S1c and d). Notably, the phenotype of the double mutant looked like a combination of the single mutants; like $\Delta mapZ$, the cells were on average smaller with large cell shape variation due to non-perpendicular division ring formation (Figure S1c), and like Δsmc , they displayed a chaining phenotype probably reflecting problems with timing of division ring formation leading to consecutive problems in timing of division and cell wall splitting (Figure S1d). These observations suggest that MapZ and SMC have independent roles in pneumococcal cell division, where SMC is important for timely localization of the division site, while MapZ is involved in placement of the division ring at a correct angle.

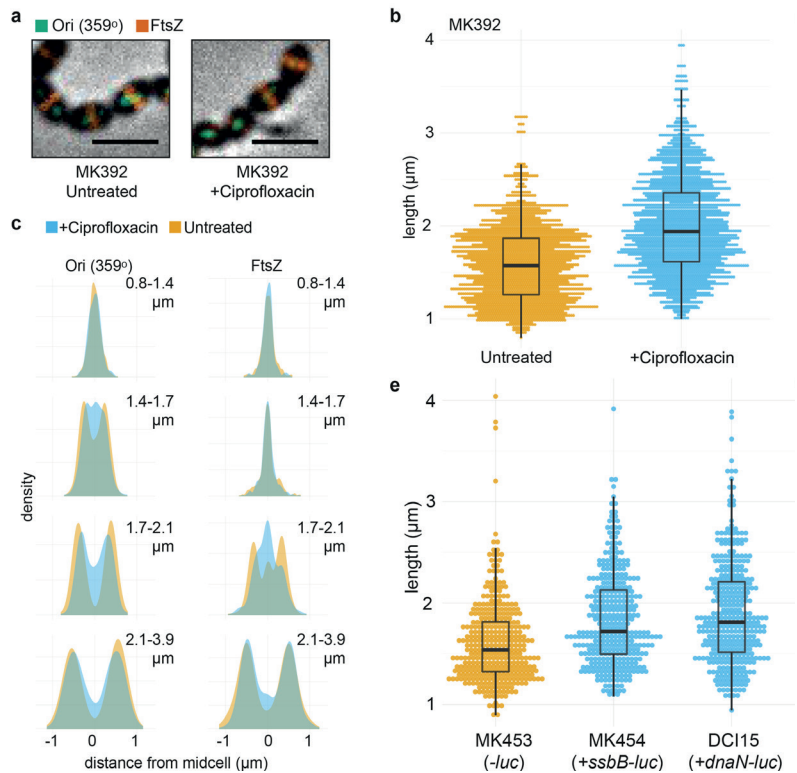
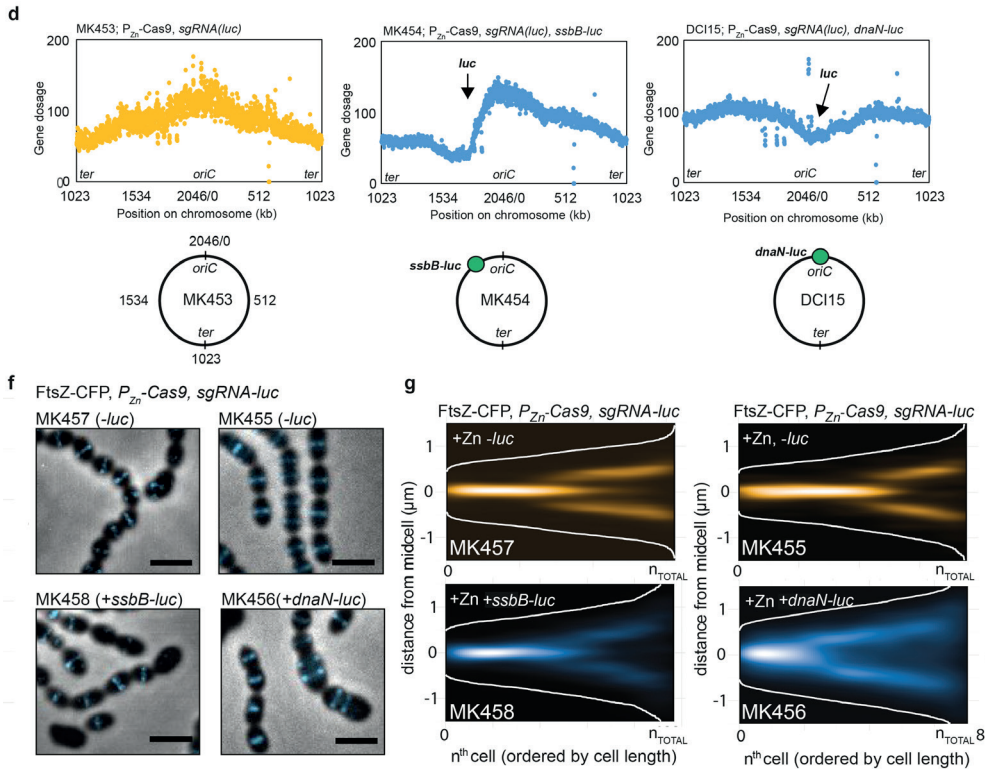


Figure 5. Perturbed chromosome segregation delays cell division. a-c. Comparison of *S. pneumoniae* D39 wild type cells treated or untreated with sublethal concentrations (0.4 $\mu\text{g/ml}$) of ciprofloxacin for 60 min. **a.** Images of strain MK392 (ParB -GFP/*parS*, at 359°, FtsZ-RFP) with overlay of phase contrast, GFP signals representing the origin and RFP signal representing FtsZ-RFP. Scale bar is 2 μm . A micrograph with more cells of the same strain can be found in Figure S9. **b.** Cell length comparison of ciprofloxacin-treated (total 1138) and non-treated (total 1402) cells (MK392). **c.** Subcellular localization of the origin (left) and FtsZ (right) in MK392 cells treated (blue) or untreated (yellow) with ciprofloxacin. Localization density plots when the cells are grouped in four quartiles by cell lengths are shown. Data represents 1138 cells/2518 RFP-localizations/2762 GFP-localizations for cells treated with ciprofloxacin and 1402 cells/2940 RFP-localizations/2540 GFP-localizations for untreated cells. **d-g.** Comparisons of cells with or without Cas9-nuclease cut chromosome. The expression of Cas9 (together with a constitutively expressed single-guide RNA directed to the *luc*-gene) was induced with Zn^{2+} for 2.5 hours in cells with or without the *luc* gene located on the chromosome. The *luc* gene was inserted either in the origin region (0°) or at the left arm (301°). In cells without the *luc* gene, no Cas9-mediated cutting will occur. **d.** Whole genome marker frequency analysis of strains without *luc* (MK453), *luc* at 301° (*ssbB-luc*, MK454) or *luc* at the origin (*dnaN-luc*, DCI15). The number of mapped reads (gene dosage) is plotted as a function of the position on the circular chromosome. The chromosomal position of the inserted *luc* gene is indicated in the plot and on the schematic chromosome maps. **e.** Cell size comparison of cells with and without cut chromosomes. The number of cells measured were 643 for the non-cut strain (MK453), 393 for the strain cut at 301° (MK454) and 383 for the strain cut near the origin (DCI15). **f.** Overlay of FtsZ-CFP signals with phase contrast images show that cell morphologies are affected in cells with cut chromosomes. Scale bar is 2 μm . **g.** Localization of FtsZ-CFP in cells with a cut chromosome (MK 458 and MK456) and intact counterparts (MK457 and MK455, respectively) shown as heat maps where cells are ordered according to cell length. The data represents 1323 cells/3117 localizations for MK455, 1128 cells/3509 localizations for MK456, 721 cells/1133 localizations for MK457 and 683 cells/1209 localizations for MK458.



Proper localization of *oriC* is crucial for division site selection in *S. pneumoniae*

We show above that deletion of *smc* caused a cell division defect in *S. pneumoniae* distinct from the $\Delta mapZ$ phenotype. To untangle whether this was a direct effect of SMC or whether it was caused by the resulting chromosome organization defect, we exposed *S. pneumoniae* to sublethal concentrations of ciprofloxacin to disturb chromosome organization while keeping *smc* intact. Ciprofloxacin is a broad-spectrum antibiotic which blocks the activities of type II topoisomerases and thereby affects DNA supercoiling and chromosome decatenation (Fernandez-Moreira, Balas, Gonzalez & de la Campa, 2000). Strikingly, when exponentially growing cells are transferred to a non-lethal concentration of ciprofloxacin (0.4 $\mu\text{g}/\text{mL}$), cells rapidly increase in cell length and form longer chains when compared to untreated cells (Figure 5a and b, Figure S9). Origin splitting was clearly delayed in ciprofloxacin treated cells, and the timing and accuracy of Z-ring formation was severely affected (Figure 5c). Moreover, localization of the replisome was less confined to the center of the cells, as was observed for Δsmc cells (Figure S10). Note that, at the ciprofloxacin concentration used in this experiment, replication elongation is reduced, but new rounds of replication are still initiated (Slager, Kjos, Attaiach & Veening, 2014).

Finally, we also perturbed the DNA biology by cutting the chromosome at two different locations; close to *oriC* (at 0°) and on the left arm (at 301°), using an inducible CRISPR/Cas9 system (see Methods). Whole-genome marker frequency analysis of these strains after induction of Cas9 for 2.5 hours, showed the expected cleavage of the chromosomal DNA at these two positions in the respective strains and major alterations in the replication patterns were observed (Figure 5d). Cutting of the chromosome had drastic effects on cell morphology and we observed frequent

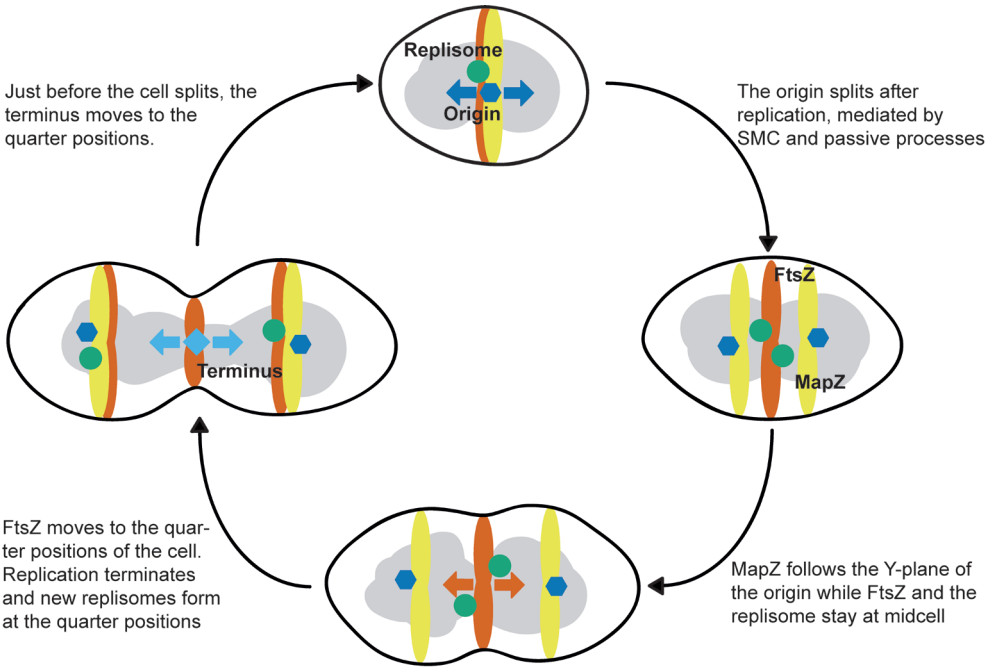


Figure 6. A schematic model for division site selection in the pneumococcus. The bulk chromosome is shown in grey, while the chromosomal origin, left/right arm and terminus are indicated as a dark blue hexagon, green circle and light blue diamond, respectively. MapZ is shown in yellow and FtsZ in orange. Four key stages of the pneumococcal cell cycle, with a newborn cell on the top, are included.

mistimed FtsZ localization (Figure 5f and g) and increased cell sizes (Figure 5e). The effects on DNA replication were more pronounced when Cas9 was targeted to *oriC* compared to the left arm location, and, subsequently, proper control of Z-ring formation was completely lost in the former case. It should be noted that generation of double strand breaks will impose severe stress on the cells, and probably elicit numerous stress responses. Nevertheless, these results further confirm that normal chromosome segregation, and origin segregation in particular, is key for well-timed Z-ring assembly and cell division progression in *S. pneumoniae*.

Discussion

By detailed mapping of DNA replication and chromosome segregation in live *S. pneumoniae* cells, we found that DNA replication initiation coincides with Z-ring formation and that proper segregation of the chromosomal origin is crucial for division site selection. We show that the pneumococcal chromosome is organized in a longitudinal fashion (ori-left/right-ter-ter-left/right-ori; Figure 3 and Figure S6) with specific subcellular addresses for each locus. In contrast to for example *B. subtilis* and *C. crescentus* (Viollier *et al.*, 2004; X. Wang *et al.*, 2014; Xindan Wang & Rudner, 2014), the origins never localize near the cell poles in *S. pneumoniae*, and the organization is in this aspect more similar to the situation in slow-growing *E. coli* (Xindan Wang, Liu, Possoz & Sherratt, 2006). Importantly, the newly replicated origins immediately mark the future cell division sites while the terminus remains at midcell. This organization is somewhat reminiscent of the chromosomal organization in *B. subtilis* and *E. coli* but is slightly simpler as every replicated locus eventually segregates to midcell before a new round of replication initiates

(Figure 6). Segregation of the chromosomal origin was dependent on SMC and deletion of *smc* caused a marked delay in origin segregation, which in turn led to alterations in the timing of localization of important cell division proteins such as MapZ and FtsZ. Mistimed MapZ and FtsZ ultimately resulted in larger cells which form chains. Importantly, the observed cell division defects are not caused by the deletion of *smc per se*; treatment of the cells with sublethal concentrations of ciprofloxacin or a CRISPR/Cas9-induced segregation block also caused similar cell division defects (i.e. larger cells and chaining). Together, this indicates that timely segregation and positioning of the chromosomal origin at the quarter position in cells is important for orchestrating pneumococcal cell division.

Recently it was found that MapZ localizes to future division sites before FtsZ and positions the Z-ring correctly via protein-protein interactions (Fleurie, Lesterlin, *et al.*, 2014; Holečková *et al.*, 2014). We found that MapZ gradually moves with a similar timing as the chromosomal origins, but MapZ is not important for correct *oriC* positioning. On the other hand, perturbation of *oriC* segregation clearly resulted in altered MapZ localization, thus indicating the pivotal role of chromosomal origin positioning for proper cell division coordination. Notably, the PG-binding protein MapZ plays an important role in establishing the correct division plane, since the angle of the Z-ring to the cell length axis was frequently skewed when *mapZ* was deleted (Figure 1d). Taken together, this suggests that while timely *oriC* positioning determines the timing of assembly and position of the cell division machinery, MapZ is a ruler for the correct angle of the division ring across the cell (Figure 6). This explains the variable size and shape of $\Delta mapZ$ cells, as well as the cell division defect resulting from the mislocalized origins in Δsmc and ciprofloxacin-treated cells. Note, however, that although *oriC* segregation is clearly delayed in both the Δsmc strain as well as in the ciprofloxacin-treated cells, it eventually segregates to the future division sites in time before Z-ring assembly. This means that there are additional cues, and not solely SMC or topoisomerases which are involved in segregation and localization of *oriC*, explaining why the cell division defects resulting from *smc* deletion or ciprofloxacin treatment are not too severe.

How the origin finds the future division site and how it can modulate the localization of division proteins such as MapZ and FtsZ is unclear. We cannot rule out an as-of-yet unknown protein factor playing a role in keeping the newly replicated origins near the future division site and MapZ or by bridging FtsZ to the DNA replication initiation complex. A link between replication and Z-ring assembly has previously been suggested for *B. subtilis* (Hajduk *et al.*, 2016), however no mechanism is yet known. In *E. coli* it has been shown that the chromosomal terminus region (Ter macrodomain) is linked to the cell division apparatus by proteins MatP, ZapA and ZapB (Bailey, Bisicchia, Warren, Sherratt & Männik, 2014; Espéli *et al.*, 2012). Similar links between the chromosome and division apparatus may also be present in other bacteria, although, in our model, we observe that segregation of the terminus occur after FtsZ re-positioning. Another hypothesis is that coupled transcription-translation-transertion of membrane proteins encoded near *oriC* aid in transitory attachment of the chromosome to the membrane (Libby, Roggiani & Goulian, 2012; Woldringh, 2002). Alternatively, physical, entropy-driven processes might be at play. In this respect, it is tempting to speculate that the origin region, which was recently shown to be highly structured and globular in shape (Marbouty *et al.*, 2015), is pushed outside the region of active DNA replication and remains rather stationary in the crowded cytoplasm (Parry *et al.*, 2014). The large globular structure of the origin can then act as a landmark for FtsZ polymerization and Z-ring formation. This hypothesis is in line with previous cytological observations demonstrating the absence of nucleoid occlusion in *S. pneumoniae* and efficient Z-ring formation over the nucleoid (Kjos & Veening, 2014; Land *et al.*, 2013). The here-described division site selection mechanism by chromosomal organization is a simple way to coordinate DNA replication, chromosome segregation and division without the need for specialized regulators of FtsZ. Future research should reveal if this mechanism is also in place in other bacteria and whether the intimate relation between chromosome segregation and cell division can be used to treat bacterial infections using combination therapy targeting both processes.

Materials and Methods

Bacterial strains, growth conditions and transformation

Bacterial strains used in this study are listed in Table S1. *S. pneumoniae* was grown in C+Y medium (Martin, Garcia, Castanié & Claverys, 1995) at 37°C without shaking. When appropriate, 0.1 mM ZnCl₂ and 0.01 mM MnCl₂ was added for induction. To reduce oxygen stress, *S. pneumoniae* was plated inside Columbia agar supplemented with 2% defibrinated sheep blood (Johnny Rottier, Kloosterzande, Netherlands). When appropriate, antibiotics were added for selection; 1 µg/ml tetracycline, 100 µg/ml spectinomycin, or 250 µg/ml kanamycin. *E. coli* was grown in LB medium with shaking. When appropriate, 100 µg/ml ampicillin or 100 µg/ml spectinomycin was used for selection.

4.

Plasmids and ligation products were transformed into *E. coli* using a standard heat shock transformation of chemically competent cells. Plasmids from *E. coli* were routinely checked by PCR and sequencing. Plasmids, ligation products or assembled DNA was transformed into *S. pneumoniae* by addition of the competence stimulation peptide (CSP) as described previously (Slager *et al.*, 2014). Constructs introduced directly into *S. pneumoniae* were checked by PCR and sequencing.

All oligonucleotides used for cloning are listed in Table S2. Construction of plasmids and strains are described in the SI Materials and Methods.

Growth assays were performed in 96-well plates using a Tecan Infinite 200 PRO instrument. OD_{595nm} was measured every 10th minute.

Preparation of cells for microscopy

S. pneumoniae cells were stored as exponential phase frozen cultures. Frozen stock were inoculated 1:100 in C+Y medium and pre-grown to OD₆₀₀ ~ 0.1. For time-lapse microscopy, cells of OD₆₀₀ ~ 0.1 were washed and diluted 1:100 in fresh C+Y and allowed to grow for 45 minutes. After this, the cells were washed once with fresh C+Y. For snap-shots, cells were diluted once again 1:100 in fresh C+Y (with inducing agent, if applicable) and grown to exponential phase to achieve balanced growth. For expression of the chromosome marker systems and CRISPR/Cas9 system, cells were induced for 2.5 hours using 0.1 mM ZnCl₂ and 0.01 mM MnCl₂ in the growth medium.

Fluorescence microscopy

For snap shots, cells were grown as described above to achieve balanced growth and subsequently concentrated and brought onto a multitest slide carrying a thin layer of 1.2% agarose in C+Y. Imaging was performed on a DV Elite microscope (GE Healthcare) with a sCMOS camera using SSI Solid State Illumination through a 100× oil immersion objective (phase contrast). The following filtersets were used: mCherry/mKate2 (Chroma, excitation at 562-588 nm, emission at 602-648 nm, Quad/mCherry polychroic), GFP (Chroma, excitation at 461-489 nm and emission at 501-559 nm, Quad polychroic) and CFP (Chroma, excitation at 400-454 nm and emission at 463-487 nm, CFP/YFP/mCherry polychroic). Several images, capturing >500 cells (exact number per experiment indicated in Figure/legends) were acquired using Softworx (Applied Precision) and further processed to TIFF files using FIJI (<http://fiji.sc>) (Schindelin *et al.*, 2012; Schneider, Rasband & Eliceiri, 2012).

Time Lapse Microscopy

A C+Y 10% polyacrylamide slide was pre-incubated for 2 hours at 37°C in C+Y (0.1 mM ZnCl₂ when applicable). 0.5 µl of the cells (prepared as described above) was spotted on the C+Y-polyacrylamide slide inside a Gene Frame (Thermo Fisher Scientific) and sealed with a cover glass. The temperature at the incubation chamber was set to 30°C to optimize fluorescence and stable growth. Time-lapse microscopy was performed as described previously (de Jong, Beilharz, Kuipers & Veening, 2011) using UltimateFocus (Deltavision) every two minutes to ensure stable imaging. The time-intervals used are indicated at each experiment. Image stacks were acquired using Softworx (Applied Precision) and further processed to TIFF files using FIJI (<http://fiji.sc>) (Schindelin *et al.*, 2012; Schneider *et al.*, 2012).

TIRF microscopy

Slides were prepared for time-lapse microscopy as described above. Imaging was done using a DV Elite microscope (Applied Precision), using the standard GFP filter sets (Chroma) and Quad polychroic. A 60x DIC oil immersion TIRF objective (1.47 NA) was used and imaging was done at 100% TIRF depth with 0.005 sec. excitation of a 50 mW laser (488 nm).

4.

Cell outline and fluorescence detection

Cell outlines were detected using Microbetracker (Sliusarenko, Heinritz, Emonet & Jacobs-Wagner, 2011) (<http://microbetracker.org>) and Oufiti (Paintdakhi *et al.*, 2016) (<http://oufti.org>). Spots were detected using Microbetracker's spotFinderZ and Oufiti's Spotfinder tool, with the exception of the experiments where interspot-distances were measured. Here we used peak fitter, a Gaussian-fit tool which is part of the ImageJ plugin iSBatch (Caldas *et al.*, 2015), to also obtain information about the full width half max (fwhm) and standard errors of the location. For larger fluorescent bodies, such as MapZ and FtsZ, Oufiti's Object Detection tool was used to obtain information about the localization and shape. For all microscopy experiments, random image frames were used for analysis and all experiments were repeated at least two times.

Plotting and statistical analysis of the localization histograms

The cell outline, object detection and spot localization data were further processed using the R-package SpotProcessR which enables plotting of the cell outlines and spot localizations (X,Y coordinates inside the cell), together with further grouping of the cells and statistical comparison of spot localizations (van Raaphorst, <https://github.com/spotprocessR>). For comparing the localization of spots inside the cell with detected objects, the centre of the minimal bounding box of the object was defined as the localization of the object and further processed the same way as spot localizations.

For the snap shots, the cells were standardly ordered by cell size and localizations on the length axis were plotted as a density plot. When two localizations were compared, the combined datasets were grouped into quartiles based on cell size and localizations on the length axis were plotted as dual colour density maps for each group. The similarity of the X and Y localization distribution shape and median were tested using a two-sided Kolmogorov-Smirnov-test, as were the similarities in cell length distribution.

Determination of the FtsZ splitting point

To compare mutant and wild type strains, the relative cell size where FtsZ splits in wild type cells was used as a fiduciary marker. To determine this, eight different datasets of in total 26986 cells

were analysed. Of these datasets, two contained the localization of FtsZ-CFP (4016 cells) and six FtsZ-RFP (22970 cells). For all datasets, the cell length was determined at which in 50% of the cells FtsZ had moved to the $\frac{1}{4}$ positions of the cells. To separate the mid-cell localizations from the $\frac{1}{4}$ position localizations, a Gaussian Mixture Model based cluster analysis was performed on the localizations per cell length for each dataset using the R package Mclust (Fraley & Rafterty, 2002; Fraley, Rafterty, Brendan Murphy & Scrucca, 2012). For each cluster, the density per cell length was determined for both the $\frac{1}{4}$ -position cluster and the midcell cluster. After correction for the number of localizations per cluster, the point in cell length was found where the density functions intercept. This point, P_{SPLIT} , is the point where 50% of the localizations are at mid-cell and 50% of the localizations are at the $\frac{1}{4}$ positions of the cell. P_{SPLIT} was determined for all eight datasets, where the weighted average (corrected for the difference in the number of cells per dataset) turned out to be $1.97 \mu\text{m} \pm 0.13 \mu\text{m}$. To be able to use P_{SPLIT} as a marker in mutant strains, where the cell size is often very different from wild type, P_{SPLIT} was normalized to a percentage of the mean cell length. For this, each splitting point of each dataset was divided by the average cell size of the dataset and multiplied by 100%. The final normalized splitting point $P_{\text{SPLIT-N}}$ was $124\% \pm 9.04\%$ of the average cell length of a dataset. This range in cell length was shaded in each graph where mutants and wild type cells are compared as a fiduciary marker.

Determination and statistical analysis of the FtsZ/septum angle

To determine the angle of either FtsZ or the septum labelled with fluorescent vancomycin, Oufi's Object Detection tool was used to outline the fluorescent bodies. The angle of the longest side of the minimal bounding box of the cell outline and the objects were determined. The difference between these two angles was determined as the angle of the fluorescent object (α_{object}). Since the direction of the angle has no meaning in this measurement (an angle of 85° is the same as an angle of 95° in a cell upside-down), the final object angles were defined as:

$$\alpha_{\text{FTSZ/SEPTUM}} = |\alpha_{\text{OBJECT}} - 90^\circ| \quad \alpha_{\text{FTSZ/SEPTUM}} = |\alpha_{\text{OBJECT}} - 90^\circ|.$$

Here, if $\alpha_{\text{FTSZ/SEPTUM}} = 0$, the angle of the fluorescent object is perpendicular to the length axis of the cell. To determine whether in the resulting non-normal distributions $\alpha_{\text{FTSZ/SEPTUM}}$ was significantly higher than zero, a one-sided sign test was performed where $H_0: \alpha = 0$ and $H_1: \alpha > 0$ and $CI=95\%$. The sample distribution shapes and medians within experiments were compared to each other using a two-sided Kolmogorov-Smirnov test.

Analysis of time-lapse movies

For the time-lapse data, Oufi was used to obtain movies of single cells and SpotprocessR was used to obtain a plot of the corresponding cells, showing the localization of the spots and cell poles over time. Using SpotprocessR, the localizations inside all individual cells were plotted as the distance from midcell for each time point in the time-lapse movie.

Colocalization analysis

For colocalization, the distances between spots in each cell were measured using SpotprocessR. Three categories were defined to determine whether two spots colocalized. (1) Colocalization was defined as an overlap of the full width half max (fwhm) of the Gaussian fit with the distance between the centres of the Gaussian fits. When the distance between the centres was smaller than their fwhm, they were defined as being at the same location. (2) Possible colocalization was defined as spots where the Gaussians fwhm's touch but do not overlap with their centres distances, making them close but not certainly colocalizing. (3) Non-colocalization was defined

as the spots where the Gaussians fwhm's do not overlap at all.

Movement analysis

The movement of the single spots in the short-interval time-lapse movies was tracked using u-track (Jaqaman *et al.*, 2008). The u-track output was combined with the cell outlines defined by Oufi using SpotprocessR, which plotted the tracks inside the individual cells and calculated and plotted the displacement of individual spots. The collective MSD was calculated using the formula:

$$MSD_{bulk} = \frac{1}{n} \sum_{i=1}^n \frac{1}{m} \sum_{j=1}^m (x_i(\tau_j) - x_i(\tau_j - t))^2$$

Where i is the n^{th} track, j is the m^{th} time point and $x(\tau)$ is the position of the tracked point at timepoint τ_j or by determining the variances of the histograms of all displacements at different time intervals. The apparent diffusion coefficient D_{app} was estimated by fitting a Gaussian curve to a histogram of all displacements as previously by Parry *et al.* (Parry *et al.*, 2014).

4.

Code availability

The exact code used for image analysis are available on www.github.com/vrrenske/spotprocessR. SpotProcessR was later converted to a full R package (BactMAP, see chapter 3).

Marker frequency analysis by whole genome sequencing

Marker frequency analysis by whole genome sequencing was performed essentially as described before (Kjos & Veening, 2014; Slager *et al.*, 2014). All strains used for marker frequency analysis (MK453, MK454, DCI15, MK350, MK422 and MK423) were grown under the same conditions as used when growing cells for microscopy analysis; cells of $OD_{600} = 0.4$ were diluted 100-fold in C+Y medium with 0.1 mM $ZnCl_2$ and incubated for 2.5 hours until $OD_{600} = 0.15$. Cells were then harvested by centrifugation for 5 min at 6500 x g at 4°C. Genomic DNA was isolated using the Wizard® Genomic DNA Purification Kit (Promega) as described previously (Kjos & Veening, 2014). Fragmentation was performed using Covaris S2 instrument, and libraries were prepared using NEBNext Ultra DNA Library Kit for Illumina prior to sequencing on an Illumina HiSeq 2000 Platform 50 bp single-end (all performed at the Genomics Core Facility, EMBL, Heidelberg, Germany). Using Rockhopper (McClure *et al.*, 2013), raw reads were mapped onto the genome of *S. pneumoniae* D39. The genome was split in segments of 1000 bp, and the number of reads mapped onto each 1000 bp segment was plotted as a function of the position on the chromosome.

Data availability

The raw sequencing data can be accessed at:
<https://seek.sysmo-db.org/investigations/93#datafiles>.

Author contributions

R.R., M.K. and J.W.V. designed the experiments. R.R. developed the SpotprocessR tool. M.K. developed tools for chromosome labeling. R.R. and M.K. performed strain construction and microscopy experiments. R.R., M.K. and J.W.V. analyzed the data and wrote the manuscript.

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4.

Supplemental Figures

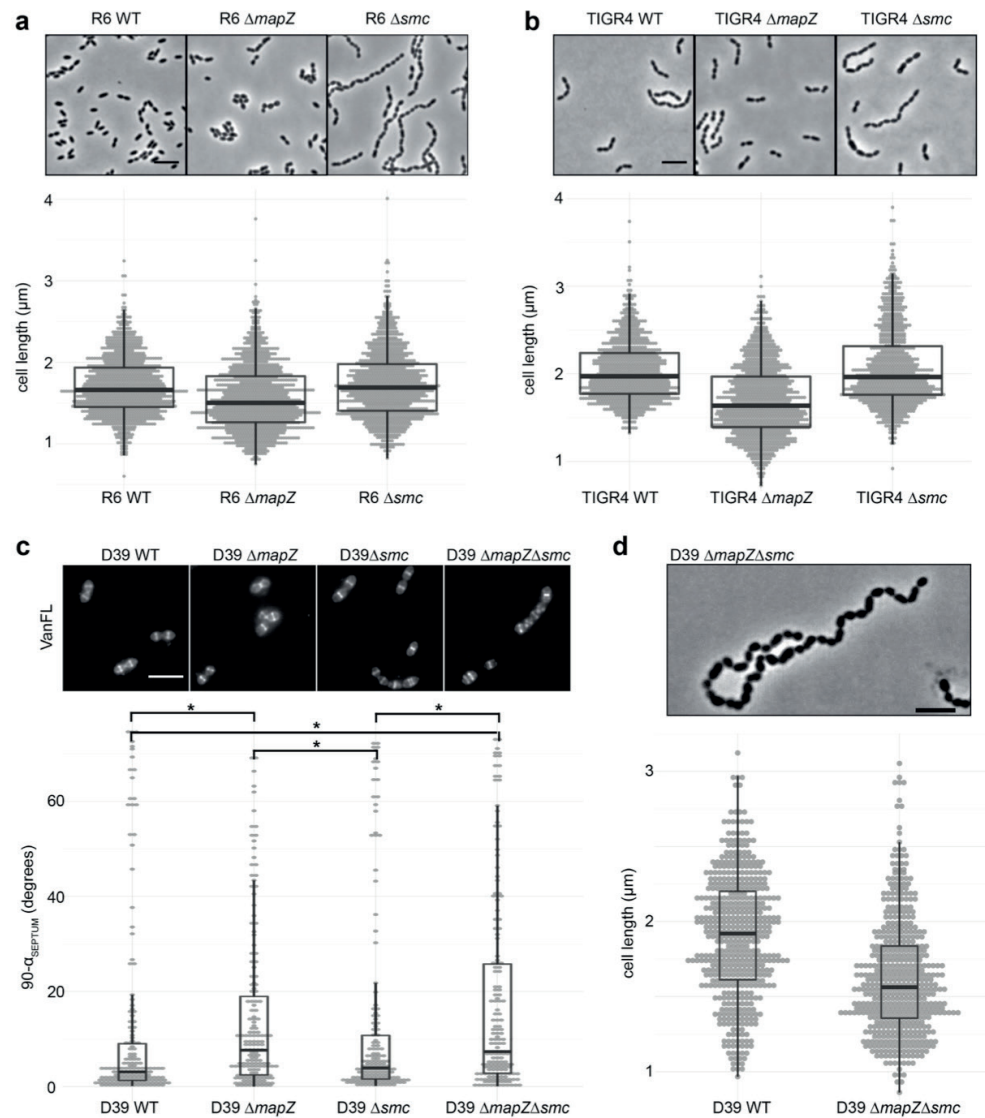


Figure S1. Deletion of *mapZ* and *smc*. **A.** Phase contrast images and box plots showing differences in cell size and morphology of *S. pneumoniae* R6 wild type (918 cells analyzed) compared to strains in which *mapZ* (strain MK448, 1319 cells analyzed) and *smc* (strain MK446, 1277 cells analyzed) were deleted. Scale bar = 5 μ m. **B.** Phase contrast images and box plots showing differences in cell size and morphology of *S. pneumoniae* TIGR4 wild type cells (1147 cells analyzed) compared to a strain in which *mapZ* (strain MK447, 1214 cells analyzed) and *smc* (strain MK445, 830 cells analyzed) were deleted. Scale bar: 5 μ m. **C.** Fluorescence microscopy images (GFP ex/em settings, 0.2 s 10% transmission) of VanFL stained wild type D39, $\Delta mapZ$ (strain RR93), Δsmc (strain AM39) and $\Delta smc \Delta mapZ$ (strain RR96) cells (top, scale bar = 4 μ m) and measured deviation of α_{SEPTUM} from 90° in the corresponding cells. The data represents 190 cells per strain. Asterisks represents statistical significant difference. **D.** Phase contrast image (scale bar = 4 μ m) of $\Delta smc \Delta mapZ$ (strain RR96, top) and cell length measurements of wild type (444 cells) vs. $\Delta smc \Delta mapZ$ cells (509 cells).

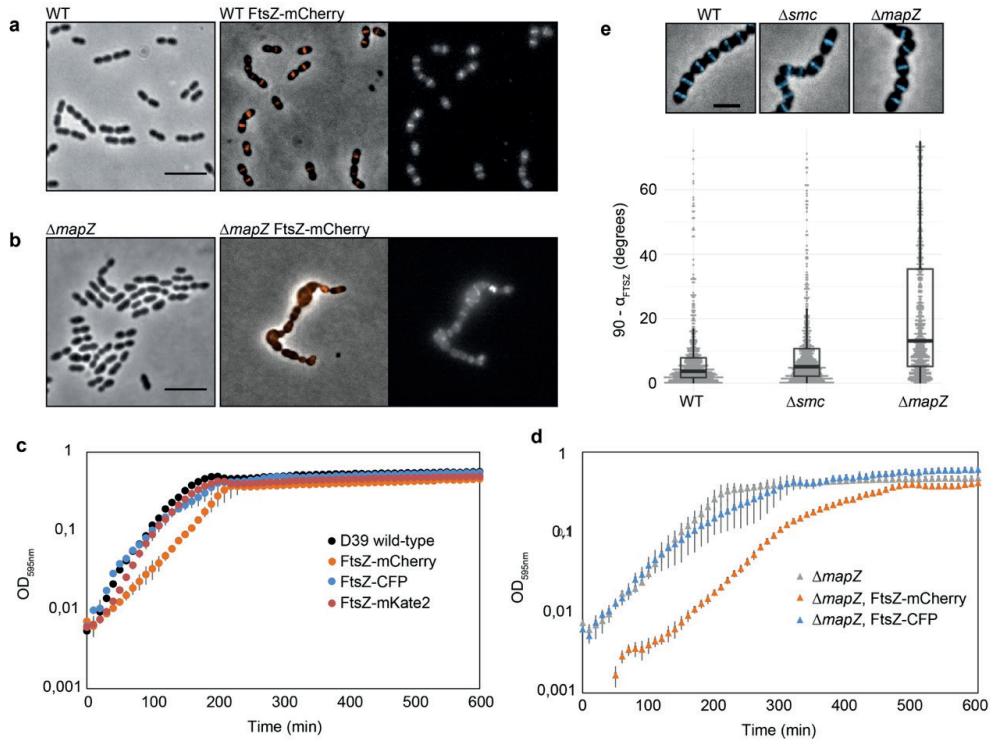


Figure S2. FtsZ-mCherry causes misformed cells in a $\Delta mapZ$ background. **A.** Left: phase contrast image of wild type D39 cells. Middle and right: overlay of fluorescence microscopy image and phase contrast image of FtsZ-mCherry in wild type cells (strain RR27), and separate fluorescence microscopy image of FtsZ-mCherry. FtsZ-mCherry does not affect the morphology of the cells in a wild type background. Scale bar = 5 μ m. **B.** Left: phase contrast image of $\Delta mapZ$ cells (strain RR93). Middle and right: overlay of fluorescence microscopy image and phase contrast image of FtsZ-mCherry in $\Delta mapZ$ cells (strain RR98) and separate fluorescence microscopy image of FtsZ-mCherry. FtsZ-mCherry expressed in $\Delta mapZ$ background gives rise to misformed cells. Scale bar = 5 μ m. **C.** Growth curves of D39 wild-type cells and strains where FtsZ has been replaced with FtsZ-mCherry fusion (strain RR27), FtsZ-CFP fusion (strain RR23) or FtsZ-mKate2 fusion (strain RR66). Grey error bars represent standard deviations from three replicates. **D.** Growth curves of D39 $\Delta mapZ$ with FtsZ wild type and $\Delta mapZ$ combined with FtsZ-mCherry fusion (strain RR98) and FtsZ-CFP fusion (RR105). Grey error bars represent standard deviations from three replicates. **E.** The angle of FtsZ is skewed in $\Delta mapZ$ cells. Top: morphology and FtsZ localization in WT (strain RR80) cells, Δsmc (RR84) cells and $\Delta mapZ$ (RR105) cells. Scale bar is 2 μ m. Bottom: the angle of FtsZ towards the long axis of the cell (α_{FtsZ}) was measured in the same manner as the angles of the septum measured in Figure 1.d. α_{FtsZ} deviates the most from 90 degrees in $\Delta mapZ$ cells (median = 13.2 degrees, se = 0.81) than in WT (median = 3.73 degrees, se = 0.41) and Δsmc (median = 5.12 degrees, se = 0.47) cells. Statistical test indicates statistical difference with $p < 0.003$ between all 3 samples. (Kolmogorov-Smirnov test).

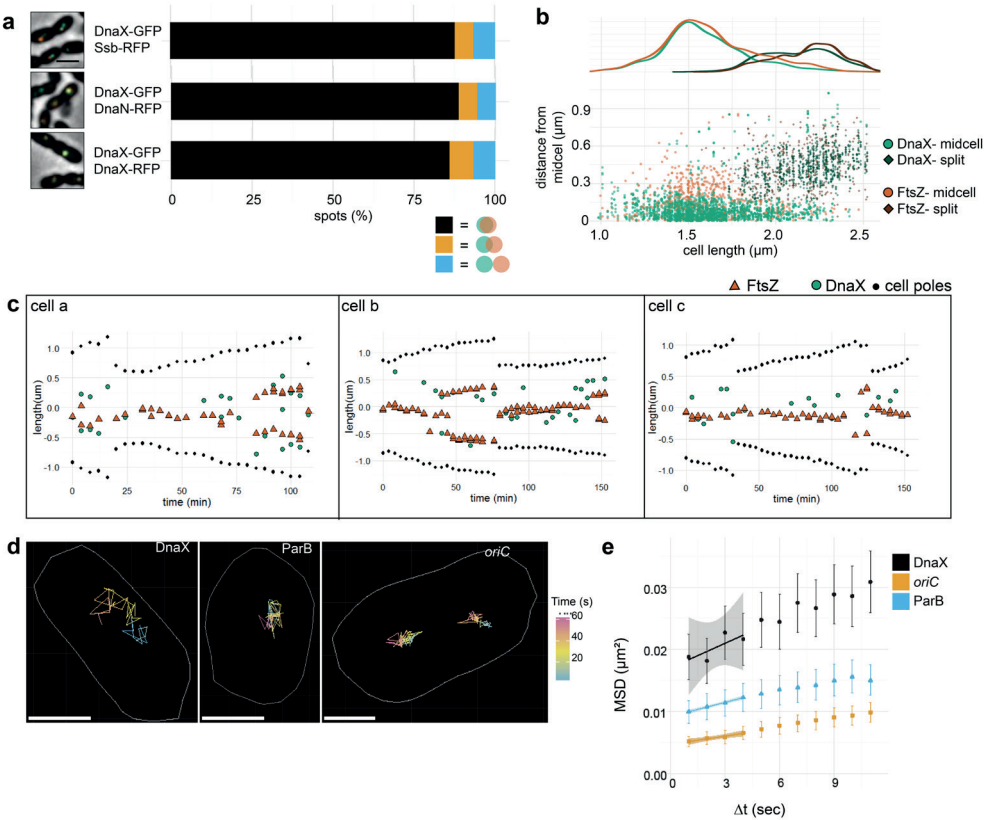


Figure S3. The replisome is dynamic around mid-cell. A. Colocalization of own-locus expressed DnaX-GFP with SSB-RFP (strain RR89), DnaN-RFP (strain RR90) and DnaX-RFP (strain RR91), respectively. For more than 88% of the spots, the full width half max of the GFP and RFP were larger than the distance between them, indicating an almost complete overlap and thus colocalization. **B.** FtsZ and DnaX split with similar timing. FtsZ and DnaX distances from midcell plotted by cell length shows that they are either localized at midcell (circles, light shaded) or around the quarter positions (diamonds, dark shaded). Both FtsZ and DnaX is mostly at midcell in small cells, while mostly at the $\frac{1}{4}$ positions in bigger cells. The moment that 50% of the localizations is either midcell or at the $\frac{1}{4}$ positions is 1.856 μm for DnaX and 1.998 μm for FtsZ, respectively. Data analysis done on images of MK396, 484 cells. **C.** Transcripts of three representative cells from the same time lapse experiment as shown in Figure 2d. The distance of FtsZ (red), DnaX (green) and the cell poles (black) to midcell is plotted against time. **D.** Tracks showing the movement of DnaX-GFP (strain RR22), ParB_c-GFP (strain MT2) and *oriC* (strain MK259), respectively, inside the ROI of the corresponding cell. The color indicates the time (s), scale bar = 0.5 μm. **E.** Plots of the collective MSD of all measured tracks of DnaX (RR22), ParB (MT2) and *oriC* (MK259) shows that DnaX is more mobile than ParB and *oriC*. The data represents 5 movies and 399 tracks (DnaX), 4 movies and 195 tracks (ParB) and 4 movies and 82 tracks (*oriC*).

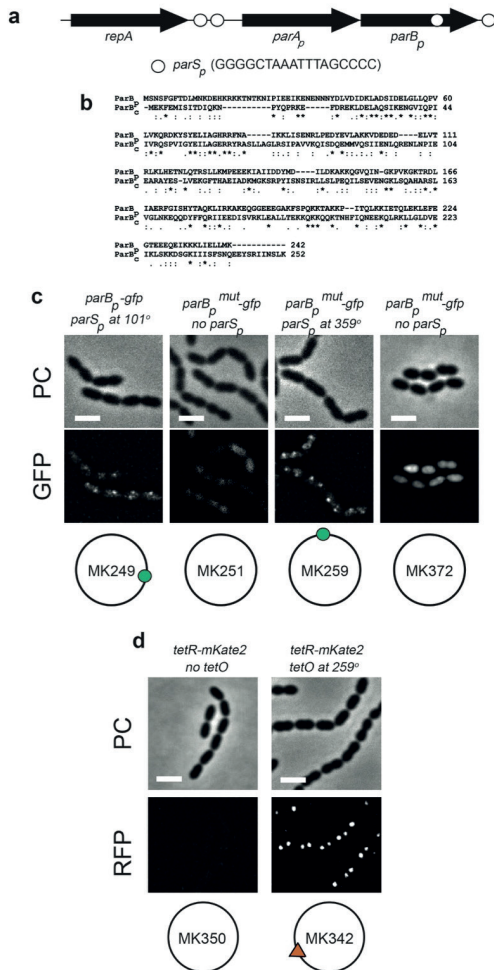


Figure S4. Construction of novel chromosome labeling systems for *S. pneumoniae*.

A. The 55-kb plasmid pLP712 (NC_019377) from *L. lactis* (Wegmann *et al.*, 2012) encodes a putative novel *parAB* system (here named *parAB*_p) located close to the plasmid's origin of replication. Close homologs to ParB_p were only found in other lactococcal plasmids, such as pKF147A, pGdh442, pVF50, pCIS7 and pQA554 (data not shown). In *E. coli*, plasmid-based ParB-*parS* systems have been used to visualize chromosomal loci by fusing plasmid-originating ParB proteins to fluorescent proteins and integrating *parS* sites at different loci in the chromosome (Nielsen, Ottesen, Youngren, Austin & Hansen, 2006; Stouf, Meile & Cornet, 2013). The *parABS* system of the lactococcal plasmids has so far not been studied, and in order to identify a putative *parS* site of ParB_p, we searched the plasmid pLP712 for palindromic sequences, a feature characteristic for *parS* sites (Livny, Yamaichi & Waldor, 2007). We identified an 18-bp palindromic sequence (GGGGCTAAATTTAGCCCC) which was found at four locations in the replication origin region of the plasmid pLP712 (circles), but not in any other location. One of the palindromes was located within the *parB*_p coding region. The same 18-bp sequence was also found at multiple locations of the origin region of the other *parB*_p harboring plasmids, but not in the chromosome of *S. pneumoniae*. **B.** The ParB_p (YP_006965824) is highly dissimilar to ParB_c of *S. pneumoniae*, although both proteins contain a domain (Pfam02195) characteristic for ParB proteins. **C.** We showed that ParB_p-*parS*_p could be used for specific chromosome labeling in *S. pneumoniae*. First, a C-terminal fusion of ParB_p to a monomeric superfolder GFP was made and integrated in the genome of *S. pneumoniae* D39 under control of a Zn²⁺ inducible promoter. Distinct fluorescent foci were formed in this strain (MK249). To find out whether the foci were caused by binding of ParB_p to the putative *parS* site within the ParB_p encoding sequence, the *parS* site was mutagenized while keeping the open reading frame intact (*parB*_p^{mut}, MK251). In the resulting strain without the 18-bp *parS*_p palindrome, no foci were observed

(MK251). Next, the 18-bp palindrome was inserted together with a kanamycin resistance cassette close to *oriC* in the strain expressing *parB*_p^{mut}, and foci appeared (MK259). As a control, we also checked that no foci appeared when only the kanamycin resistance cassette was inserted (MK372). Thus, the 18 bp *parS*_p serves as binding site for ParB_p (and *parB*_p^{mut}) and the *parB*_p/*parS*_p complex is visible as a distinct diffraction limited spot by fluorescence microscopy. **D.** As a second, non-related, chromosome labeling system, we adapted the TetR/*tetO* fluorescence repressor-operator system (FROS) which previously has been used in other bacteria, including *E. coli*, *B. subtilis* and *Caulobacter crescentus* (Lau *et al.*, 2003; Viollier *et al.*, 2004; Xindan Wang, Possoz & Sherratt, 2005). Our initial attempts to directly transfer the TetR-mCherry/*tetO* system used in *B. subtilis* (Veening, Murray & Errington, 2009) to *S. pneumoniae* were not successful (data not shown). In order to optimize the system, we used a *tetR* gene which was codon optimized for *S. pneumoniae* and fused this to *mKate2*. In addition, we reduced the size of the *tetO*-array from 240 repeats to 48 repeats. Such short *tetO* arrays have also been shown to be sufficient for imaging in *B. subtilis* (X. Wang, Montero Llopis & Rudner, 2014) and are probably less prone to recombinational instability. The *tetO*48-array was integrated in the *cep*-locus (259°) and upon induction of *tetR-mKate2* expression, bright foci were formed (strain MK342).

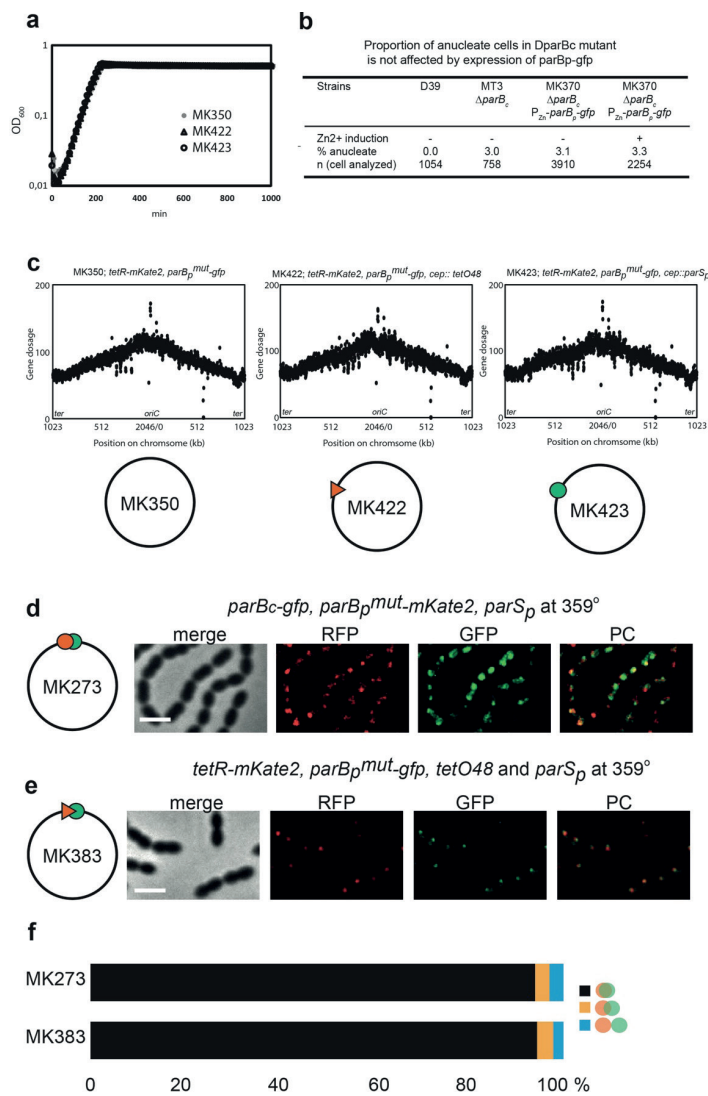


Figure S5. Controls for novel chromosome marker systems. A. Binding of ParB_p-GFP or TetR-mKate2 do not affect growth of *S. pneumoniae*. Growth curves of strains expressing *parB_p-gfp* and *tetR-mKate2* without binding sites (strain MK350), with *parS_p* at 295° (strain MK423) or *tetO* at 295° (strain MK422). **B.** The *parB_p-parS_p* system does not promote chromosome segregation in *S. pneumoniae*, and cannot complement the chromosomal *parB_c-parS_c* system. By using a $\Delta parB_c$ deletion strain (chromosomal *parB* deleted), containing Zn²⁺-inducible *parB_c-gfp* and an origin-proximal *parS_p* (strain MK370), we showed that the proportion of anucleate cells in strain MT3 ($\Delta parB_c$, 3.0% anucleate cells, in line with previous reports (Minnen *et al.*, 2011) such as the oval-shaped human pathogen *Streptococcus pneumoniae*, efficiently segregate their chromosomes is poorly understood. Here we show that the pneumococcal homologue of the DNA-binding protein ParB recruits *S. pneumoniae* condensin (SMC) was not reduced in strain MK370, neither without nor with induction of *parB_c-gfp* expression (3.1 % and 3.3 % anucleate cells, respectively). **C.** Whole genome marker frequency analysis shows that DNA replication is not affected when ParBp-GFP or TetR-RFP is bound to the

chromosome. Sequencing data is available at <https://seek.sysmo-db.org/investigations/93#datafiles>. **D.** To verify the localization pattern of the novel ParB_p-*parS_p* system, we constructed a double labeled strain (strain MK273) in which the chromosomal ParB_c was fused to GFP (Kjos & Veening, 2014; Minnen *et al.*, 2011) such as the oval-shaped human pathogen *Streptococcus pneumoniae*, efficiently segregate their chromosomes is poorly understood. Here we show that the pneumococcal homologue of the DNA-binding protein ParB recruits *S. pneumoniae* condensin (SMC) and the introduced ParB_p was fused to the red fluorescent protein *mKate2*. In previous studies we have shown that ParB_c of *S. pneumoniae* binds to *parS_c* sites close to the origin of replication, and that a ParB_c-GFP fusion can be used as marker for localization of *oriC* (Kjos & Veening, 2014; Minnen *et al.*, 2011) such as the oval-shaped human pathogen *Streptococcus pneumoniae*, efficiently segregate their chromosomes is poorly understood. Here we show that the pneumococcal homologue of the DNA-binding protein ParB recruits *S. pneumoniae* condensin (SMC). **E.** To further verify the localization patterns of the TetR/tetO system, we constructed a strain containing both *parS_p* and *tetO* in the *ori*-region (strain MK383, *tetO* and *parS_p* both at -1°, separated by 1 kb), in which *parB_p-gfp* and *tetR-rfp* are expressed in parallel. **F.** In both the double labeled strains MK273 and MK383, the two markers co-localized, verifying that the ParB_p-*parS_p* system and the TetR/tetO systems are reliable markers for analyzing chromosome localization in *S. pneumoniae* and potentially other organisms.

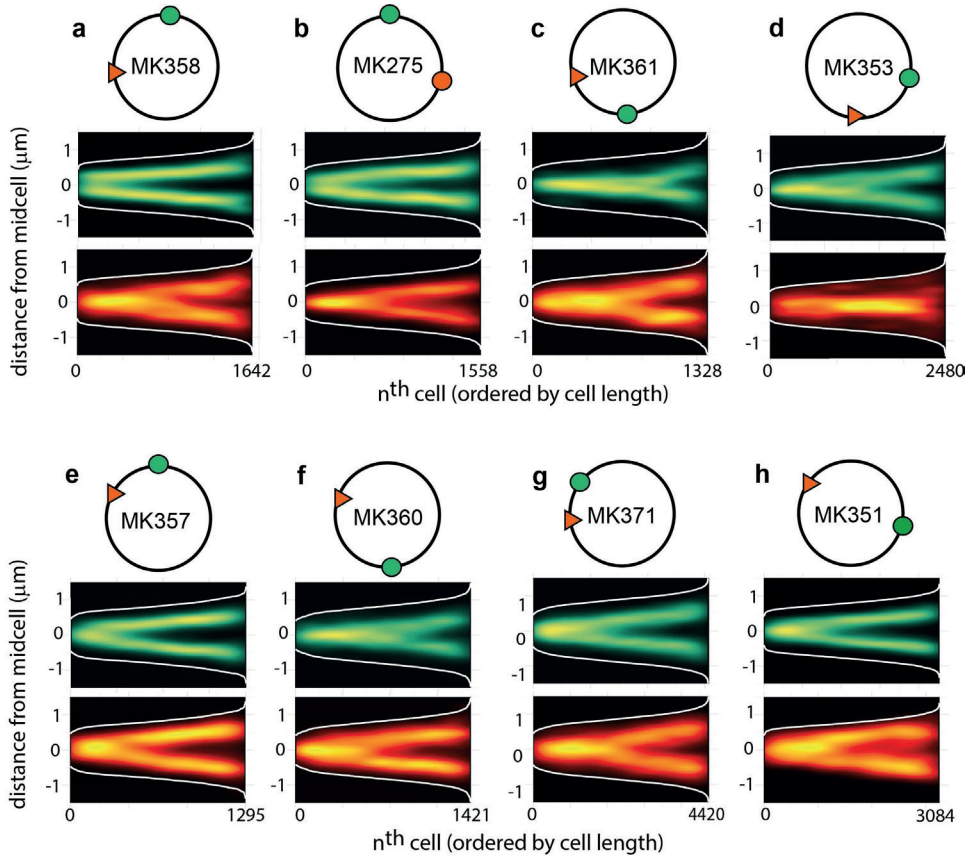


Figure S6. Organization of the pneumococcal chromosome. Analysis of chromosome organization in strains with two labeled chromosomal loci. Pneumococci were exponentially growing at 37°C (balanced growth) in C+Y medium and directly imaged by fluorescence microscopy (see Methods). Localizations are shown as heat maps with cell sorted according to cell length. **A.** Strain MK358, origin and 259°. The data represents a total of 1642 cells/2868 GFP-localizations/1928 RFP-localizations. **B.** Strain MK275, origin and 101°. The data represents a total of 1558 cells/2198 GFP-localizations/1789 RFP-localizations. **C.** Strain MK361, terminus and 259°. The data represents a total of 1328 cells/1486 GFP-localizations/1603 RFP-localizations. **D.** Strain MK353, terminus and 101°. The data represents a total of 2480 cells/3222 GFP-localizations/2626 RFP-localizations. **E.** Strain MK357, origin and 295°. The data represents a total of 1295 cells/1900 GFP-localizations/1498 RFP-localizations. **F.** Strain MK360, terminus and 295°. The data represents a total of 1421 cells/1840 GFP-localizations/1858 RFP-localizations. **G.** Strain MK371, 259° and 295°. The data represents a total of 4220 cells/6137 GFP-localizations/4737 RFP-localizations. **H.** Strain MK351, 101° and 295°. The data represents a total of 3082 cells/4536 GFP-localizations/3247 RFP-localizations.

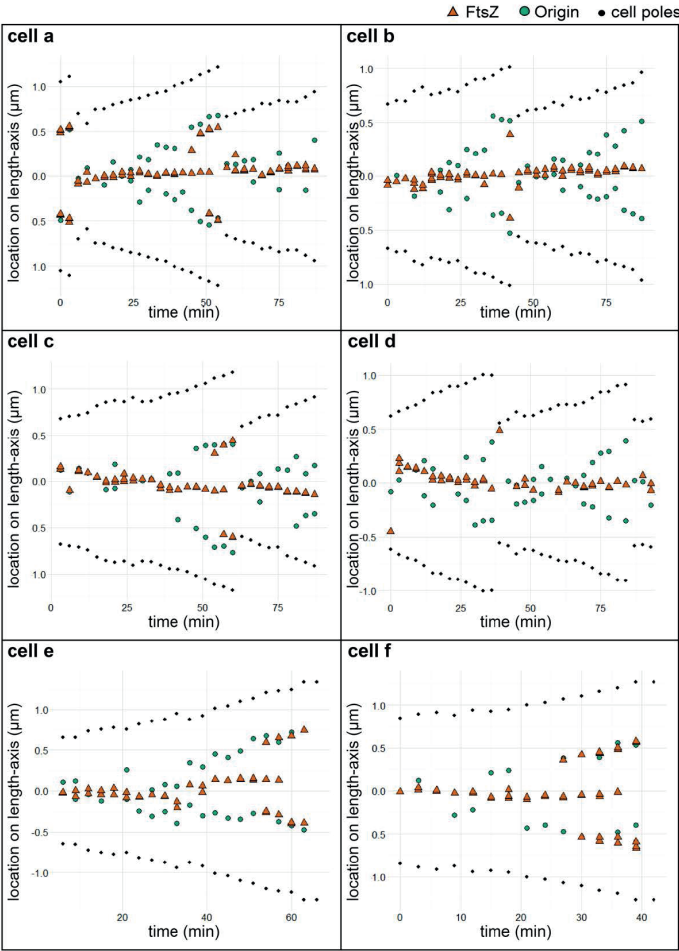


Figure S7. The origin moves to mid-cell before FtsZ assembles at the future division site. Six transcripts of cells (strain MK392; $\text{ParB}_p\text{-GFP}/\text{parS}_p$ at 359° and FtsZ-RFP) from the same time lapse experiment as shown in Fig 3f. For comparison, the cell from Fig 3f is shown in panel a. Distances of FtsZ, the origin and the cell poles relative to midcell in a single cell are shown.

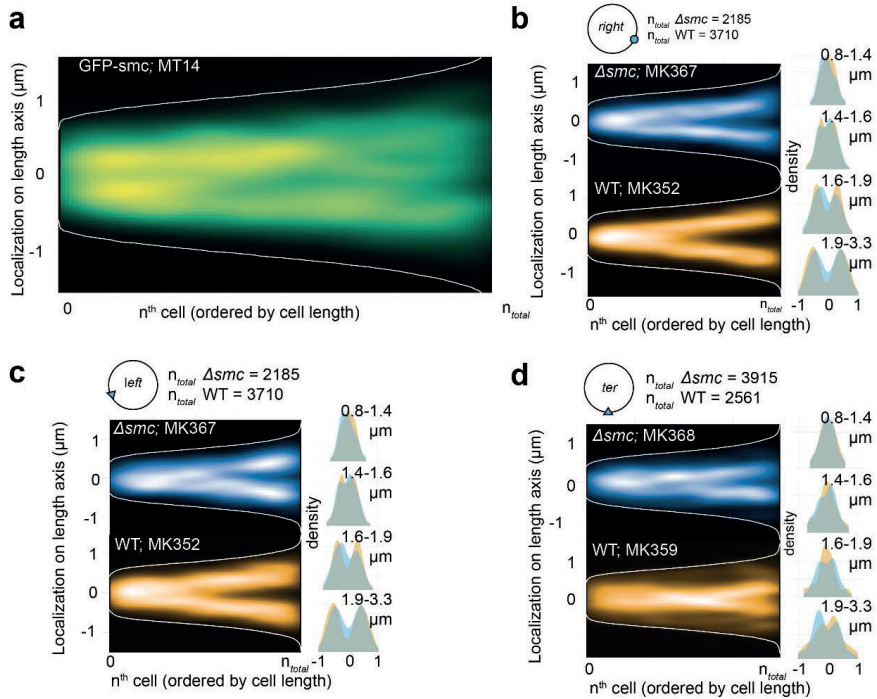


Figure S8. Chromosome organization in the absence of SMC. SMC localization in wild type **A.** and organization of the right arm of the chromosome **B.**, left arm **C.** and the terminus **D.** in Δsmc compared to wild type. **A.** GFP-SMC localization in wild type cells (strain MT14). The data represents 1006 cells/1942 GFP localizations. **B.** and **C.** Δsmc strain MK367 compared to parental strain MK352. The data represents 2185 cells/2821 GFP-localizations/2236 RFP-localizations for MK367 and 3710 cells/5288 GFP-localizations/4372 RFP-localizations for MK352. **D.** Δsmc strain MK368 compared to original strain MK359. The data represents 3915 cells/3963 RFP-localizations for MK368 and 2561 cells/2793 RFP-localizations for MK359.

4.

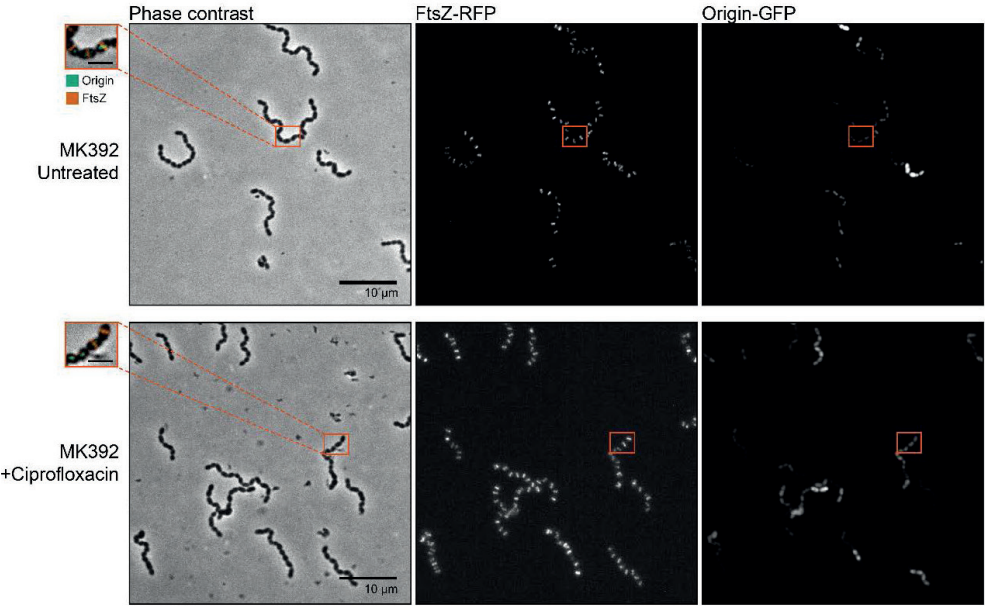


Figure S9. Ciprofloxacin-treated cells. Zoomed-out version of Figure 5a showing strain MK392 ($ParB_p$ -GFP/ $parS_p$ at 359° , FtsZ-RFP) with and without treatment with sub-inhibitory concentrations of ciprofloxacin. From left to right; original zoomed-in Figure 5A. (scale bar = $2\mu m$), phase contrast (scale bar = $10\mu m$), RFP and GFP microscopy image. Untreated cells are shown in the top row and cells treated with $0.4\mu g/mL$ ciprofloxacin for 60 minutes are shown at the bottom.

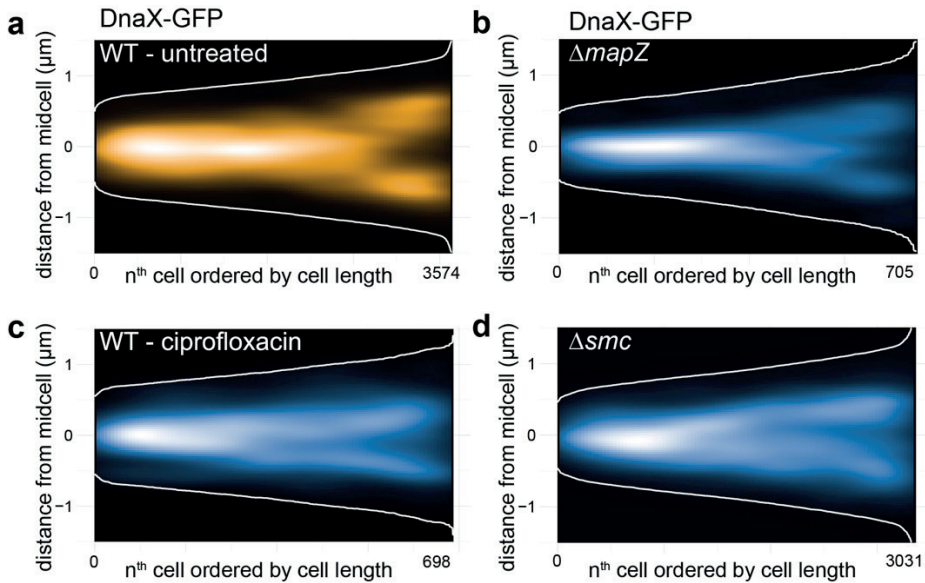


Figure S10. DnaX is mislocalized in Δsmc cells and in cells treated with ciprofloxacin, but not in $\Delta mapZ$ cells. **A.** DnaX localization as judged by imaging localizations of DnaX-GFP in wild type cells (strain RR22, 3574 cells, 4145 localizations). **B.** DnaX localization in $\Delta mapZ$ cells (strain RR95, 705 cells, 841 localizations). **C.** DnaX localization in wild type cells treated with 0.4 $\mu\text{g}/\text{mL}$ ciprofloxacin for 60 minutes (strain RR22, 668 cells, 1181 localizations). **D.** DnaX localization in Δsmc cells (strain RR86, 3031 cells, 3361 localizations).

Supplementary Materials and Methods

Strain construction

Construction of plasmids pMK14, pMK15, pMK17 (for zinc-inducible expression of C-terminal protein fusions to superfolder GFP, mKate2, monomeric superfolder GFP).

(*sf*)*gfp* (superfolder *gfp*) was amplified from pKB01-sfgfp(Bs)(Overkamp *et al.*, 2013) with primers pr21 and pr22, *mKate2* (codon optimized for *S. pneumoniae*) was amplified from pKB01-mKate(Sp)(Beilharz, van Raaphorst, Kjos & Veening, 2015) using primers pr23 and pr24, monomeric superfolder *gfp*, *m(sf)**gfp* with the monomer mutation V206K was amplified from plasmid pNZ-MS2-GFPdsm (van Gijtenbeek, Robinson, van Oijen, Poolman & Kok, 2016) using primers pr21 and pr22. The forward primers (pr21 and pr23) all contain an overhang with a multiple cloning site and a domain breaking linker (Arai, Ueda, Kitayama, Kamiya & Nagamune, 2001), -RGSGGEAAAKGSS-. The resulting PCR fragments were cut with restriction enzymes EcoRI and NheI and ligated into the EcoRI and SpeI sites of plasmids pMK11 downstream of a Zn²⁺-inducible promoter (Kjos *et al.*, 2016). The ligations were transformed into *E. coli* DH5 α . The resulting plasmids are suitable for construction of C-terminal FP fusions (pMK14 for *sf**gfp*, pMK15 for *mKate2* and pMK17 for *m(sf)**gfp*). The plasmids contain an ampicillin resistance gene for selection in *E. coli*. Flanking the construct region, the plasmids contain a tetracycline resistance gene as well as sequences homologous to the *bgaA* locus of *S. pneumoniae*. Upon transformation of the resulting plasmids into *S. pneumoniae*, the construct of interest will therefore integrate into the *bgaA* locus by double crossover, and transformants can be selected on Columbia agar plates containing tetracycline.

Construction of $\Delta bgaA::P_{Zn}-parB - m(sf)gfp$

The *parB_p* gene was cut out of plasmid pSEUDO39-mKate2 (J. Siebring, unpublished) using restriction enzymes BglII and XbaI and first subcloned into the BamHI and SpeI sites of plasmid pMK14 to generate plasmid pMK14-02 ($P_{Zn}-parB_p-(sf)gfp$). *parB_p* was then cut out from this plasmid using enzyme XbaI ligated into XbaI-digested pMK17 to generate plasmid pMK17-01 where the *parB_p-m(sf)gfp* fusion is encoded downstream of P_{Zn} .

Mutagenesis of *parB* to *parB^{mut}* and construction of $\Delta bgaA::parB^{mut}-gfp$.

The *parS_p* site inside *parB_p* was mutagenized using 'round PCR' with primers parBj-mut-F and parBj-mut-R on plasmid pMK14-02, generating plasmid pMK14-13. These primers anneal to the *parS_p* sequence and introduces mutations replacing 8 out of 18 bp in *parS_p* (GGGGCTAAATTTAGCCCC was changed to GGGAGCAAAAGTTCTCTCCAC, mutated positions underlined). By introducing only synonymous codons, the *ParB_p* sequence remained unchanged. For construction of pMK17-02 ($P_{Zn}-parB_p^{mut}-m(sf)gfp$), the *parB_p^{mut}*-gene was cut out from pMK14-03 using XbaI and ligated into the XbaI-digested pMK17.

Construction of $\Delta bgaA::P_{Zn}-parB^{mut}-mKate2$.

The *parB-mKate2* fusion (the two fused proteins are separated by a flexible linker -RGSGGEAAAKGSS- (Arai *et al.*, 2001)) was cut out from plasmid pSEUDO39-mKate2 (J. Siebring, unpublished) using BglII and SpeI and ligated into pKB01-sfgfp(Sp)(Overkamp *et al.*, 2013) digested with BamHI and SpeI to generate plasmid pMK103. Using this intermediate plasmid as a template, *parB-mKate2* was amplified with primers sPG88 and pPP2-R, digested with EcoRI and SpeI and ligated into the corresponding sites of pMK11 to create plasmid pMK11-01 where *parB_p-mKate2* is located downstream of a Zn²⁺-inducible promoter. The *parS_p* site inside *parB_p* was then mutagenized by round-PCR to *parB_p^{mut}* as described above, to create plasmid pMK11-02 ($P_{Zn}-parB_p^{mut}-mKate2$).

Table S1. Strains and plasmids used in this study

Strain	Description ^{a, b, c}	Reference
<i>S. pneumoniae</i>		
D39V	Serotype 2 strain	Avery, MacLeod & McCarty, 1944
R6	D39 derivative, unencapsulated	Hoskins <i>et al.</i> , 2001
TIGR4	Serotype 4 strain	Tettelin <i>et al.</i> , 2001
DCI15	D39, <i>DbgaA::P_{cas9}-cas9, tet^R; dnaA-dnaN-luc, ery^R; Dcep::P3-sgRNA_{luc1}; spc^R</i>	van Kessel and Veening, unpublished
AM39	D39, <i>Dsmc::trmp^R</i>	Minnen <i>et al.</i> , 2011
MT2	D39, <i>parB-gfp, spc^R</i>	Minnen <i>et al.</i> , 2011
DLA14	D39, <i>comCDE, kan^R</i>	Slager <i>et al.</i> , 2014
DJS2	D39, <i>bgaA::gfp-dnaN, tet^R</i>	Slager <i>et al.</i> , 2014
MK113	D39, <i>iga-ery^R</i>	Kjos & Veening, 2014
MK119	D39, <i>hlpA_hlpA-mKate2, cam^R</i>	Kjos & Veening, 2014
MK249	D39, <i>DbgaA::P_{zn}-parB_p-gfp, tet^R</i>	This study
MK251	D39, <i>DbgaA::P_{zn}-parB_p^{mut}-gfp, tet^R</i>	This study
MK259	D39, <i>DbgaA::P_{zn}-parB_p^{mut}-gfp, tet^R; comCDE, kan^R, parS_p</i>	This study
MK273	D39, <i>parB-gfp_spc^R, DbgaA::P_{zn}-parB_p^{mut}-mKate2, tet^R; comCDE, kan^R, parS_p</i>	This study
MK275	D39, <i>DbgaA::P_{zn}-parB_p-mKate2; tet^R; parB_c-(sf)gfp_spc^R</i>	This study
MK342	D39, <i>relA-tetO48, spc^R; DbgaA::P_{zn}-tetR-mKate2, tet^R</i>	This study
MK350	D39, <i>DbgaA::P_{zn}-tetR-mKate2_parB_p^{mut}-gfp, tet^R</i>	This study
MK351	D39, <i>DbgaA::P_{zn}-tetR-mKate2_parB_p-gfp, tet^R; Dcep::tetO48, spc^R</i>	This study
MK352	D39, <i>DbgaA::P_{zn}-tetR-mKate2_parB_p-gfp, tet^R; relA-tetO48, spc^R</i>	This study
MK353	D39, <i>DbgaA::P_{zn}-tetR-mKate2_parB_p-gfp, tet^R; pulA-tetO48, spc^R</i>	This study
MK357	D39, <i>DbgaA::P_{zn}-tetR-mKate2_parB_p^{mut}-gfp; tet^R; comCDE-parS_p-kan^R; Dcep::tetO48, spc^R</i>	This study
MK358	D39, <i>DbgaA::P_{zn}-tetR-mKate2_parB_p^{mut}-gfp; tet^R; comCDE-parS_p-kan^R; relA, tetO48, spc^R</i>	This study
MK359	D39, <i>DbgaA::P_{zn}-tetR-mKate2_parB_p^{mut}-gfp; tet^R; comCDE, parS_p, kan^R; pulA, tetO48, spc^R</i>	This study
MK360	D39, <i>DbgaA::P_{zn}-tetR-mKate2_parB_p^{mut}-gfp, tet^R; cep::tetO48, spc^R; iga, parS_p, ery^R</i>	This study
MK361	D39, <i>DbgaA::P_{zn}-tetR-mKate2_parB_p^{mut}-gfp, tet^R; relA-tetO48, spc^R; iga, parS_p, ery^R</i>	This study
MK367	D39, <i>DbgaA::P_{zn}-tetR-mKate2_parB_p-gfp, tet^R; relA, tetO48, spc^R; Dsmc::trmp^R</i>	This study
MK368	D39, <i>DbgaA::P_{zn}-tetR-mKate2_parB_p^{mut}-gfp; tet^R; comCDE, parS_p, kan^R; pulA, tetO48, spc^R; Dsmc::trmp^R</i>	This study
MK370	D39, <i>DbgaA::P_{zn}-tetR-mKate2_parB_p^{mut}-gfp; tet^R; comCDE, parS_p, kan^R; pulA, tetO48, spc^R; DparB::cm^R</i>	This study
MK371	D39, <i>DbgaA::P_{zn}-tetR-mKate2_parB_p^{mut}-gfp, tet^R; relA-tetO48; spc^R; cep::parS_p, ery^R</i>	This study
MK372	D39, <i>DbgaA::P_{zn}-parB_p^{mut}-gfp, tet^R; comCDE, kan^R</i>	This study
MK383	D39, <i>DbgaA::P_{zn}-tetR-mKate2_parB_p^{mut}-gfp; tet^R; comCDE, tetO48, kan^R, parS_p</i>	This study
MK387	D39, <i>DbgaA::P_{zn}-parB_p^{mut}-gfp; tet^R; comCDE, parS_p, kan^R; dnaX-dnaX-mKate2, ery^R</i>	This study
MK392	D39, <i>comCDE, parS_p, kan^R, DbgaA::P_{zn}-parB_p^{mut}-gfp, tet^R, ftsZ-mKate2, kan^R</i>	This study
MK396	D39, <i>ftsZ-mKate2, kan^R, dnaX-gfp, ery^R</i>	This study
MK422	D39, <i>DbgaA::P_{zn}-tetR-mKate2_parB_p^{mut}-gfp, tet^R; Dcep::tetO48, spc^R</i>	This study
MK423	D39, <i>DbgaA::P_{zn}-tetR-mKate2_parB_p^{mut}-gfp, tet^R; Dcep::parS_p, spc^R</i>	This study
MK445	TIGR4, <i>Dsmc::trmp^R</i>	This study
MK446	R6, <i>Dsmc::trmp^R</i>	This study

Continuation of Table S1. Strains and plasmids used in this study

Strain	Description ^{a,b,c}	Reference
<i>S. pneumoniae</i>		
MK447	TIGR4, <i>DmapZ::cm^R</i>	This study
MK448	R6, <i>DmapZ::cm^R</i>	This study
MK453	D39, <i>DbgaA::P_{zn}-cas9, tet^R; Dcep::P3-sgRNA_{luc1}, spc^R</i>	This study
MK454	D39, <i>DbgaA::P_{zn}-cas9, tet^R; Dcep::P3-sgRNA_{luc1}, spc^R; ssbB-luc, kan^R</i>	This study
MK455	D39, <i>DbgaA::P_{zn}-cas9, tet^R; Dcep::P3-sgRNA_{luc1}, spc^R; ftsZ-cfp, kan^R</i>	
MK456	D39, <i>DbgaA::P_{zn}-cas9, tet^R; Dcep::P3-sgRNA_{luc1}, spc^R; dnaN-luc, ery^R; ftsZ-cfp, kan^R</i>	This study
MK457	D39, <i>DbgaA::P_{zn}-cas9, tet^R; Dcep::P3-sgRNA_{luc1}, spc^R; ftsZ-cfp, ery^R</i>	This study
MK458	D39, <i>DbgaA::P_{zn}-cas9, tet^R; Dcep::P3-sgRNA_{luc1}, spc^R; ssbB-luc, kan^R; ftsZ-cfp, ery^R</i>	This study
RR22	D39, <i>dnaX-gfp-ery^R</i>	This study
RR23	D39, <i>ftsZ-cfp-ery^R</i>	This study
RR27	D39, <i>ftsZ-mCherry-ery^R</i>	Beilharz <i>et al.</i> , 2015
RR31	D39, <i>DbgaA::P_{zn}-dnaX-gfp, tet^R</i>	This study
RR33	D39, <i>DbgaA::P_{zn}-ssb-gfp, tet^R</i>	This study
RR55	D39, <i>dnaX_dnaX-mKate2-ery^R</i>	This study
RR66	D39, <i>ftsZ-mKate2, kan^R</i>	This study
RR67	D39, <i>hlpA_hlpA-mKate2, cm^R; dnaX-gfp, ery^R</i>	This study
RR70	D39, <i>ftsZ-cfp, kan^R</i>	This study
RR82	D39, <i>ftsZ-mKate2, kan^R; dnaX-gfp, ery^R; Dsmc::trmp^R</i>	This study
RR84	D39, <i>ftsZ-cfp; kan^R; Dsmc::trmp^R</i>	This study
RR86	D39, <i>dnaX-gfp; ery^R; Dsmc::trmp^R</i>	This study
RR89	D39, <i>dnaX-gfp; ery^R; DbgaA::P_{zn}-ssb-mKate2, tet^R</i>	This study
RR90	D39, <i>dnaX-gfp; ery^R; DbgaA::P_{zn}-dnaN-mKate2, tet^R</i>	This study
RR91	D39, <i>dnaX-gfp; ery^R; DbgaA::P_{zn}-dnaX-mKate2, tet^R</i>	This study
RR93	D39, <i>DmapZ::cm^R</i>	This study
RR96	D39, <i>DmapZ::cm^R; Dsmc::trmp^R</i>	This study
RR98	D39, <i>ftsZ-mCherry, ery^R; DmapZ::cm^R</i>	This study
RR99	D39, <i>DbgaA::P_{zn}-parB^{mut}-gfp, tet^R; comCDE, kan^R, parS_p; DmapZ::cm^R</i>	This study
RR101	D39, <i>ΔbgaA::P_{zn}-gfp-mapZ, tet^R</i>	This study
RR105	D39, <i>ftsZ-cfp, kan^R; ΔmapZ::cm^R</i>	This study
RR110	D39, <i>gfp-mapZ, tet^R; Δsmc::trmp^R</i>	This study
RR127	D39, <i>mKate2-mapZ; cm^R</i>	This study
RR128	D39, <i>DbgaA::P_{zn}-parB^{mut}-gfp, tet^R; comCDE, kan^R, parS_p; mKate2-mapZ, cm^R</i>	This study
VL528	D39, <i>DbgaA::P_{zn}-parB^{mut}-gfp, tet^R; comCDE, kan^R, parS_p; mKate2-mapZ, cm^R; PEP::P_{mapZ}-mKate2-mapZ, cm^R</i>	This study

^aIn the *S. pneumoniae* strains; the *gfp*-variant used was the monomeric superfolder GFP, *m(sf)gfp*, unless otherwise is indicated. ^bAbbreviations: MCS, multiple cloning sites; *ery^R*, erythromycin resistance; *cam^R*, chloramphenicol resistance; *kan^R*, kanamycin resistance; *trmp^R*, trimethoprim resistance; *tet^R*, tetracycline resistance; *spc^R*, spectinomycin resistance. ^cDifferent constructs in the same strain are separated by semicolons (;), different elements in a construct are separated by comma (,), translational fusion indicated by dash (-), and transcriptional fusions indicated by underscore (_). The loci used for chromosome tagging: *comCDE* (359°), *bgaA* (101°), *iga* (178°), *pulA* (182°), *relA* (259°), *cep* (295°) and *ssb* (301°).

Construction of $\Delta bgaA::P_{zn}$ -tetR-mKate2

A *tetR* gene codon-optimized for *S. pneumoniae* was amplified from plasmid pUC57-tetR-tetO1 (Sorg, Gallay & Veening, 2019) using primers TetR-F-RBS-EcoRI-BglII and TetR-R-fus-SpeI, digested with restriction enzymes BglII and SpeI and ligated into the BamHI and SpeI sites of plasmid pMK15 to generate pMK15-01 with P_{zn} -tetR-mKate2.

Continuation of Table S1. Strains and plasmids used in this study

Strain/Plasmid	Description ^{a,b,c}	Reference
Plasmids		
pAM33	Used as source for <i>tetO</i> sites	Lab collection
pKB01_sfgfp(Sp)	<i>amp^R, bgaA', P_{Zn}-(sf)gfp, 'bgaA</i>	Overkamp <i>et al.</i> , 2013
pLP712	Megaplasmid from <i>Lactococcus lactis</i> NCDO712	Wegmann <i>et al.</i> , 2012
pMK14	<i>amp^R, bgaA', tet^R, P_{Zn}-MCS-linker-(sf)gfp, 'bgaA</i>	This study
pMK14-02	<i>amp^R, bgaA', tet^R, P_{Zn}-parB_p-(sf)gfp, 'bgaA</i>	This study
pMK14-03	<i>amp^R, bgaA', tet^R, P_{Zn}-parB_p^{mut}-(sf)gfp, 'bgaA</i>	This study
pMK15	<i>amp^R, bgaA', tet^R, P_{Zn}-MCS-linker-mKate2, 'bgaA</i>	This study
pMK17	<i>amp^R, bgaA', tet^R, P_{Zn}-MCS-linker-m(sf)gfp, 'bgaA</i>	This study
pMK17-01	<i>amp^R, bgaA', tet^R, P_{Zn}-parB_p-m(sf)gfp, 'bgaA</i>	This study
pMK17-02	<i>amp^R, bgaA', tet^R, P_{Zn}-parB_p^{mut}-m(sf)gfp, 'bgaA</i>	This study
pMK11	<i>amp^R, bgaA', P_{Zn}-MCS, 'bgaA</i>	Kjos <i>et al.</i> , 2016
pMK11-01	<i>amp^R, bgaA', P_{Zn}-parB_p-mKate2, 'bgaA</i>	This study
pMK11-02	<i>amp^R, bgaA', P_{Zn}-parB_p^{mut}-mKate2, 'bgaA</i>	This study
pMK103	<i>amp^R, bgaA', P_{Zn(short)}-parB_p^{mut}-mKate2, 'bgaA</i>	This study
pMK109	<i>spc^R, cep', tetO48, 'cep</i>	This study
pMK110	<i>amp^R, bgaA', P_{Zn}-tetR-mKate2_{parB_p}-m(sf)gfp, 'bgaA</i>	This study
pMK111	<i>amp^R, bgaA', P_{Zn}-tetR-mKate2_{parB_p}^{mut}-m(sf)gfp, 'bgaA</i>	This study
pMK112	<i>spc^R, cep', parS_p, ery^R, 'cep</i>	This study
pMK15-01	<i>amp^R, bgaA', tet^R, P_{Zn}-tetR-mKate2, 'bgaA</i>	This study
pMK17-X	<i>amp^R, bgaA', tet^R, P_{Zn}-dnaX-m(sf)gfp, 'bgaA</i>	This study
pMK17-N	<i>amp^R, bgaA', tet^R, P_{Zn}-dnaN-m(sf)gfp, 'bgaA</i>	This study
pMK17-S	<i>amp^R, bgaA', tet^R, P_{Zn}-ssb-m(sf)gfp, 'bgaA</i>	This study
pMK15-X	<i>amp^R, bgaA', tet^R, P_{Zn}-dnaX-mKate2, 'bgaA</i>	This study
pMK15-N	<i>amp^R, bgaA', tet^R, P_{Zn}-dnaN-mKate2, 'bgaA</i>	This study
pMK15-S	<i>amp^R, bgaA', tet^R, P_{Zn}-ssb-mKate2, 'bgaA</i>	This study
pNZ-MS2-GFPdsm	template for <i>m(sf)gfp</i>	van Gijtenbeek <i>et al.</i> , 2016
pPEP1	<i>spc^R, cep', MCS, 'cep</i>	Sorg <i>et al.</i> , 2014
pSEUDO39-mKate2	used as source of <i>parB_p</i> for cloning	J. Siebring, unpublished
pUC57-cfp	<i>cfp</i> (cerulean) gene codon optimized for <i>S. pneumoniae</i>	Genscript
pUC57-tetR-tetO1	<i>tetR</i> -gene codon optimized for <i>S. pneumoniae</i>	Genscript

Construction of double chromosomal marker cassettes; $\Delta bgaA::(P_{Zn}-tetR-mKate2_{parB_p}-m(sf)gfp)$ and $\Delta bgaA::(P_{Zn}-tetR-mKate2_{parB_p}^{mut}-m(sf)gfp)$

The *tetR-mKate2*_p fusion was amplified from plasmid pMK15-01_p using primers TetR-F-RBS-EcoRI-BglII and pMK-down-R+XhoI, and ligated into the EcoRI and XhoI sites of plasmids pMK17-01 and pMK17-02 to generate plasmids pMK110 and pMK111, respectively.

Construction of *comCDE-kan^R-parS_p* (*parS_p* at 359°, origin) and *comCDE-kan^R* (control, no *parS_p*)

The *comCDE-kan^R* construct has been described for strain DLA14 (Slager, Kjos, Attaiech & Veening, 2014). For construction of *comCDE-kan^R-parS_p*, *comCDE-kan^R* was amplified from DLA14 using primers comC1F and kan-R+NotI. A downstream fragment was amplified from the same strain using primers insparS-comCDE-F-NotI and comC4R, in which the *parS_p* site was introduced by the forward primers. The two fragments were digested with NotI, ligated and transformed into *S. pneumoniae*. Transformants were selected on Columbia blood agar plates with kanamycin.

Construction of $\Delta_{iga-parS_p-spc^R}$ ($parS_p$ at 178°, terminus)

The strain MK113, containing an erythromycin marker near the terminus (*iga* locus), was used as a template for the PCR. An upstream fragment of 1.2 kb was amplified using primers *rbgA*-up-F and *rbgA*-up-R-BamHI. A downstream 2-kb fragment was amplified with primers insert-ter-1parSp-BamHI and *rbgA*-down-R, in which a $parS_p$ site is introduced in the forward primer. The latter fragment contains the erythromycin marker. The two fragments were digested with BamHI, ligated and transformed into *S. pneumoniae*. Transformants were selected on Columbia blood agar with erythromycin.

Construction of $\Delta_{cep::parS-ery^R-spc^R}$ ($parS_p$ at 295°, left arm)

A fragment containing $parS_p-ery^R$ was amplified from the $\Delta_{iga-parS_p-ery^R}$ construct using primers insert-ter-1parSp-BamHI and PRR66. This fragment was digested with BamHI and XhoI and ligated into the corresponding sites of plasmid pPEP1 (Sorg, Kuipers & Veening, 2014). The ligation was transformed to *E. coli* and transformants were selected on LB agar with spectinomycin. A correct plasmid was named pMK112, and contains homology regions for integration at the *cep*-locus. The plasmid was transformed to *S. pneumoniae* and transformants were selected on Columbia blood agar with erythromycin.

Construction of $\Delta_{cep::tetO48-spc^R}$ (*tetO48* at 295°, left arm)

A 1.6 kb fragment containing 48 *tetO* sites (*tetO48*) were cut out from plasmid pAM33 (laboratory collection, containing the *tetO240* sites of plasmid pPSL6 (Lee & Grossman, 2006)) using restriction enzymes EcoRI and BglII. The *tetO48* fragment was ligated into the EcoRI and BamHI sites of plasmid pPEP1 (Sorg *et al.*, 2014). The ligation was transformed to *E. coli* and transformants were selected on LB agar with spectinomycin. The plasmid, named pMK109, contains homology regions for integration at the *cep*-locus. *S. pneumoniae* transformants were selected on Columbia blood agar with spectinomycin.

Construction of $\Delta_{relA-tetO48-spc^R}$ (*tetO48* at 259°, left arm)

Using chromosomal DNA from *S. pneumoniae* D39 as template, up- and downstream sequences were amplified using primer pairs insert-left-up-F/insert-left-up-R-AscI and insert-left-down-F-XhoI/insert-left-up-R, respectively. The *tetO48-spc^R* cassette was amplified from plasmid pMK109 using primers pPEP-down-spc+AscI and NCS-R. Digestion with AscI (up-fragment), XhoI (down-fragment) or AscI and XhoI (*tetO48-spc^R* fragment) was performed and the three fragments were ligated. The construct was transformed into *S. pneumoniae*, and correct transformants were selected on Columbia blood agar with spectinomycin.

Construction of $\Delta_{pula-tetO48-spc^R}$ (*tetO48* at 182°, terminus)

Using chromosomal DNA from *S. pneumoniae* D39 as template, up- and downstream sequences were amplified using primer pairs insert-ter-up-F/insert-ter-up-R-XhoI and insert-ter-down-F-AscI/insert-ter-up-R, respectively. The *tetO48-spc^R* cassette was amplified from plasmid pMK109 using primers pPEP-down-spc+AscI and NCS-R. Digestions with XhoI (up-fragment), AscI (down-fragment) or AscI and XhoI (*tetO48-spc^R* fragment) were performed and the three fragments were ligated. The construct was transformed into *S. pneumoniae*, and correct transformants were selected on Columbia blood agar with spectinomycin.

Construction of $\Delta_{comCDE-tetO48-kan^R-parS_p}$ (*tetO48* and $parS_p$ at 359°, origin)

An up-fragment was amplified from chromosomal DNA of *S. pneumoniae* D39 using primers comC1F and comC2R+AscI. A down-fragment including the $parS_p-kan^R$ construct was amplified from the $\Delta_{comCDE-parS_p-kan^R}$ construct using primers kan-F+SpeI and comC4R. The *tetO48* sequence was amplified from pMK109 using primers MCS-BF and pPEP-down-AscI. The three fragments were digested (up-fragment with AscI, down-fragment with SpeI and *tetO48* sequence

with both *AscI* and *SpeI*) and ligated. The ligation product was transformed into *S. pneumoniae* and transformants were selected on Columbia blood agar with spectinomycin.

Construction of *parB-gfp* fusion

The *parB-gfp* fusion was described previously as strain MT2 (Kjos & Veening, 2014; Minnen, Attaiech, Thon, Gruber & Veening, 2011).

Construction of pMK17-X, pMK15-X, pMK17-N, pMK15-N, pMK17-S and pMK15-S

dnaX, *dnaN* and *ssb* were amplified from *S. pneumoniae* D39 genomic DNA using primer pairs PRR68/PRR99 (*dnaX*), PRR67/PRR98 (*dnaN*) and PRR69/PRR97 (*ssb*), containing overhangs with restriction sites for *XbaI* and *NotI*. Amplified products were digested by corresponding restriction enzymes and purified plasmids pMK17 and pMK15 were digested with *SpeI* and *NotI*. The inserts and vectors were ligated and transformed in *E. coli*. Transformants were selected on ampicillin, and the resulting plasmids were named pMK17-X (with *dnaX*), pMK15-X (with *dnaX*), pMK17-S (with *ssb*), pMK15-S (with *ssb*), pMK17-N (with *dnaN*) and pMK15-N (with *dnaN*).

4.

Construction of *hlpA_hlpA-mKate2, cm^R*

This fusion was described previously (Beilharz *et al.*, 2015; Kjos *et al.*, 2015; Kjos & Veening, 2014). For construction of RR67, *hlpA_hlpA-mKate2, cm^R* was amplified from strain MK119 using primers *hlpA-up-F* and *hlpA-down-R*. The fragment was transformed into RR22 and transformants were selected on Columbia blood agar with chloramphenicol to generate RR67.

Construction of *dnaX-m(sf)gfp-ery^R*

dnaX-m(sf)gfp was amplified from plasmid pMK17-X using primer pair PRR118/PRR107, where PRR107 contained an overhang for *eryR*. *eryR* was amplified from strain RR27 encoding *ftsZ-mCherry-ery^R* (Beilharz *et al.*, 2015), with primers PRR109 and PRR110 containing overhangs for *dnaX-m(sf)gfp* (forward) and *dnaX-down* (reverse). *dnaX-down* was amplified from genomic DNA of *S. pneumoniae* D39 using forward primer PRR111, containing an overhang for *eryR*, and reverse primer PRR12. The three parts were assembled by Gibson Assembly and directly transformed into *S. pneumoniae* D39 as described (Beilharz *et al.*, 2015), resulting in strain RR22.

Construction of *dnaX_dnaX-mKate2-ery^R*

dnaX-mKate2 was amplified from pMK15-X using primers PRR118 and PRR75, *eryR* was amplified from the *ftsZ-mKate2-ery^R* strain RR28 (Beilharz *et al.*, 2015) using primers PRR74 and PRR110, *dnaX-up* was amplified from genomic DNA of *S. pneumoniae* D39 using primers PRR13 and PRR117 and *dnaX-down* was amplified from genomic DNA using primers PRR12 and PRR111. All PCR products contained overlaps for their neighbouring products in the order *dnaX-up*, *dnaX-mKate2*, *eryR* and *dnaX-down*. The four parts were assembled by Gibson Assembly and directly transformed into *S. pneumoniae* D39, resulting in strain RR55.

Construction of *ftsZ-mKate2-kan^R*, *ftsZ-cfp-ery^R* and *ftsZ-cfp-kan^R*

For *ftsZ-cfp-ery^R*, the *ftsZ-up* fragment was amplified from *S. pneumoniae* D39 genomic DNA using primers PRR46 and PRR47. *cfp* was amplified from pUC57-*cfp* using PRR116 and PRR49 and *ery-ftsZ-down* was amplified from the *ftsZ-mCherry-ery^R* strain RR27 (Beilharz *et al.*, 2015) using PRR44 and PRR115. The three parts contained overlapping regions for the neighbouring parts. The parts were assembled using Gibson Assembly and directly transformed into *S. pneumoniae* D39, resulting in strain RR23. The *cfp* (cerulean) gene was designed based on the *m(sf)gfp*(Sp) gene published before (Overkamp *et al.*, 2013), with the following mutations to convert it to *cfp* (cerulean): Y66W, S72A, F145A, H148N and N149I. For *ftsZ-mKate2-kan^R*, *ftsZ-mKate2* and *ftsZ-*

Table S2. Oligos used in this study

Primer name	Sequence (5' -> 3') †
comC1F	GCATGCAGATGGCAATTGAC
comC2R+Ascl	CCCGGGCGCGCCTGCTAATTGTCAATCACTTT
comC4R	AATGCCATCTTCCAATCCC
hlpA-down-R	CGTGGCTGACGATAATGAGG
hlpA-up-F	AACAAGTCAGCCACCTGTAG
insert-left_down_F+XhoI	TAATCTCGAGGACCAGAACCTGCCAGTAG
insert-left_down_R	GGAGCGTCTGCTTAAGAATG
insert-left_up_F	GCGCCTTGCTAGTCTTGACC
insert-left_up_R+Ascl	TTATGGCGCGCCAGGCAGCAAACCTCCTAACG
insert-ter-1parSp-BamHI	ATGGATCCGGGGCTAAATTTAGCCCCAACAGCAAAGAATGGCGGA
insert-ter-1parSp-BamHI	ATGGATCCGGGGCTAAATTTAGCCCCAACAGCAAAGAATGGCGGA
insert-term-down-F-Ascl	TAATGGCGCGCCCTATGACAGTTATAGTAGGC
insert-term-down-R	GAAGGTATAGCCATGCTCTC
insert-term-up-F	CAATCGGTGCTGGAGTTTG
insert-term-up-R-XhoI	CCGCCTCGAGTTGTTGAAACAAAGTCTAGG
insparS-comCDE-F-NotI	AGCGCGGCCGCGGGGCTAAATTTAGCCCCCTGATATAATGGTTATATCTGCTAC
kan-R-NotI	AATAGCGGCCCGACAGTTGCGGATGTACTTC
MCS-BF	GCATCGGTCTCAGTGTACTGCAGAAAACCTAGTCGC
NCS-R	GCAAGGCATGCGTTACCATACGTAGGTGTC
parBj-mut-F	GAGGGAGCAAAGTTCTCTCCACAAAAGAAACCGCTAAAAAGC
parBj-mut-R	TTGTGGAGAGAACTTTGCTCCCTCTCTTACCACCTTGTC
PB012	CGTCGCTCTTTGCATTTGAGG
PB015	CGAGGCATGATAGAAGGACCTTC
pMK-down-R+XhoI	ACGTCCTCGAGGCAGAATTGCCGACCTTGAC
pPEP1-down-spc-Ascl	ACGTGGCGCGCCACCAGATCATGGCTAGAAAGCT
pPEP-down-Ascl	ACGTGGCGCGCCCTCGAGTTACTGGAGGGATC
pPP2-R	CGAAATACGGGCAGACATGG
pr21 (EcoRI)	GGAATTCGGATCCGCGGCCGCGAGATCTACTAGTTCTAGAGGATCTGGTGAGAAGC TGCAGCTAAAGCTGGATCAAAAGGAGAAGAGCTGTTTC
pr22 (NheI)	GCGTAGCTTACTTATAAAGCTCATCCATGC
pr23 (EcoRI)	GGAATTCGGATCCGCGGCCGCGAGATCTACTAGTTCTAGAGGATCTGG TGGAGAAGCTGCAGCTAAAGCTGGA TCAGAACTTATCAAGGAAAATATG
pr24 (NheI)	GCGTAGCTTAACGGGTGCCAATTTACTAG
PRR12	CTTTAAGAGCGTCAATATCAAG
PRR13	TTACTGGCACCATTAGCCTACC
PRR44	ATCAAAACCGAACTCACCTGTTGAT
PRR46	CCTGTATTGCTCGTATGCCAAA
PRR47	GCTTCTCCACCAGATCCGGAACGATTTTGA AAAATGGAGGTG
PRR66 (XhoI)	AGCTCTCGAGTTATTTCCTCCCGTTAAATAATAG
PRR67 (XbaI)	CGTCTAGAATTGTACGAACTGGTGTAAATGAG
PRR68 (XbaI)	CGTCTAGAAGCGTCTTCTCTACCTTCACTTTATC
PRR69 (XbaI)	CGTCTAGAGAATGGTAAATCATCATCTGAAATA
PRR74	GTAATTGGGACACCGTTAATAACCCGGAGGAATTTTCATATGAAC
PRR75	GTTCAATGAAAATTCCTCCGGGTTATTAACGGGTGCCCAATTTAC
PRR80	AGTGGTGCCGATATGG
PRR81	TGACAGCGAAGGCTAC
PRR97 (NotI)	GCGCGGCCCGCAGGAGGTAAACTATGATTAACAATGTTGTACTTG
PRR98 (NotI)	GCGCGGCCCGCAGGAGGAGATCCATGATTCATTTTC
PRR99 (NotI)	GCGCGGCCCGCAGGAGGATCTTAATGTATCAAGCACTTTATCG
PRR107	GTTCAATGAAAATTCCTCCGGGCCACCTTGACTAGCTTAC
PRR109	GTAAGCTAGTCAAGGTGCGCCCGGAGGAATTTTCATATGAAC
PRR110	CTTATTGTATCATGAAATCTTCTTTACGGCCGCTTATTTCCTCCC
PRR111	GGGAGGAAATAAGCGGCCGTAAAGAAAGATTTTCATGATACAATAAG
PRR115	GGTATGGACGAACTTTATAATAACCCGGAGGAATTTTCATATGAAC
PRR116	GTTCAATGAAAATTCCTCCGGGTTATTATAAAGTTCGCCATACC
PRR118	GATAAAGTGAAGGTAGAGGAAGACGAGGAGATCTTAATGTATCAAGC

down were amplified from DRR28 using primers PRR46 and PRR121 (*ftsZ-mKate2*) and PRR124 and PRR44 (*ftsZ-down*), leading to *ftsZ-mKate2* with a 3' overhang for *kan^R* and *ftsZ-down* with a 5' overhang for *kan^R*. *kan^R* was amplified from *S. pneumoniae* D39 Δ *cpsE::kan^R* (Kjos *et al.*, 2015) using PRR122 and PRR123, leading to a product with overhangs for the other two amplified products. The three products were assembled by Gibson Assembly and directly transformed into *S. pneumoniae* D39, resulting in strain RR66. For *ftsZ-cfp-kan^R*, the same *kan^R* and *ftsZ-down* fragments were used, but *ftsZ-CFP* was amplified from *ftsZ-cfp-ery^R* using primers PRR121 and PRR46. The three parts were combined and assembled using Gibson Assembly and directly transformed into *S. pneumoniae* D39, resulting in strain RR70. All strains were checked for integration via double-crossover using primers PRR80 and PRR81.

Deletion of *mapZ* (Δ *mapZ::cm^R*)

mapZ-up and *mapZ-down* fragments were amplified from *S. pneumoniae* D39 genomic DNA using primers PB012 (Holečková *et al.*, 2014) and PRR133 for *mapZ-up* and PB015 and PRR137 for *mapZ-down*, creating a 3' overlap for *cmR* on *mapZ-up* and a 5' overhang for *cmR* on *mapZ-down*. *cmR* was amplified from MK119 (Kjos & Veening, 2014) using primers PRR134 and PRR135, creating overhangs for *mapZ-up* and *mapZ-down*. The three products were assembled by Gibson Assembly and directly transformed into *S. pneumoniae* D39, resulting in strain DRR93.

Construction of *mKate2-mapZ*, *cm^R*

An *mKate2-link* fragment was amplified from pMK15 using primers PRR143 and PRR146. A fragment *up-cam* was amplified from strain RR93 using primers PRR145 and PRR136. *mapZ-down* was amplified from D39 genomic DNA using primers PRR138 and PRR144. The three fragments contained overlaps for their neighbouring products in the order *up-cam*, *mKate2-link* and *mapZ-down*. The three parts were assembled by Gibson Assembly and directly transformed

Continuation of Table S2. Oligos used in this study

Primer name	Sequence (5' -> 3') †
PRR121	GTGATATTCTCATTTTAGCCATATGAAAATTCCTCCGGGTTA
PRR122	TAACCCGGAGGAATTTTCATATGGCTAAAAATGAGAATATCAC
PRR123	CTTTTACATTCATCGGCCGCTTCTAAATCTAGGTACTAAAAACAATTC
PRR124	GAATTGTTTTAGTACCTAGATTAGAAGCGGCCGATGAATGTAAAAG
PRR133	CTACTAGTGCTCATTAGAATTCTCGTCTTTTTTACTCATGAGTATC
PRR134	GATACTCATGAGTAAAAAAGACGAGAATTCTAATGAGCACTAGTAG
PRR135	TTAGTAGTCCAAGTCATCCGCATGACCGGCCGCTTATAAAGCCAG
PRR137	CTGGCTTTTATAAGCGGCCGGTCATGCGGATGACTTGGACTACTAA
PRR139	GCGTCTAGAATGAGTAAAAAAGACGAAATC
PRR140	GAGCGGCCGCTTAGTAGTCCAAGTCATCCGATGACC
PRR143	CTTCTCCACCAGATCTCTAGAACGGTGTCCCAATTTAC
PRR144	GTAAATTGGGACACCGTTCTAGAGGATCTGGTGAGAGAAG
PRR145	CCTTGATAAGTTCTGACATATTGCCTCCTCGGCCGCTTATAAAGCCAG
PRR146	CTGGCTTTTATAAGCGGCCGAGGAGGCAAATATGTCAGAACTTATCAAGG
<i>rbgA-down-R</i>	GACCACGACCAACCTCATCA
<i>rbgA-up-F</i>	CAGATCTTCAGAACTATGTCCA
<i>rbgA-up-R-BamHI</i>	CCCGGGATCCAGCCTATCTTTTACCCTATATAGA
sPG88	CGAGTCTGGTGGTTGGTAAG
TetR-F-RBS-EcoRI-BgIII	GAGAATTCAGATCTAAGGAGGAGGCAAATATGTCTCGTTTGATAAGAGT
TetR-R-fus-SpeI	AGTACTAGTACTACCACTTTCACATTTAAGTTG
OVL33 (NheI)	GCGCTAGCAGCTCTATGCTGAG
OVL34 (BglII)	CGAGATCTATCATTTTACTTTTG
LK049	CTACATGAAAAGAAACGTCAAATG
LK354	TGGTCTCCGATCGTGACTG

† Restriction sites are underlined.

into *S. pneumoniae* D39. For VL528, the full P_{mapZ} -*mKate2-mapZ*, *cm^R* fragment was amplified by PCR using primers OVL33 and OVL34. The fragment was cut with BglII and NheI and ligated into plasmid pPEPY. The ligated plasmid was directly transformed in *S. pneumoniae* D39 and checked by colony PCR using primers LK049 and LK354.

Construction of $\Delta bgaA::m(sf)GFP-mapZ$; *tet^R*

mapZ was amplified from D39 genomic DNA using primers PRR139 and PRR140. The fragment digested with XbaI and NotI and pCG6 (Liu *et al.*, n.d.) was digested with XbaI and NotI as well. The fragments were ligated and transformed into *S. pneumoniae* D39.

Movies

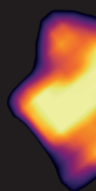
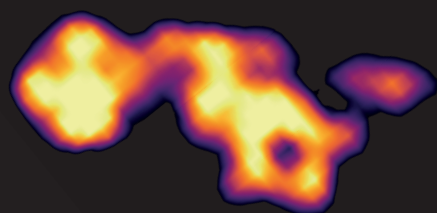
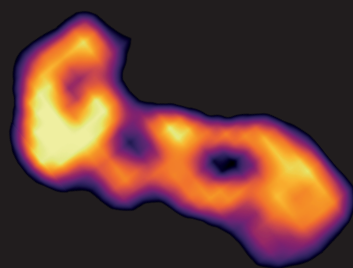
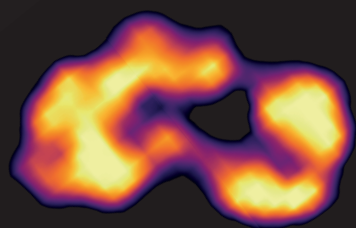
All movies can be found online at: <https://www.pnas.org/content/114/29/E5959>

4.

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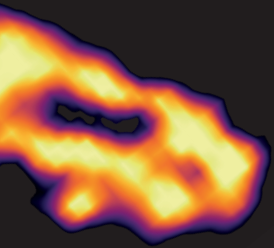
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Chapter 5:

Investigations into the condensation and segregation dynamics of the pneumococcal nucleoid

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Abstract

In the opportunistic pathogen *Streptococcus pneumoniae* (the pneumococcus), the regulation and timing of cell division and chromosome segregation are intertwined. During chromosome segregation, the origin of replication positions at the new mid-cell position and may act as a landmark for FtsZ ring formation. How the chromosome is positioned at mid-cell during the cell cycle remains unclear. The pneumococcus lacks a nucleoid occlusion system. On top of that, chromosome segregation proteins SMC and ParB can both be deleted without major problems. Therefore, we decided to look at the role of the essential histone-like protein HU (aka HlpA) during chromosome segregation. HU is the only known nucleoid-associated protein in *S. pneumoniae* and has recently been shown to be involved in DNA supercoiling. Using Chromatin Immuno-Precipitation Sequencing (ChIP-Seq) we show that HU binds the whole chromosome with a preference for regions with a high GC content. Using a newly developed degron system for *S. pneumoniae*, we show that rapid depletion of HU results in arrested growth, decondensed nucleoids and the formation of anucleate cells. Finally, we used Photo-Activated Localization Microscopy (PALM) to follow HU localization on the chromosome. Combined with Structured Illumination Microscopy (SIM), we could present a detailed mapping of the condensation, movement and shaping of the pneumococcal chromosome during the cell cycle.

5.

Introduction

Unlike eukaryotes, where DNA compaction, replication and segregation is tightly controlled and separated by check-points, bacteria seemingly divide, replicate, translate, compact and segregate their DNA simultaneously. Rather than a lack of organization, this points to an intimate connection between all these processes in the bacterial cell cycle. In the last decade, different key players in this connection have been discovered in different bacteria. There is a strikingly great variability of cell cycle regulation between bacterial species. At the same time, key processes are identified over a range of different bacterial species

The opportunistic pathogen *Streptococcus pneumoniae* is no exception to this. *S. pneumoniae* or the pneumococcus is a small, oval-shaped Gram-positive bacterium that forms diplococci or small chains in laboratory conditions. It contains one relatively small circular genome of 2 Mbp, and the commonly used strain D39V lacks plasmids (Slager, Aprianto & Veening, 2018). The pneumococcus has a division time of approximately 30 minutes in optimal conditions and does not perform multi-fork replication (Slager, Kjos, Attaiech & Veening, 2014). During cell division, DNA replication takes place in the center area of the cell at mid-cell, where FtsZ has formed the Z-ring. The replication forks are not fixed on one location, but are rather dynamic (Raaphorst, Kjos & Veening, 2019; van Raaphorst, Kjos & Veening, 2017). Besides the active process of DNA replication taking place at mid-cell, another mechanism important for driving chromosome segregation is the *parS*/ParB system. ParB (partial partition system B) binds to *parS* sites on the chromosome, which are located close to the origin of replication. ParB recruits structural maintenance of the chromosome complex SMC to the chromosome (Minnen, Attaiech, Thon, Gruber & Veening, 2011). Since *S. pneumoniae* lacks a clear cognate ParA, it has been suggested that the structural integrity of the chromosome, influenced by ParB and SMC, is enough to facilitate proper segregation. The recently identified membrane-binding protein RocS is another player in chromosome segregation, of which deletion is leading to a similar phenotype (Mercy *et al.*, 2019). RocS localizes at the origin of replication, and while ParB and SMC can be deleted simultaneously, they become essential upon RocS deletion and vice versa. How RocS influences chromosome segregation remains unclear.

While chromosomes are segregating, MapZ, a membrane-bound protein that forms a ring at mid-cell, is moving together with the origin of replication to the $\frac{1}{4}$ positions of the cell (Fleurie *et al.*, 2014). MapZ is positioned at the forefront of the newly formed peptidoglycan (Manuse *et al.*, 2016) and ensures the positioning of the FtsZ-ring is at a 90° angle opposed to the cell length axis (van Raaphorst *et al.*, 2017). FtsZ filaments gradually move from mid-cell to the $\frac{1}{4}$ -positions (Perez *et al.*, 2019). Replication termination, full formation of the Z-ring and replication initiation happen almost simultaneously (Gallay *et al.*, 2019; Raaphorst *et al.*, 2019), where the replication forks are fired when colocalizing with the Z-ring, mediated by cell cycle protein CcrZ (Gallay *et al.*, 2019).

One of the questions that remain is how the pneumococcus manages to segregate its chromosomes when many of the known players can be deleted from the chromosome with only mild phenotypes. There are two proteins important for chromosome structure that are essential. While the new Z-ring is fully formed, the replication terminus of the chromosome is located at the closing septum. FtsK, which acts as a motor to pull through near-segregated DNA in this latest stage and localizes at the septum in *E. coli* (Stouf, Meile & Cornet, 2013), is essential in *S. pneumoniae*. Another essential protein in chromosome structure is HU (or Histone-like protein A, HlpA), which is the only known nucleoid associated protein in *S. pneumoniae*. HU is important for DNA supercoiling, and overexpression of HU is shown to compensate the blockage of DNA gyrase by novobiocin (Ferrándiz, Carreño, Ayora & de la Campa, 2018).

HU is highly conserved among bacteria. It generally binds non-specifically to DNA and forms

a dimer. However, its role in chromosome structuring is different among species. In *E. coli*, HU consists of two subunits ($\text{hup}\alpha/\beta$), of which deletion only leads to minor growth defects. Hi-C shows that $\Delta\text{hupa}/\beta$ mutants are deficient in long-range DNA interactions and show an increase in short-range interactions, similar to *E. coli* deficient of *mukBEF*, the functional SMC homologue of *E. coli* (Lioy *et al.*, 2018). Superresolution imaging of enlarged, circular *E. coli* confirms that large subdomains are substituted for smaller subdomains in $\Delta\text{hupa}/\beta$ mutants (F. Wu, Japaridze, *et al.*, 2019). Interestingly, in *Caulobacter crescentus*, removing HU has an opposite effect. Hi-C studies show a decrease in short-range interactions (Tran, Laub & Le, 2017). The authors suggest that HU stabilizes plectonemes during transcription. Interestingly, in both Hi-C studies, HU binding as measured by ChIP-seq did not correlate with the contact subdomains found by Hi-C.

In many model bacteria, the nucleoid has a dynamic, helical shape (Berlatzky, Rouvinski & Ben-Yehuda, 2008; Fisher *et al.*, 2013a; Gorle *et al.*, 2017). Both Fisher *et al.*, 2013 and Wu *et al.*, 2019 propose that this helical conformation and compaction is due to molecular crowding and the mixture of DNA and crowders (e.g. ribosomes) being spatially confined inside a tube. This concept received recent experimental support by a study on cell division of L-form bacteria inside microfluidic channels (L. J. Wu *et al.*, 2019). Together, these findings suggest that the shape and form of the bacterial nucleoid might be highly dynamic during the cell cycle.

5.

Here, we investigated the role of pneumococcal HU in maintaining the chromosome shape and assess its role in chromosome segregation. For this, we performed ChIP-seq and showed that HU binds chromosomal DNA uniformly with a slight preference for GC-rich DNA. We developed a ClpX/A *sspB*-based degron system for use in *S. pneumoniae* and used it to rapidly degrade HU and look at the phenotypic consequences of reduced cellular HU levels. We also investigated the effect of disrupting chromosome integrity by antibiotics and deletion of SMC on HU localization and the nucleoid-cell size ratio. Finally, we used HU as a marker for the chromosome to be able to investigate the shaping and mobility of the chromosome during cell division using superresolution microscopy. Together, we present new insight into the different stages of the chromosome cycle of *S. pneumoniae*.

Results & Discussion

HU binds to the whole chromosome

In our previous studies, we have used fusions of HU to various fluorescent proteins to visualize the chromosome during the cell cycle or to identify pneumococcal strains during infection by FACS or microscopy. HU-FP colocalizes with DNA-staining such as DAPI, giving the brightest fluorescent signal we know of in *Streptococcus pneumoniae* (Kjos *et al.*, 2015). HU is highly transcribed under many environmental conditions (Aprianto, Slager, Holsappel & Veening, 2018), and with an estimated number of 67.240 molecules per cell (Ferrándiz *et al.*, 2018), one would expect one HU dimer to bind every 60 basepairs when there is an even, homogeneous distribution.

To find out whether HU is homogeneously distributed along the chromosome, we performed Chromatin ImmunoPrecipitation-sequencing (ChIP-seq) of pneumococcal cells in early exponential phase carrying a functional fusion of HU to superfolder GFP (HU-sfGFP). The resulting enrichment profile showed that HU-GFP indeed binds rather uniformly to the chromosome. Strikingly, while there are no large peaks of enrichment (a maximum relative fold change of 3), there are small regions where there is less binding (“troughs”), and this pattern is conserved between the two replicates. The largest region with low HU-binding is close to the replication terminus (1 – 1.04 Mbp, Figure 1.B).

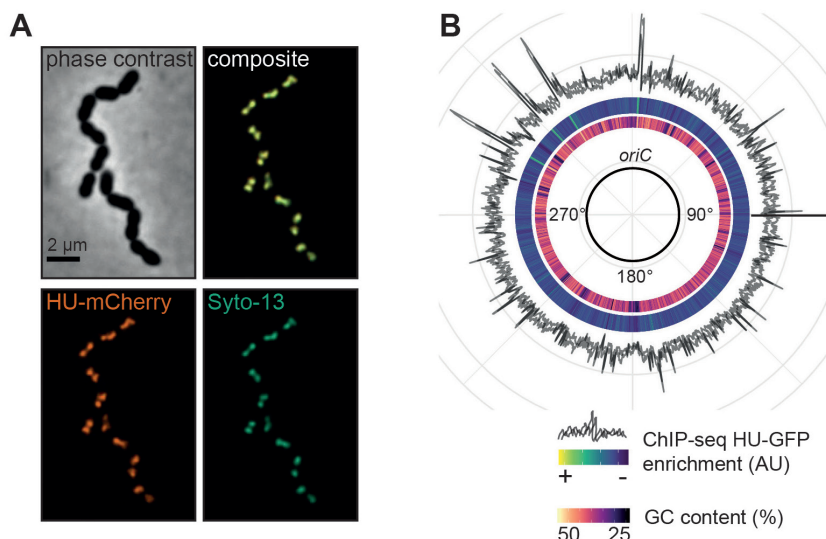


Figure 1. HU binds the whole chromosome of *S. pneumoniae*. **A.** Deconvolved epi-fluorescence microscopy images of HU-mCherry and the chromosome stained with the intercalating DNA dye Syto-13 show near-perfect overlap. **B.** Enrichment profile of ChIP-seq results of HU-GFP over the chromosome shows binding everywhere (both line profile and blue-green color profile), with less binding at regions with low GC content (yellow-purple color profile), for instance at the terminus region.

Since different NAPs in other bacteria, including HU homologs, have been reported to prefer AT-rich regions, we correlated the HU binding pattern with the GC content of the DNA. As shown in Figure 1B, HU-binding is generally low at AT-rich regions, while many of the regions with high HU occupancy are GC-rich. However, the global correlation between HU-binding and GC content is poor (Figure S1). What could also influence the HU-binding pattern is the accessibility of the DNA. There are several actors identified to influence chromosome conformation, from passive processes as molecular crowding, to DNA-binding proteins (such as Structural Maintenance of the Chromosome complex SMC and HU itself), transcription/translation and DNA methylation.

To see if there was a correlation with transcription/translation, we also performed ChIP-seq on *S. pneumoniae* with GFP bound to the beta subunit of RNA polymerase (RpoB-(sf)GFP). Since RpoB is binding to regions where it will transcribe DNA, these would directly correlate to the regions of transcription. ChIP-seq of RpoB-GFP indeed showed enrichment at highly transcribed regions (Figure S1). While the highest HU-binding regions, which are localized around the ribosomal RNA and tRNA loci, are also high in RpoB-binding, there was no correlation between HU-binding and RpoB-binding over the rest of the chromosome.

Partially degrading HU in live cells lead to decondensed chromosomes and chromosome segregation problems.

(Ferrándiz *et al.*, 2018) showed that HU-depleted cells have growth defects, but not nearly as severe as one might think. We reasoned that depletion on a transcriptional level with inducible promoters might be too slow for the cells to respond or might induce stress responses that could buffer the effects. Therefore, we decided to utilize a depletion method on protein level using a SspB/ClpX recognition system (see Figure 2A). Upon induction of P_{Zn} -*sspB* with 0.1 μM

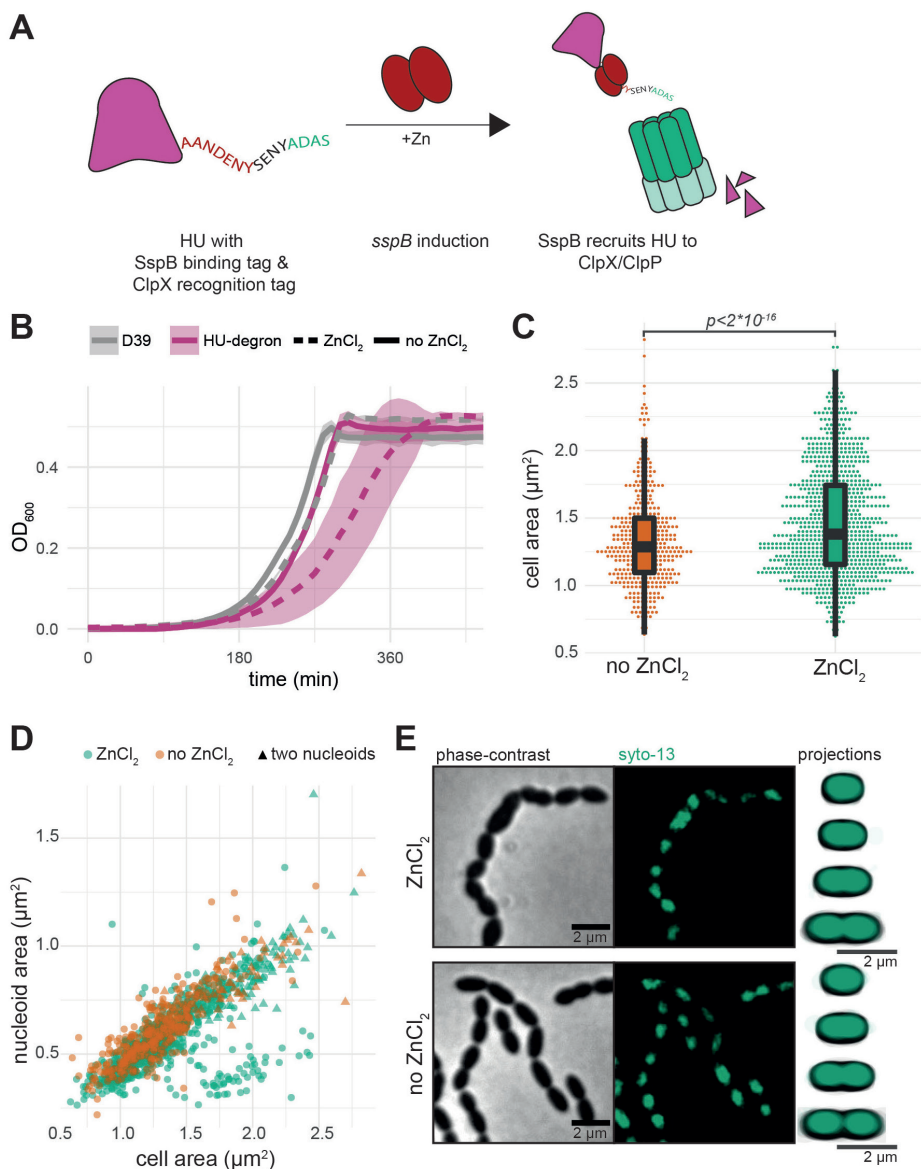


Figure 2. Degradation of HU leads to slower growth, larger cells and decondensed chromosomes.

A. Schematic representation of the ClpX-ssrA degradation system. The modified *ssrA*-tag (red and black) is recognized by SspB which is placed under a Zn-inducible promoter. SspB leads HU to ClpX, that recognizes the ClpX tag (green) and degrades HU. **B-D** HU-degtron cells grown with and without 0.1 mM $ZnCl_2$ for four hours. **B.** Growth curves of wild-type *S. pneumoniae* D39 and cells carrying *hu::hu-degtron*; *cep::P_{ssrB}-ssrB* (HU-degtron cells). Dashed lines: induction with 0.1 mM $ZnCl_2$ from $t=0$. **C.** Cell size distribution in HU-degtron cells induced with $ZnCl_2$ is significantly different from uninduced HU-degtron cells (paired Wilcoxon-rank sum test). Sizes recorded from phase-contrast using Outfi. **D.** The nucleoid to cell area (N/C) ratio in induced and uninduced HU-degtron cells. **E.** Cell and nucleoid shape is altered by degrading HU. Cells were stained with 0.5 μM syto-13 and imaged immediately afterwards. Right: cell projections show segregated chromosomes in uninduced cells, but not in cells induced with $ZnCl_2$. Projections grouped by cell length ($ZnCl_2$: group 1: 0.26-0.49 μm , group 2: 0.49-0.61 μm , group 3: 0.61-0.76 μm , group 4: 0.76-1.7 μm . No $ZnCl_2$: group 1: 0.22-0.52 μm , group 2: 0.52-0.62 μm , group 3: 0.62-0.72 μm , group 4: 0.72-1.33 μm).

ZnCl₂, HU with a C-terminal SspB-ClpX recognition tag (HU-degron) is recognized by SspB and degraded by ClpX. While we expected the degradation to be fast, tests with HU-mCherry-degron showed that the mCherry signal was fully depleted after four hours (Figure S1). Similar to the transcriptional depletion strains described in Ferrandiz *et al.*, induced HU-degron had a clear growth defect, but cultures reached the same final OD as wild type cells suggesting that cells still managed to survive and utilize all the nutrients from the growth medium (Figure 2).

HU-degron cells induced with ZnCl₂ for four hours were irregularly shaped and had a larger nucleoid area (Figure 2 and S1). When plotting nucleoid area over cell area, the nucleoid to cell (N/C) ratio was constant at 0.5 in both induced and uninduced cells (Figure 2), with the exception of a small group of induced cells where the N/C ratio was exactly twice as small. These cells are nearly divided cells with a condensed nucleoid in only one cell half, almost producing an anucleoid cell. Interestingly, to maintain the N/C ratio, induced cells are larger (Figure 2). While there is no difference in cell length distribution, induced cells are considerably wider (Figure S1). This suggests that the nucleoid itself could act as a nucleoid-occlusion factor controlling division.

Sub-MIC concentrations of ciprofloxacin lead to HU accumulation and distorted nucleoid-cell size (N/C) ratios

5.

HU binds the complete nucleoid and is expressed at an even level by different cells (Figure 3B). Its working might change when the integrity of the nucleoid is compromised by external factors. Ciprofloxacin is a quinolone which targets topoisomerase IV (ParC) and DNA gyrase (GyrA/B) in *Streptococcus pneumoniae* (Pan, Ambler, Mehtar & Fisher, 1996), affecting DNA supercoiling and chromosome segregation. HPURa (6-p-hydroxyphenylazo-uracil) stalls replication by reversibly binding to DNA Polymerase III (PolC) after intracellular conversion to a deoxyguanosine triphosphate analog (Brown, 1970). Previously, we observed that permissive concentrations of both antibiotics lead to the formation of anucleate cells, mislocalized chromosome loci and replication forks, and larger, chained cells (van Raaphorst *et al.*, 2017).

We measured the nucleoid size using HU-mKate2 as a marker. Strikingly, when grown with ciprofloxacin, the N/C ratio of cells is distorted in both directions. There is a fraction of small cells ($\leq 1 \mu\text{m}^2$) with relatively large nucleoids ($\sim 0.7 \mu\text{m}^2$) which are often found next to anucleate cells (Figure 3B, right panel). Next to this, there are large cells with highly condensed nucleoids at mid-cell or close to the old septum, which are likely to produce a pair of small cells – one anucleate and one with a relatively large nucleoid – after cell division. Cells treated with HPURa show similar defects, though the phenotype is much less severe. Here, the N/C ratio remains intact, though more variable than in untreated cells. Cells lacking *smc* show a phenotype very similar to HPURa treatment (Figure S2).

What is intriguing, is that while some of the nucleoids have a less defined boundary and are therefore probably less condensed after treatment with ciprofloxacin, the average nucleoid size is smaller than in untreated cells. On top of that, in some cells, some nucleoids seem condensed and confined in only one region of the cell. The intensity of HU-mKate2 per nucleoid is higher and generally more variable in treated cells. One explanation for the variability in nucleoid size and intensity is the mode of action of ciprofloxacin. Ciprofloxacin targets both gyrase, which is responsible for negative supercoiling of DNA, and topoisomerase IV, which is involved in DNA relaxation and decatenation of the DNA close to replication termination (El Houdaigui *et al.*, 2019). Ciprofloxacin is thought to have a preference for topoisomerase IV in the pneumococcus, which should lead to segregation problems in the late segregation stage and could possibly even lead to positive supercoiling. On top of this, the high HU-mKate2 intensity might be due to *hu* upregulation to counteract the loss of function of DNA gyrase. This could be tested with a promoter fusion of P_{HU} to a luciferase (*luc*) gene, or a quantitative Western blot.

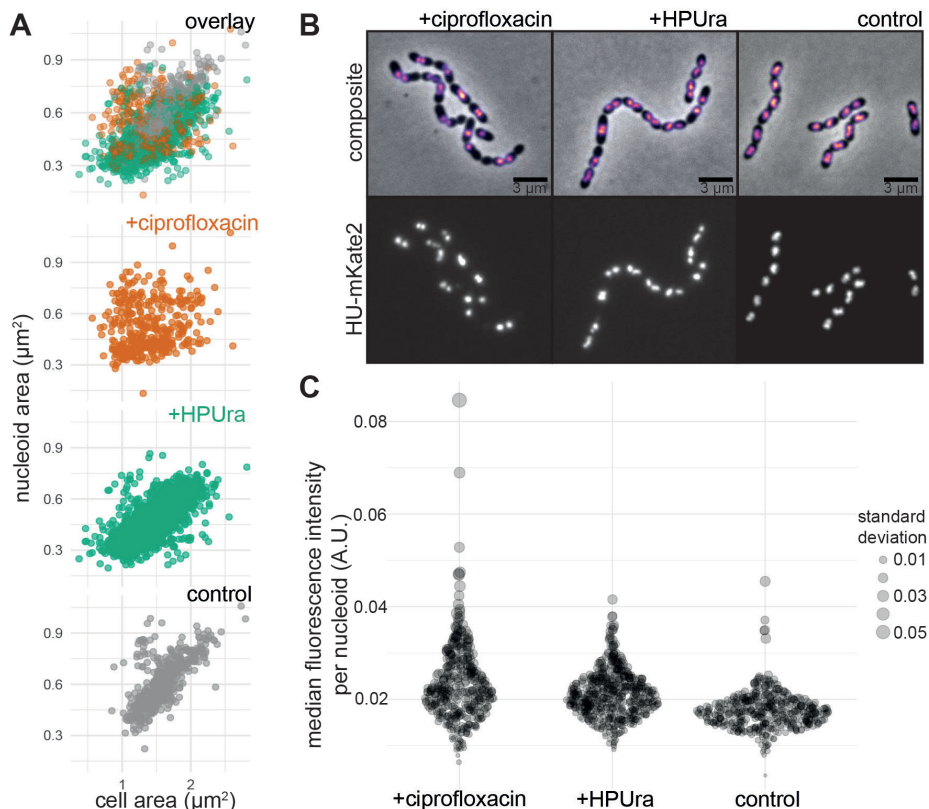


Figure 3. Sub-inhibitory concentrations of ciprofloxacin lead to both decondensed and highly condensed nucleoids and variable HU-mKate2 intensities. Cells carrying *hu::hu-mKate2* (strain RR67) grown at sub MIC concentrations of ciprofloxacin (0.4 $\mu\text{g}/\text{mL}$) or HPUra (0.15 $\mu\text{g}/\text{mL}$). **A.** Nucleoid-cell size (N/C) ratios are distorted in cells treated with ciprofloxacin. Cell area and nucleoid area were segmented from phase-contrast (cells) and fluorescence images (nucleoids) with Oufiti. **B.** Cutout from images used to generate data for (A) and (C). Composite of phase-contrast and HU-mKate2 image (top) and deconvolved HU-mKate2 only (bottom). Scale bar: 3 μm . **C.** Median fluorescence intensity (A.U.) of HU-mKate2 per nucleoid is higher and more variable in cells treated with ciprofloxacin or HPUra. Dot size indicates the standard deviation of each median intensity.

Superresolution microscopy shows two nucleoid morphologies

Since HU is binding the whole chromosome, we decided to use fluorescent fusions to HU to further investigate the morphology and dynamics of the nucleoid during chromosome segregation. Using conventional fluorescence microscopy, our view on the pneumococcal nucleoid is limited. Being $\sim 0.5 \mu\text{m}^2$ as measured by wide-field fluorescence microscopy of HU-RFP (see Figure S3A), the nucleoid dimensions are close to the resolution limit. To distinguish the shape of the nucleoid in different stages of the cell cycle, we used PALM (Photoactivated Localization Microscopy).

Live *S. pneumoniae* carrying a functional *hu-meos3.2*, *cm^R* at its native locus as only copy were immobilized on an acrylamide patch with PBS and subsequently imaged using a 405 laser for activation and a 568 laser for excitation (see Materials & Methods). 5000 images were taken for each PALM sequence, leading to an imaging time of ten minutes per experiment. To test if the cells were still viable after PALM imaging, a PALM experiment was carried out with cells on an

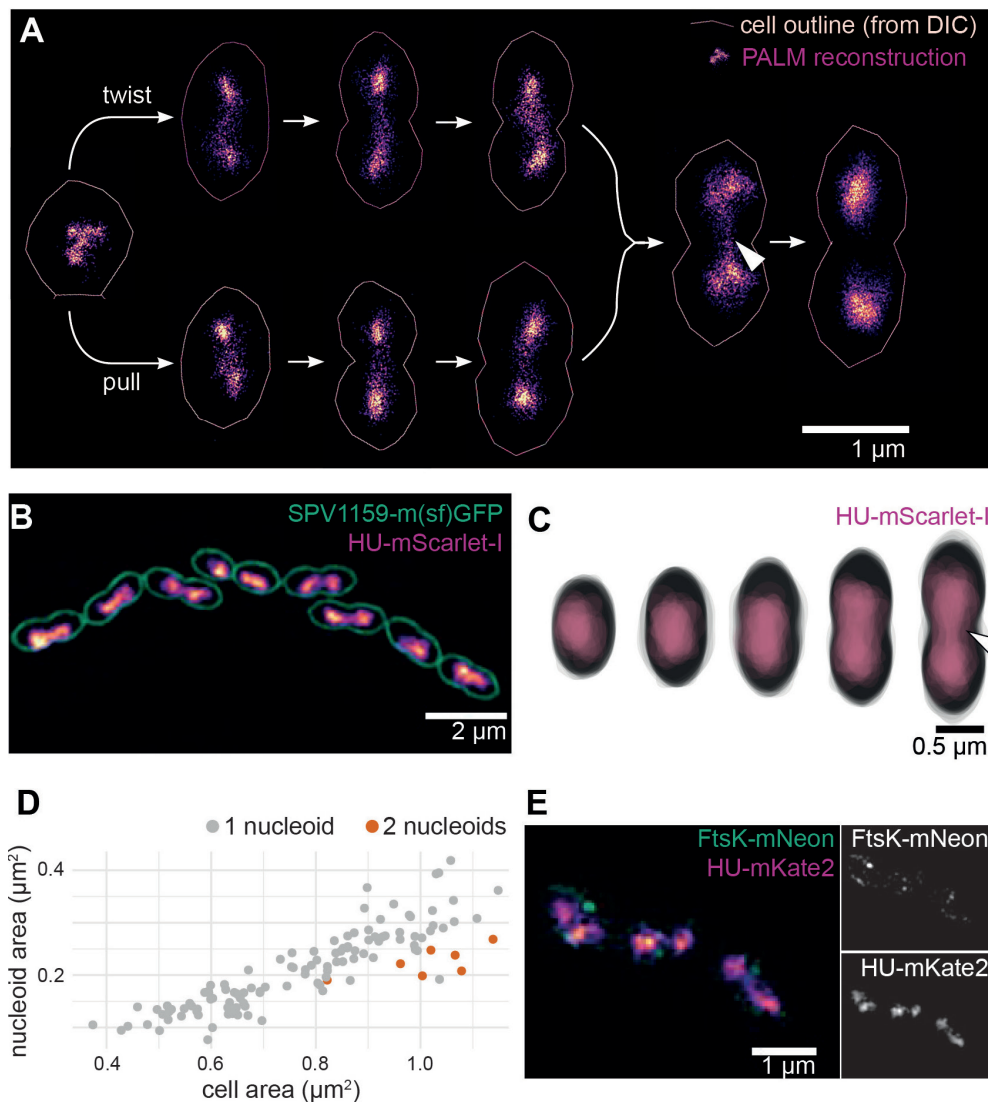


Figure 4. Superresolution microscopy analysis of nucleoid shape and cell measurements. **A.** PALM reconstructions of HU-meos3.2 representative for six cell size groups show helical nucleoids ('twist') and non-helical nucleoids ('pull'). Arrowhead points to the thinner nucleoid region between the larger nucleoid domains (average width 163 ± 45 nm). Cell outlines drawn by hand from DIC outlines. **B-D.** Analysis from SIM imaging of HU-mScarlet-I and membrane-bound protein SPV1159-(sf)GFP. **B.** SIM reconstruction. **C.** Cell projections from segmented nucleoids and membranes, both segmented using morphometrics (Ursell *et al.*, 2017). **D.** Nucleoid to cell ratio. Only a small fraction of the cells has two separate nucleoids. **E.** SIM reconstruction of pneumococcus carrying *hu::hu-hu-mKate2; bgaA::P_{loc}-ftsK-mNeon; prs1::lacI* induced with 1 mM IPTG shows FtsK foci at the septum where the chromosomes are segregating.

acrylamide patch with C+Y medium. After PALM imaging, a time-lapse experiment was started, imaging the cells every 5 minutes for 5 hours. While most cells grow and divide after PALM imaging, 30% of the cells were too severely damaged to grow. This, and the fact that 10 minutes is a considerably long time when imaging a potentially dynamic structure, is important to keep in mind when looking at the results.

In total, 160 pneumococcal cells carrying *hu-meos3.2* were imaged, with a Nyquist resolution of 18 nm and an average number of 3.990 ± 2.730 HU dimers per cell. Taking into account that the average number of HU dimers per cell as estimated before (Ferrándiz *et al.*, 2016) is nearly ten times as large (32.000 dimers/cell), many HU-meos3.2 dimers were missed, probably due to incomplete meos3.2 maturation, bleaching or being too close to other HU-meos3.2 dimers.

The images of pneumococcal cells were grouped in six groups based on cell length as measured from DIC images taken along with each PALM reconstruction. Representatives of the six groups are shown in Figure 3A. In the three mid-sized groups (groups 2-4), we observed two different nucleoid morphologies. In 80% of the cells, the nucleoid consisted of two domains located opposed to each other, with a less dense region connecting them, as if the two segregated chromosomes are pulled apart (Figure 4A, bottom). In the other 20% of the cells, the two dense domains are forming a helical shape together (Figure 3A, top), as observed previously for nucleoids of *E. coli* and *B. subtilis* (Berlitzky *et al.*, 2008; Fisher *et al.*, 2013b; Jung & Ha, 2019; F. Wu, Swain, *et al.*, 2019). Interestingly, in the fifth group, the region between the dense domains of the nucleoid became a thin line of 163 ± 45 nm. This possibly reflects the point in cell division just before replication termination, where the last part of the chromosome is channeled through the closing septum by the DNA translocase FtsK (Jean, Rutherford & Löwe, 2019).

Using DIC images, it is difficult to estimate the boundaries of the cell. Therefore, to confirm the observed nucleoid morphologies and cell cycle stages, we used 3D SIM (Structured Illumination Microscopy) to co-image HU-mScarlet-I (RFP) with SPV1159, a small membrane protein, bound to GFP under a constitutive promoter (*hu::hu-hu-mScarlet-I; cep::P3-spv_1159-(sf)GFP*). With SIM, the resolution is considerably lower (~ 100 nm in x/y direction) than PALM, but SIM has the advantage that it needs less images and imaging time: a typical two-color 3D experiment takes ~ 300 images in a total time of ~ 3 seconds. Because the Z-resolution of a SIM experiment is ~ 250 nm and the cell widths as we measured previously with phase-contrast microscopy are between 0.5 and 1 μm (Figure S1), we decided to use only the 2D images in the middle of each reconstructed Z-stack for our analysis.

Moreover, the membrane marker (SPV_1159-GFP) allowed for a precise measurement of the cell shape and determination of septum closure. Compared to phase-contrast microscopy, cells are estimated to be nearly twice as small when measured from SIM images. To confirm that the measured cell sizes are not a SIM artefact, we measured cell lengths and widths in electron micrographs (wild type D39V, images taken previously for Gallay *et al.*, 2019). This indeed showed that strain D39V, grown exponentially in C+Y medium at 37°C, are on average 1.3 μm in length (pole to pole) when measured from EM pictures taking the membrane as point of reference. In SIM images, the average cell length is closer to the EM measurements with 1.4 μm compared to 1.8 μm as observed by using phase contrast images. The difference in measured nucleoid size between wide-field fluorescence microscopy and SIM is even larger (Figure S3). These differences in measurement, however, seem to be consistent, leading to a lower but constant N/C ratio of 0.25 (Figure 3D). Figure 4D also shows that in almost all cells, even the nearly divided ones, there is only one nucleoid present.

The SIM reconstructions of the nucleoids showed similar morphologies compared to the PALM reconstructions, with helical morphologies as well as nearly segregated nucleoids connected by a thin region close to the septum (Figure 3B). A cell projection of different cell size groups also shows that even in the largest cell group, the two chromosomes are connected (Figure 4C,

arrowhead), something we could not observe in the projections from widefield fluorescence microscopy (Figure 2D). We wondered if this thin region of connected DNA coincides with the final stages of cell division. To test this, we constructed an ectopically inducible fusion of FtsK to mNeogreen and introduced this construct into a strain carrying HU fused to mKate2. As shown in Figure 4E, this thin nucleoid region indeed colocalized with FtsK. With the image resolution obtained with SIM combined with precise segmentation due to the membrane localization, we can conclude that the final stage of chromosome segregation and septum closure happen simultaneously and that FtsK plays an important role in this.

The nucleoid and HU are dynamic

Time-lapse experiments using both wide-field fluorescence microscopy and SIM showed the ‘pull’ and ‘twist’ morphologies observed by PALM are rather transient, following each other up, as the nucleoid is a dynamic structure. While the global nucleoid moves outwards in bulk during segregation (Figure 5B), time-lapse SIM shows that the shape of the nucleoid is changing constantly (Figure 5A) even within 1 minute time intervals (Figure 5B). Time-lapse SIM of HU- (sf)GFP shows a remarkable level of detail. Here, substructures and domains resembling chromosome arms are visible (Figure 5A, 30 and 40 minutes). Unfortunately, no other fluorescently reported tested were abundant and photostable enough to use for co-imaging, making it impossible to do a time-lapse SIM of HU together with membrane protein SPV_1159 or ParB/*parS* bound to known chromosomal loci.

To see whether HU stays bound to a region for a long time or is rather mobile, we used PALM imaging to determine the mobility of HU. As shown in Figure 5C, HU-meos3.2 is relatively mobile with a D_{app} of $5.3 \cdot 10^{-2} \mu\text{m}^2\text{s}^{-1}$ measured over short time intervals (< 0.5 s). This is more than 20-fold more mobile than ParB_{*lactis*}-GFP bound to a single *parS*_{*lactis*}-site at the origin of replication, which we measured previously using TIRF microscopy (van Raaphorst *et al.*, 2017), and 10-fold more mobile than DnaX-GFP, however, more confined than free diffusing GFP (Eldar & Elowitz, 2010). This means that when using HU to study the dynamics of the nucleoid at short time intervals (seconds), one has to take into account that HU is probably moving independently of the chromosome, since the speeds measured are faster than those of the chromosomal loci we tested previously.

Conclusion

While HU is the only known NAP in *S. pneumoniae*, it is not singlehandedly responsible for the shaping and compaction of the chromosome. Even when removing HU proteins, many cells still manage to have compacted, segregating nucleoids. One explanation could be that removing HU with a degradation tag is just not efficient enough. While this needs to be confirmed by Western blotting, it is certainly conceivable that the expression of HU is so high that it is impossible to degrade everything. However, as Ferrándiz *et al.*, 2018 showed, depletion mutants of HU had a comparable phenotype. To rule out that this phenotype is also due to residual HU or leaky expression, one could try a combination approach of either depletion and degradation, or one combined with Crispr interference.

Given that SMC, ParB and RocS deletions all have a similar phenotype as HU degradation, it would not be surprising that even fully deleting HU gives a relatively mild phenotype. However, recent synthetic lethal screens in our laboratory (Morten Kjos and Julien Denereaz, unpublished) showed that HU is indeed synthetic lethal with SMC, ParB, and also FtsK. These findings are in favor of a model where ParB, SMC and HU are important for chromosome segregation, but maybe even more for maintenance of chromosome shaping and flexibility, which in turn facilitates proper chromosome segregation.

This model, with very late chromosome segregation when the chromosome shaping is perturbed by mutations or antibiotics, is in line with the current view that chromosome segregation is facilitated by being rod or tube-shaped. The pneumococcus is a small ovococcus which is nearly round upon the start of a new cell cycle. After peripheral peptidoglycan synthesis, the cell becomes ovococoid, which is the moment that most chromosomes segregate even when normal segregation is perturbed.

Of course, apart from the influences of the geometry of the cell on chromosome segregation, the essentiality of FtsK in the pneumococcus, in contrast to for instance *B. subtilis* where FtsK is not essential (Koo *et al.*, 2017), also suggests that regulation of the final stage is necessary in the pneumococcus. Using PALM and 3D-SIM, it was possible to distinguish a thin nucleoid region between nearly segregating chromosomes and FtsK-mNeon colocalizes with these thin regions.

Combining different microscopy techniques can be very powerful to study both the shape and the dynamics of the chromosome. The chromosome of pneumococcus is a very dynamic structure. While helical shapes are observed throughout the whole cell cycle, it seems that the pneumococcal chromosome alternates between twisting (helical shapes) and pulling (more straight shapes). Since this is observed in many bacteria, this is most probably not an active process, but rather a physical property of the segregating and replicating nucleoid itself.

5.

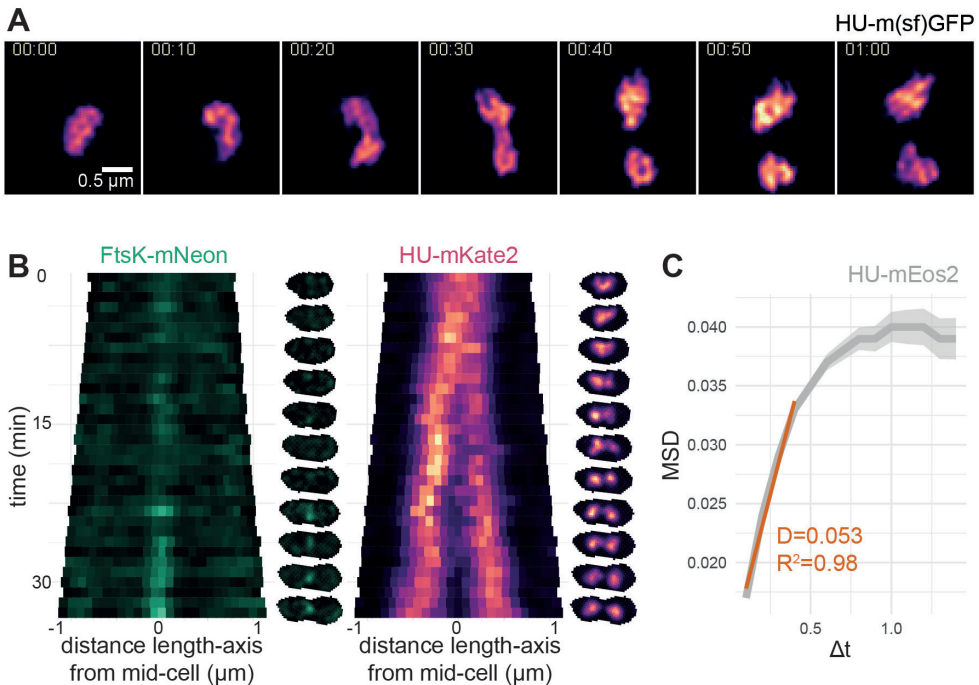


Figure 5. The nucleoid and HU are dynamic. **A.** Cutout of a single cell expressing HU-(sf)GFP. Mid-section of 3D-SIM experiment, imaged every 10 minutes. **B.** Kymograph of a representative single cell carrying *bgaA::P_{lac}-ftsK-mNeon*; *hu::hu-hu-mKate2*. Induced with 1 mM IPTG. Time-lapse wide-field microscopy experiment, imaged every minute. Right to kymographs: cell tower showing the fluorescence signal of the same cell as the kymograph, every 4th image frame. **C.** Mean Square Displacement (MSD) over time of HU-meos3.2. HU-meos3.2 recorded using PALM (same experiment as Figure 3A), particles were tracked and collective MSD was calculated using iSBatch (Caldas *et al.*, 2015). Apparent diffusion coefficient for the short time intervals was calculated by determining the linear slope between 0-0.5 s. Shade = standard error.

Now that we have a working tool for imaging the chromosome over time at a high resolution, it is time to use this technique to examine in high detail the different locations on the chromosome. Can we distinguish subdomains and chromosome arms at this resolution? One of the greatest challenges for this to work is to find a fluorescent protein in the red spectrum stable enough to do these type of colocalization experiments, but it is possible to get started without a time resolution. Alternatively, the use of clickable bright dyes such as the Janelia dyes to genetic tags, such as HALO and SNAP might be a viable approach compared to the use of fluorescent proteins.

Finally, having a membrane marker so precise that we know exactly which cells are separated and which are not, might change statistics on cell sizes in mutants and cells in different conditions. It can be interesting and possibly important to see whether the measurements done on phase-contrast images are reflecting the actual cellular processes at play.

To conclude, HU is important for the maintenance of chromosome shaping and flexibility. The nucleoid itself is a very dynamic and a flexible structure, while HU is very mobile and binds aspecifically to the whole chromosome. As SMC and ParB, the contribution of HU to chromosome shaping is important for proper chromosome segregation.

Materials & Methods

Strain Construction

Table 1. Strains and Plasmids used in this study

Strain/Plasmid	Description	Reference
<i>Streptococcus pneumoniae</i>		
VL1	<i>S. pneumoniae</i> D39V	Slager <i>et al.</i> , 2018
VL1449 (MK108)	D39V, <i>hu::hu-meos3.2-cm^R</i>	This study
VL358 (RR01)	D39V, <i>hu::hu-meos3.2-cm^R</i> , <i>cps2E::kan^R</i>	This study
VL2226 (JWV500)	D39V, <i>hu::hu-(sf)GFP-cm^R</i>	Kjos <i>et al.</i> , 2015
VL1800	D39V, <i>hu::hu-hu-mScarlet-l-cm^R</i> , <i>cep::P3-spv1159-(sf)GFP-spc^R</i>	Kurushima and Veening, unpublished
VL426 (RR139)	D39V, <i>hu::hu-hu-mKate2-cm^R</i> , <i>bgaA::P_{lac}-ftsK-mNeon-tet^R</i> , <i>psrA::lacI-gent^R</i>	This study
VL440 (MK119)	D39V, <i>hu::hu-hu-mKate2-cm^R</i>	Kjos <i>et al.</i> , 2015
JaS01	D39V, <i>hu::hu-degron-cm^R</i> , <i>bgaA::P_{Zn}-sspB-spc^R</i>	This study
JaS02	D39V, <i>hu::hu-mCherry-degron-cm^R</i> , <i>bgaA::P_{Zn}-sspB-spc^R</i>	This study
VL447 (KB04-28)	D39V, <i>bgaA::P_{Zn}-sspB-tet^R</i> , <i>cep::P32-(sf)GFP-degron, spc^R</i>	This study
VL382 (RR67)	D39V, <i>hu::hu-hu-mKate2-cm^R</i> , <i>dnaX-(sf)GFP-ery^R</i>	van Raaphorst <i>et al.</i> , 2017
VL396 (RR85)	D39V, <i>hu::hu-hu-mKate2-cm^R</i> , <i>dnaX-(sf)GFP-ery^R</i> , <i>smc::trmp^R</i>	This study
VL3056 (PDU-20)	D39V, <i>RpoB-(sf)GFP-kan^R</i>	Utari and Veening, unpublished
VL486 (BW05)	D39V, <i>hu::hu-meos3.2-cm^R</i> , <i>cps2E::kan^R</i> , <i>smc::spc^R</i>	This study
Plasmids		
pRRneon iptG	<i>bgaA::P_{lac}-MCS-linker-mNeon-tet^R</i> ; <i>amp^R</i>	This study
pMK11	<i>bgaA::P_{Zn}-MCS-tet^R</i> ; <i>amp^R</i>	van Raaphorst <i>et al.</i> , 2017
pMK11-sspB_{EC}	<i>bgaA::P_{Zn}-sspB_{EC}-tet^R</i> ; <i>amp</i>	This study
pPEPx	<i>cep::spc^R</i>	Sorg, Kuipers & Veening, 2014

VL1449: *hu::hu-meos3.3-cm^R*, VL358: *hu::hu-meos3.2-cm^R*, *cps2E::kan^R* and VL486: *hu::hu-meos3.2-cm^R*, *cps2E::kan^R*, *smc::trmp^R*
hlpA-up was amplified from *S. pneumoniae* D39V using OVL43 and *hlpA-R*-BamHI. *camR-down* was amplified from VL440 using *camR-up-F* and OVL46. *mEos3.2* was amplified from pNZ-MS2S(D)-*mEos3.2* (Lieke van Gijtenbeek) using *mk77* and *mk78*. The fragments were digested with BamHI, EcoRI and BamHI/EcoRI, respectively, ligated and transformed in *S. pneumoniae* D39V, resulting in strain VL1449. *cps2E::kan^R* was amplified from strain MK127 (Kjos *et al.*, 2015) using primers JWV46 and *rpsL-R*. The product was transformed into strain MK1449, resulting in VL358. *Smc::trmp^R* was amplified from AM39 (Minnen *et al.*, 2011) using primers MT10 and MT13. The PCR product was transformed in strain VL1449, resulting in VL486.

pRRneon_iptG and VL426: *hu::hu-hu-mKate2-cm^R*, *bgaA::P_{lac}-ftsK-mNeon-ter^R*, *psrA::lacI-gent^R*
hu::hu-hu-mKate2-cm^R was amplified from strain VL440 (Kjos & Veening, 2014) using primers OVL43 and JWV60. The PCR product was directly transformed in D39V carrying *psrA::lacI-gent^R* (Sorg, 2016). To make *bgaA::P_{lac}-ftsK-mNeon-ter^R*, *ftsK* was amplified from *Streptococcus pneumoniae* D39 using primers pr36 and pr37. *linker-mNeon* was amplified from pUC-mNeongreen (GenScript) using primers RR162 and RR163. The resulting PCR product was restricted with EcorI and NheI and plasmid pMK11 with EcorI and SpeI. After ligation, the mixture was transformed in *E. coli* dh5 α . The plasmid was purified and cut with EcorI and KpnI, while *PiptG* was amplified from pPEPX-plac (Sorg *et al.*, 2014) using primers BW01 and BW02 and subsequently restricted with the same enzymes. The two parts were ligated and transformed into *E. coli* dh5 α and the plasmid was subsequently purified, resulting in plasmid pRRneon_iptG. This plasmid was cut with XbaI and NotI, while *ftsK* was cut with NotI and SpeI. The parts were ligated overnight and directly transformed into the strain carrying *hu-hu-mKate2-cm^R* and *psr1::lacI-gent^R*, resulting in strain RR139.

Table 2. Oligos used in this study

Primer	Sequence
OVL43	AACAAGTCAGCCACCTGTAG
OVL46	AACAAGTCAGCCACCTGTAG
OVL1573	CGGTCTCGTAACCTTAGGATGCGTCAGCATAATTTTCGCTATAATTTTCATCA TTGCAGCTTTAACAGCGTCTTTAAGAGC
OVL1574	CGGTCTCGTAACCTTAGGATGCGTCAGCATAATTTTCGCTATAATTTTCATCAT TTGCAGCTTTGTAAAGCTCATCCATTC
OVL625	GACGATGGTCTCCGTTAATAATAAGAATTCTAATGAGCACTAGT
Pr36	GAGCGCGGCCGCTTATGGTTACCTACGCAAACCCAC
Pr37	CGCCACTAGTTTGTGTGAACACTTTTCGAGGTTTG
MT10	ACAACACCCTCGTCTGAAAG
MT13	CAGGTCTTGGTGTGCTATG
JWV46	GGCCAATCAGGTTTCAGATAC
rpsL-R	AGCCGATATGAGCTGGAACGGGATG
RR162	GCGAATTCGGATCCGCGGCCGCGAGATCTACTAGTTCTAGAGGATCTGGTG GAGAAGCTGCAGCTAAAGCTGTCTCTAAAGGTGAAGAAG
RR163	GCGCTAGCTTATTATACAATTCATCCATAC
BW01	CGGGTACCCTAGTAATTGTGAGGCGGATAAC
BW02	CGGAATTCAATTGTGAGCGCTACAATTAG
sspB-F+RBS+EcoRI	GCGCGAATTCAGGAGGCAAATATGGATTTGTACAGCTAACACC
sspB-R+SpeI	GCGCACTAGTTTACTTCACAACGCGTAATGC
KB02-F-BamHI	GCGCAGGATCCATTGATATCATATTC
GFPsf-ssrA-ALGG-F+SpeI	GCGCACTAGTTTAGCCGCCAAGAGCATAATTTTCGCTATAATTTTCATCATTT GCAGCTTTATAAAGTTTCGTCCATACCGTGAG
P32-F-BglII	GCGCAGATCTGGCCGGCCGATATGATAAG
ssrA-DAS4-R+XhoI	GCGCCTCGAGTTAGGATGCGTCAGCATAATTTTCGCTATAATTTTC
hlpA-R-BamHI	CTGCGGATCCTTTAACAGCGTCTTTAAGAGCTTTACCAGC
camR-up-F	ACTCGGAATCTAATGAGCACTAGTAGGAGGCATATC
mk77	CGATGGATCCGGATCTGGTGGAAGCTGCAGCTAAAGGATCA AGTGCG ATTAAGCCAGACATGAAG
mk78	ACTCG GAATTC TTATCGTCTGGCATTGTCTAGGCAATC

pMK11-ssb_{EC} and VL447: *bgaA::P_{Zn}-ssbB-tet^R, cep::P32-(sf)GFP-deg^R, spc^R*
 To construct pMK11-ssb_{EC} *ssbB* was amplified from *E. coli* K12 using primers *ssbB*-F+RBS+EcoRI and *ssbB*-R+SpeI, and the PCR product and pMK11 were restricted using EcoRI and SpeI. pMK11 and *ssbB* were ligated and transformed in *E. coli* dh5 α , resulting in pMK11-ssb_{EC}. *gfp-ssrA_{EC}-ALGG* was amplified from KB01-*P₃₂-(sf)GFP* (pPEPx-*P₃₂-(sf)GFP*, Veening Lab collection) using primers KB02-F-BamHI and GFPsf-ssrA-ALGG-F+SpeI and subsequently restricted with BamHI and SpeI and ligated into pPEPx. The ligation product KB04-21 was amplified in *E. coli*. Subsequently, *gfp-ssrA_{EC}-DAS+4* was amplified from KB04-21 using primers P32-F-BglII and ssrA-DAS4-R+XhoI, subsequently restricted using XhoI and BglII and ligated into pPEPx, resulting in pPEPx-*gfp-ssrA_{EC}-DAS+4*. The product was directly transformed in *S. pneumoniae* D39V, and subsequently, pMK11-ssb_{EC} was transformed in the resulting strain, creating the final strain VL447.

JaS01: *hu::hu-deg^R-cm^R, bgaA::P_{Zn}-ssbB-tet^R* and JaS02: *hu::hu-mCherry-deg^R-cm^R, bgaA-P_{Zn}-ssbB-tet^R*

Genomic DNA from strain VL447 was transformed in *S. pneumoniae* D39V, selecting for tetracyclin. Proper insertion of *bgaA::P_{Zn}-ssbB-tet^R* and absence of *cep::P32-(sf)GFP-deg^R, spc^R* were checked for by sequencing. *Upstream-hu-deg^R* was amplified from the *Streptococcus pneumoniae* D39 genome using primers OVL43 and OVL1573, *upstream-hu-mcherry-deg^R* was amplified from the genome of strain VL1559 using primers OVL43 and OVL1574, and *cm^R-downstream* was amplified from strain JWV500 using primers OVL625 and OVL46. All PCR products were restricted with BsaI, and *Upstream-hu-deg^R* and *upstream-hu-mcherry-deg^R* were both ligated to *cm^R-downstream*. The resulting mixtures were transformed directly in the intermediate strain carrying *bgaA::P_{Zn}-ssbB-tet^R*, resulting in strains JS01 and JS02.

VL369: *hu::hu-hu-mKate2-cm^R, dnaX::dnaX-(sf)GFP-ery^R, smc::trmp^R*
Smc::trmp^R was amplified from strain AM39 (Minnen *et al.*, 2011) using primers MT10 and MT13. The PCR product was transformed in strain VL382 (van Raaphorst *et al.*, 2017), resulting in strain VL369.

Growth conditions

Pneumococcal cells were grown without shaking at 37°C in C+Y medium (Domenech, Slager & Veening, 2018). When inducing expression of *P_{Zn}*, C + Y medium supplemented with 100 μ M ZnCl₂ + 10 μ M MnCl₂ was used. For *P_{lac}* expression, C+Y medium supplemented with 1 mM IPTG was used. For growth in permissive concentrations of antibiotics, 0.15 μ g/mL of HPURa and 0.4 μ g/mL ciprofloxacin was used. For cloning, cells were plated on Columbia Blood Agar containing the appropriate antibiotics.

Wide-Field Microscopy

Cells were grown up to an OD of 0.2, diluted 100 times and allowed to regrow for 1 hour. For snap shots, cells were spun down and spotted on microscopy slides covered with 0.4 % agarose in PBS.

For time lapse microscopy, cells were spotted on a pre-heated 10% polyacrylamide slide containing C+Y medium (as described in (van Raaphorst *et al.*, 2017)). The slide was left in the pre-heated (37°C) microscopy chamber for 30 minutes to allow it to adjust to the temperature. After this, three spots were imaged every minute for 4 hours.

Cells shown and analyzed for Figures 1, 2, S1 where acquired on a Leica DMi8 microscope with a DFC9000 GT camera, using a 100x/1.40 NA phase-contrast objective (Leica). Images were

acquired using a Lumencor SpectraX light engine with the following filters: mKate2/mCherry: SpectraX-Quad cube (Chroma #89000) ex: 575/35, em: 595/40, GFP/syto-13: SpectraX-Quad cube (Chroma #89000) ex: 470/24, em: 515/40.

Cells shown and analyzed for Figures 3 where acquired on a Deltavision Elite microscope (GE Healthcare) with a 100x/1.42 NA phase-contrast objective (Olympus) using a sCMOS camera and SSI Solid State Illumination. mKate2 was acquired with a Chroma filter set (ex: 562/588 nm, em: 602-548 nm, Quad/mCherry polychroic). mNeongreen and (sf)GFP images were acquired using a Chroma filter set (ex: 461-489 nm, em: 463-487 nm, Quad polychroic). Images were acquired using Softworx (Applied Precision) and converted to TIFF files using FIJI (Schindelin *et al.*, 2012).

SIM Microscopy

Samples were prepared as for time-lapse wide field microscopy, with the exception that for snap shots, the C+Y medium was substituted for PBS, and a high-precision 17 μ m coverslip was used (VWR). 3D SIM experiments were performed on a Deltavision OMX SR (GE Healthcare) microscope equipped with 488 nm and 568 nm lasers. 9-12 Z-stacks of 125 nm distance were acquired in 3D-SIM mode. For time lapse imaging, a 3D-stack was taken every 10 minutes, while a constant temperature of 37°C was maintained. Reconstruction and channel alignment was done in SoftworX (GE Healthcare) with 'force modulation amplitude' checked on and furthermore standard settings.

PALM Microscopy

PALM microscopy was performed on a Deltavision Elite microscope equipped with DLM (Deltavision Localization Microcopy), using a 60x oil 1.49 NA TIRF objective (Olympus), 405 nm, 588 and 568 nm lasers, a sCMOS camera. PALM imaging was done in Hi-Lo mode at 37°C. Cells were prepared as for Wide-Field time-lapse microscopy, where C+Y medium was substituted with PBS. Before each acquisition, a green fluorescence image (excitation: 488 nm laser, emission 525/50 nm) and DIC image of the cells was made. After this, PALM images were acquired in the following sequence: activation (405 nm) – 4x red excitation (568 nm), while red emission (632/60 nm) was recorded. The activation power was set to very low in the beginning of the acquisition and manually increased over time. 5000 images were taken for each PALM acquisition.

PALM analysis

The open-source ImageJ plugin Thunderstorm (Ovesný, Křížek, Borkovec, Svindrych & Hagen, 2014) was used to generate the PALM reconstructions. In short, the images were filtered using a wavelet filter (B-Spline, order 3, scale 2.0). The local maximum method (threshold: std(Wave.F1), connectivity: 8-neighbourhood) was used to approximate the localization of the molecules. The following settings were used for sub-pixel localization: PSF: Integrated Gaussian; fitting radius: 3; Maximum likelihood fitting; initial sigma: 6; enabled multi-emitter fitting analysis; max. 5 molecules per fitting region, the same intensity for all molecules and a model selection threshold of $p=1 \times 10^{-6}$. The Base level (A/D counts) was determined by taking the minimal value of the image histogram for each individual PALM acquisition. After analysis, the results were filtered by removing too low and high intensities, applying a density filter, removing duplicates, merging molecules and applying drift corrections. Finally, the PALM localizations were visualized using the averaged shifted histogram method. In order to place the reconstructed nucleoid images correctly on the DIC images, Fiji (Schindelin *et al.*, 2012) was used to overlay the 488nm image of HU-meos3.2 and the PALM reconstruction.

Particle tracking

To determine the mean square displacement (MSD) of HU-meos3.2, iSBatch (Caldas *et al.*, 2015) was used to track HU-meos3.2 in the images acquired using PALM. For this, firstly the regions containing the cells were selected in imageJ, after which the Peak Fitter was used to find the fluorescent spots. Using the Particle Tracker, allowing for distances traveled not longer than 4 pixels/frame, only the spots appearing in multiple frames were kept. Tracks longer than 15 frames were removed. Finally, the collective MSD was calculated. This procedure was repeated for 10 PALM movies (25 cells). All MSD tables were collected and plotted in R.

Cell and nucleoid segmentation and analysis

Cells and nucleoids imaged using SIM were segmented using Morphometrics (Ursell *et al.*, 2017). Cells and nucleoids imaged using phase-contrast / wide-field fluorescence microscopy were segmented using Oufiti (Paintdakhi *et al.*, 2016), where the nucleoids were segmented in object detection mode. To determine the cell and nucleoids dimensions in the PALM experiments, cell outlines were drawn by hand three times from DIC images (cells) and PALM reconstructions (nucleoids) using FIJI (Schindelin *et al.*, 2012) and subsequently averaged.

5.

After segmentation, the wide-field and SIM microscopy data was analyzed and visualized in R (<https://cran.r-project.org/>) using the R packages BactMAP (van Raaphorst, Kjos, and Veening 2019) and ggplot2 (Wickham, 2016). To determine nucleoid size, dimensions and intensity, firstly, the intensities from the TIFF values were added to the segmented nucleoids using BactMAP::extr_OriginalCells(). This returned the dimensions and size of the nucleoids and a list of pixel values per nucleoid. Of these values, the median and standard deviation was determined. The cell dimensions were imported from the Oufiti or Morphometrics segmentations.

ChIP-Seq

Protocol adjusted from (last chip paper from Gruber lab). Cells were grown in 2 mL of C+Y, 37°C up to $OD_{600} \sim 0.4$, diluted 50 times in 30 mL of pre-heated C+Y (37°C) and allowed to grow up to an OD_{600} of 0.2. The cells were harvested and fixated in 1% formaldehyde. The cells were washed several times in ice-cold PBS and the pellets were snap-frozen and stored in -80°C. Subsequently, the cells were thawed and sheared in a Covaris S2 sonicator using the following protocol: 200 cycles, 100 W, 10% load, 12 min. A fraction of the cell mixture was taken apart (input fraction).

Polyclonal anti-GFP (ThermoFisher A-6455) was bound to magnetic beads (Dynabeads Protein G 10004D). The remaining sheared cells (IP fraction) were added to the beads and the mixture was incubated for two hours on a rotating wheel for two hours. After immunoprecipitation, the beads were washed. Cross-linking was reversed in both the input and IP fraction by shaking 1400 rpm overnight at 65°C in 1% SCS, 10 mM EDTA and 50 mM Tris (pH 8). The DNA was purified using phenol-chloroform extraction followed by a PCR purification kit (Qiagen QIAquick).

Libraries were prepared by the Lausanne Genomic Technologies Facility (LGTF) using an Ovation Ultralow V2-DNA-Seq library preparation kit (NuGEN) and 100 nt paired-end sequencing was done at the LGTF on an Illumina HiSeq.

ChIP-Seq Analysis

Sequence trimming, alignment and peak annotation was done using a snakemake pipeline (<https://github.com/veeninglab/chip-seq>). In short, sequence files were gunzipped and sequence quality

was assessed using fastqc (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Data was trimmed using Trimmomatic (Bolger, Lohse & Usadel, 2014) and sequences were aligned to the reference genome (D39V, GenBank CP027540) using Bowtie2 (Langmead & Salzberg, 2012) and converted to .Bam-files using SamTools (Li *et al.*, 2009) and filtered to remove false positive peaks due to duplicated regions in the chromosome using Sambamba (Tarasov, Vilella, Cuppen, Nijman & Prins, 2015). Peaks were annotated using MACS2 (Liu, 2014).

Sequence read depths were binned by 500 bp and normalized per sample. Relative enrichment was plotted in R by dividing the normalized input over the normalized IP. The relative enrichment of HU per binned region (1000 bps) was plotted against RpoB enrichment and GC content (0-1) per 1000 basepairs.

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Author contributions

RR, BW, JS and JWV designed the experiments. JS and RR performed ChIP-seq experiments and analysis. BW did the PALM experiments and reconstructions. KB developed the degron-tag system. RR performed experiments and RR, JS, BW and JWV analyzed the data. RR and JWV wrote the manuscript.

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Supplemental Figures

5.

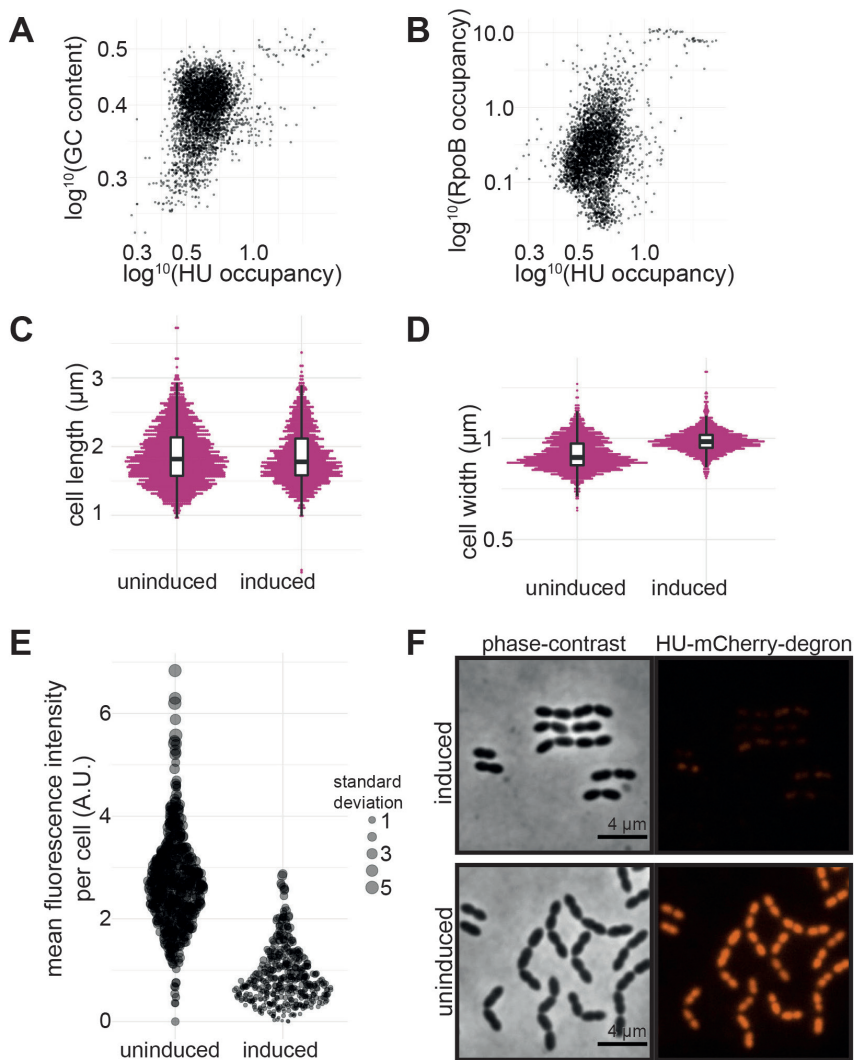


Figure S1. **A.** Correlation between HU occupancy (IP/input reads, normalized for total reads) and GC content. **B.** Correlation between RpoB occupancy and HU occupancy (IP/input reads, normalized for total reads). **C/D.** Cell lengths (C) and widths (D) of cells carrying *hu::hu-degtron* (strain JaS01) after four hours of induction with 0.1 mM ZnCl_2 . **E.** Mean fluorescence intensity (background corrected) per cell of HU-mCherry-degtron after four hours of induction with 0.1 mM ZnCl_2 . Each dot is one cell, the dot size represents the standard deviation of the mean fluorescence intensity. **F.** Cut-out of the images analyzed for (E). The same contrast settings were used for the fluorescence images. Scale bar: 4 μm .

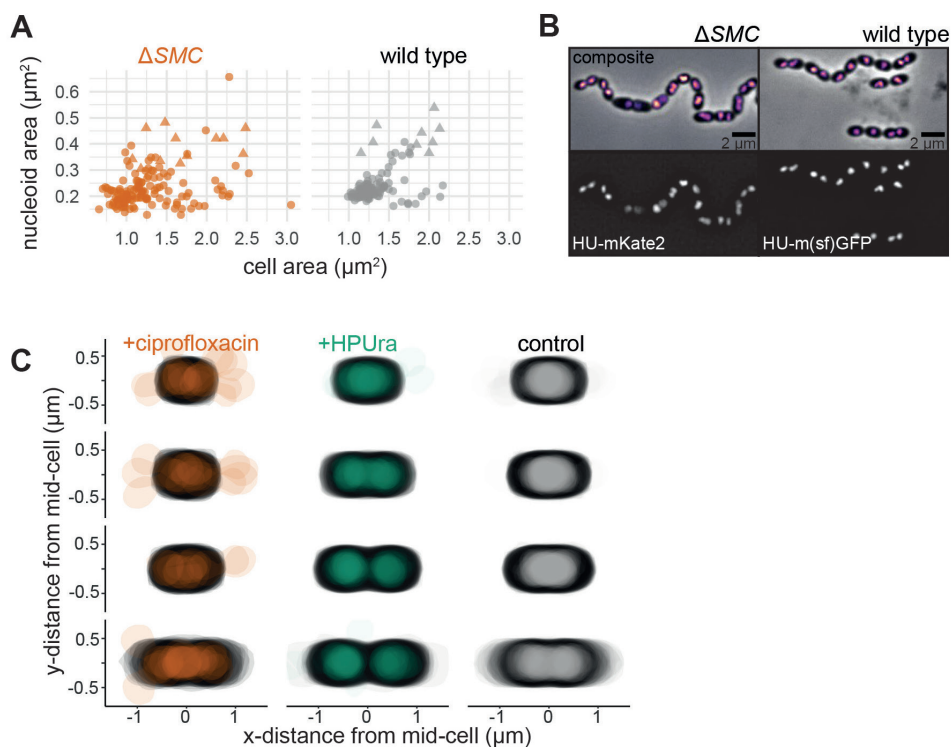


Figure S2. A/B. Comparison of nucleoid shape and N/C ratio in wild type cells and Δsmc mutants. **A.** N/C ratio in Δsmc cells (strain VL396) and WT cells (strain VL2226) carrying either hu-mKate2 or hu-(sf)GFP. **B.** Δsmc mutants are more irregularly shaped, have anucleate cells and the intensity of HU-FP is more variable. Scale bar: 2 μm . **C.** Cell projections (grouped by equally-sized groups based on cell length). Complementary analysis of experiment shown in Figure 3.

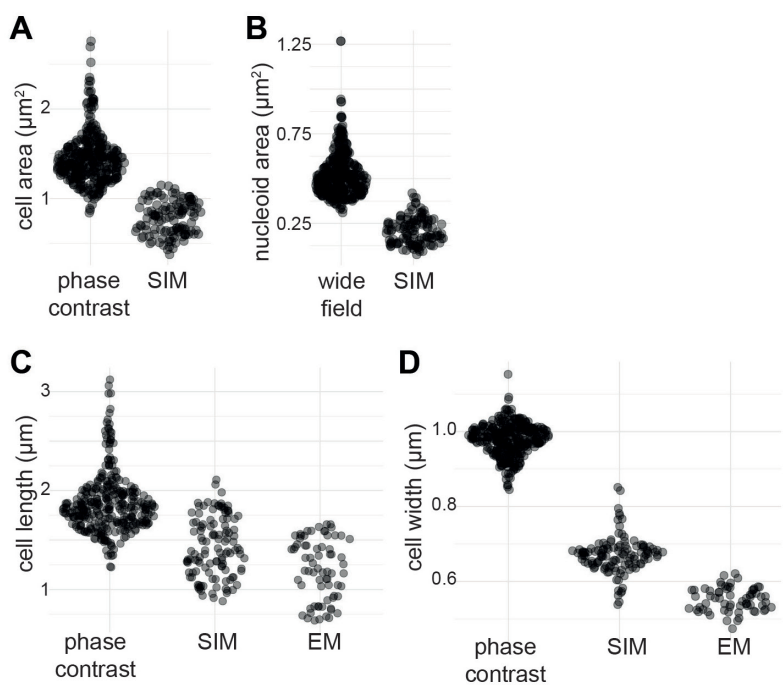
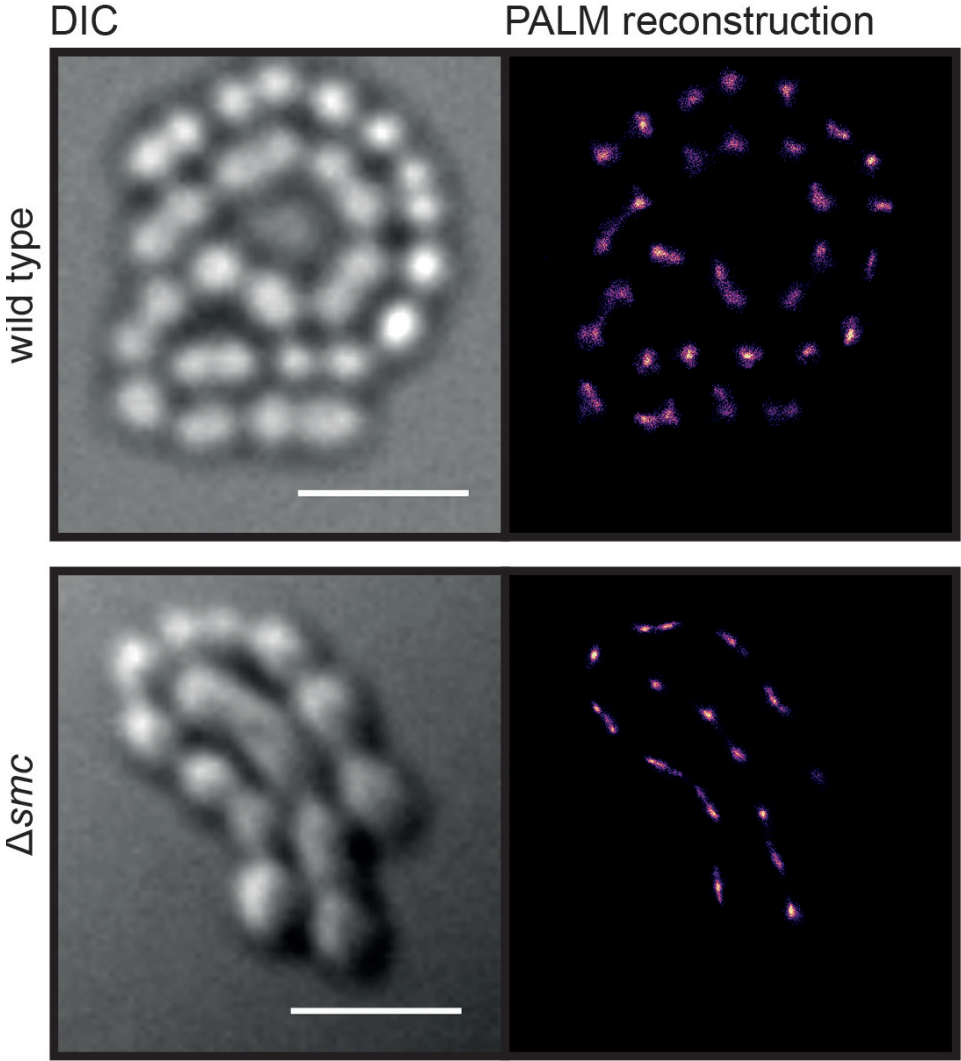


Figure S3. Comparison of cell area (A), length (C) & width (D) and nucleoid area (B) measured with SIM (SPV1159-(sf)GFP (cell) and HU-mScarlet-I (nucleoid) segmented with morphometrics) to phase contrast microscopy images of cells carrying HU-mKate2, segmented with Oufiti (Paintdakhi *et al.*, 2016), and Electron Micrographs of wild type D39V cells of which the cell width and length was measured by hand using Fiji (Schindelin *et al.*, 2012).



5.

Figure S4. PALM reconstruction and DIC images of strains VL358 (*hu::hu-meos3.2*) and VL486 (*hu::hu-meos3.2, Δsmc*) shows cells and nucleoids in different stages of the cell cycle. In Δsmc cells, there are less blinking events, leading to less clear nucleoid shapes. Scale bar: 3 μm .



Chapter 6:

General Discussion

This thesis presents the mapping of the pneumococcal chromosome cycle, as well as an exploration of the available tools for fluorescence microscopy and analysis of pneumococcal cells. In line with the order of the chapters, I will first discuss the technical advances and challenges related to this thesis, in the order as they appeared: from the assessment of fluorescent proteins, to analysis and visualization of microscopy data, to superresolution microscopy. After this, I will point out the major biological conclusions from the previous chapters and look ahead for future directions of research.

Benchmarking fluorescent proteins for pneumococcal cell biology

While bacterial cell biology has been studied for nearly a century, pneumococcal cell biology as a separate field of study just got started about ten years ago. Now, our lab and many others have a range of tools for visualizing the pneumococcal cell cycle at their disposal. In our lab alone, we have used and tried out a plethora of fluorescent proteins and continue to test the latest variants. There is a considerable turnover of new fluorescent proteins, since the news of new, promising proteins travels fast through preprints and social media. While the red fluorescent protein mScarlet-I is winning in popularity in our lab (see Keller, Rueff, Kurushima & Veening, 2019), the value of a systematic comparison of fluorescent proteins for different purposes still stands and might even be more important with a high turnover of new tools. Results like the partial degradation of mCherry fused to HU (HlpA) and FtsZ, or the slow maturation times of the other red fluorescent proteins show that there are several things to consider when designing experiments with fluorescence. Since the publication of chapter 2 (Beilharz, van Raaphorst, Kjos & Veening, 2015), we added a range of new proteins to our toolbox (for instance mNeonGreen, mScarlet-I, mTurquoise2, tagBFP2, spDendra and mEos3.2), and it might be wise to systematically compare their functionality for protein and promoter fusions to the previous benchmarked GFPs (Overkamp *et al.*, 2013) and RFPs.

Depicting reality

Similarly, it is necessary to be critical when looking at microscopy data. As fluorescent proteins have evolved, a lot has changed in the processing and analysis of the data as well over the last ten to twenty years. Since it is possible to do automated cell segmentation, statements on subcellular localization or cell morphology are generally backed up by measurements of hundreds to thousands of cells, providing insight in the heterogeneity of the population. This is a large improvement in the field. Probably every microbiologist has looked at a microscopy image thinking: “are these cells larger than wild type, or do I just think they are?” Automatic measurements can prevent bias.

However, it is dangerous to think of automatic segmentation or fluorescence detection as bias-free. Researchers have to check the segmentation process, set the parameters and manually edit the results when needed. This might not necessarily be a bad thing: to depict the biology, human eyes tend to be better than most segmentation programs (up to now). The differences in segmentation results in our laboratory were a striking and slightly alarming example of how not only different segmentation software, but also differences in decisions made by our lab members largely influenced the segmentation results (chapter 3). So what can we do to depict and quantify biology in the best possible way when doing cell measurements?

Supervised machine learning algorithms might be a solution for the problem of having different lab members choosing different parameters. Still, we would need to find a consensus of what

outline of a phase-contrast image reflects the real cell parameters the best. As shown in chapter 5, using SIM images of a membrane marker for cell measurements comes closer to reality and, in contrast to cell sizes measured by phase contrast imaging, cell sizes as determined by SIM highly correlate with transmission electron microscopy images (see chapter 5 of this thesis). The distribution of cell lengths is reflecting reality in a better way, not only because the cell outline is more clear, but also because it is possible to determine when the septum is closed. Not only do we seem to overestimate the cell widths proportionately when using phase contrast, we also do not estimate septum closing correctly (a problem highlighted earlier for *B. subtilis* in Syvertsson *et al.*, 2016). Using a membrane marker could therefore significantly improve the accuracy in cell segmentation data. When it is not possible to use another fluorescent marker, it is important to double-check data in other ways, for instance by using time-lapse microscopy. When this is not an option, using another marker – for instance FtsZ (chapter 4) – to know the point in the cell cycle of each single cell, can be a method to compare different conditions or mutants. Either way, it is important to be careful when interpreting data with a time component when it is impossible to directly measure over time. Figure 2.A from the introduction of this thesis is a good reminder for that.

Visualization Strategies

For the development of BactMAP (chapter 3), it was important to think about the way visualization influences the way people think about their data. I tried to make visualization strategies that show as much raw data as possible, because it is easy to forget about the noisiness of data when the noise is out of sight. While there can be a use for bar plots (for instance, chapter 3, Figure S1), it is better to avoid them when there is more information hiding behind the bars (see for instance Evanko, 2013).

Visualizations that are more difficult are the density-kymographs presented in chapter 4, since they show no raw data, but localization data. This is vastly improving the resolution of the localization, making it easier to follow noisy fluorescence data. Without plotting techniques like this, it would not have been possible to track the low-fluorescence signals obtained by labelling chromosome loci in live cells in chapter 4. On the other hand, ordering the cells by length gives the incorrect impression that you follow localization over time, while cell size distribution is influencing the shape of the graphs. In exponentially growing cells, the overall distribution is similar, but cell chaining, addition of Zn^{2+} , centrifugation of cells before microscopy and many other factors can potentially change the distribution of (measured) cell sizes. I anticipate that using more membrane markers and time-lapse microscopy will help reduce the effect of cell chaining on the final cell length distributions in the future. Until then, it is important to be critical on what a data visualization like a density plot can and cannot tell.

What I hope to include in a new version of BactMAP, are visualizations that take into account the single cell information available in each dataset. Instead of plotting grouped localizations based on cell size, we can visualize distances between proteins or groups of cells on the relative septal closure. Both MicrobeJ and SuperSegger have made great progress in the last years, providing software updates with options for grouping and filtering cells based on many parameters (Cass, Stylianidou, Kuwada, Traxler & Wiggins, 2017; Ducret *et al.*, 2016, see <https://microbej.com> for recent updates). The analysis on DnaX localization in chapter 3 already showed that there is large heterogeneity in the movement of the replisome and the timing of replication initiation. Using single cell information on for instance cell length and septum closure might help the understanding of the heterogeneity and, at the same time, make it easier to group cells appropriately. It becomes easier to build interactive visualizations in R with tools as Shiny (<https://shiny.rstudio.com>), Plotly (<https://plot.ly>) and D3 (<https://rstudio.github.io/r2d3/>). This will be of good use when exploring cell-to-cell heterogeneity using R.

Using superresolution microscopy to understand chromosomal shaping

In chapter 5, we use both PALM and SIM to image the pneumococcal chromosome. Rather than being interchangeable, these techniques complement each other to better understand the shaping and dynamics of the chromosome. Where PALM is useful for single-molecule dynamics, SIM can reveal the dynamics of the complete chromosome over a longer time span. The high resolution of the PALM reconstructions could reveal thin chromosome regions at a late cell cycle stage, while dual-color SIM could confirm that the septum is indeed near closing at this point.

Nevertheless, both SIM and PALM are technically challenging and prone to artefacts. In both cases, the preparation of the sample is the greatest bottleneck for a good image. In the pneumococcus, the signal intensity obtained from samples for fluorescence microscopy is generally not very high. Until now, only HU-mEos3.2 fusions led to enough blinking events for a reliable PALM reconstruction in our laboratory. Jaqc *et al.*, 2015 showed good results using the also highly abundant FtsZ protein fused to spDendra. For SIM imaging, the greatest problem is the stability of the red fluorescent proteins when doing dual color imaging, even using the latest mScarlet-I. However, the stability of (sf)GFP and mNeonGreen in the pneumococcus is sufficient. With this, SIM could become a routine technique for localization in the pneumococcus. Note that it will always be an addition and not a replacement to wide-field fluorescence microscopy, where samples are easier to prepare and the phototoxicity is much lower.

6.

From tools to insight

The benchmarking of fluorescent proteins, development of microscopy analysis tools and assessment of superresolution techniques together form a powerful toolset for a better understanding of the pneumococcal chromosome cycle. To discuss the major biological conclusions of this thesis, I will go up in scale: from the localization of DNA replication and the chromosomal origin towards the global organization of the chromosome. Finally, I will describe the order of events in the pneumococcal chromosome cycle (see Figure 1).

Replication at the new septum

To understand the dynamics of the replication fork, we had to look beyond bulk imaging and into single cell measurements. While bulk images in chapter 4 showed unspecific replisome localization, time-lapse imaging showed that the replisome was moving in a zone of the cell that also accommodates the Z-ring. Finally, in chapter 3, clustering analysis and colocalization with FtsZ made clear that replication indeed initiates at the Z-ring position. It would be good to repeat the localization of the replication fork using SIM, to pinpoint the timing of replication termination and initiation compared to septum closure.

The observation that there is barely a moment in the pneumococcal cell cycle where the replisome is completely dissociated is consistent with the ori-ter ratio of pneumococcal cells of 1.4-1.8 in optimal growth conditions: the pneumococcus is literally replicating its DNA, segregating its chromosome and building its cell wall at the same time. The recent findings from our lab that replication firing through DnaA is connected to the formation of the Z ring (Gallay *et al.*, 2019) fits the observations in this thesis. At the same time, modeling and experimental studies on the regulation of cell size homeostasis also puts DNA replication as key in cell size control in bacteria (Si *et al.*, 2019).

The replisome is mislocalized in *smc* mutants and cells treated with ciprofloxacin, where the replisome is still very dynamic, but not confined to mid-cell. This is similar to experiments in *B. subtilis* where they showed that the replication forks in decondensed nucleoids are not as confined to mid-cell anymore (Mangiameli, Veit, Merrikh & Wiggins, 2017). While the phenotype of *smc* mutants in the pneumococcus is leading to anucleate cells and mislocalized chromosomes, the replisome mislocalization during replication does not seem to have great influence on the cell cycle.

Until now, it has been technically very challenging to follow replication initiation in the pneumococcus because of the fast replication turnover, but it would be interesting to see if it is possible to follow and disturb proper localization of the origin before replication initiation. If DnaA firing at the mid-cell position is indeed key, this should be detrimental for DNA replication in single cells.

Localization of the chromosomal origin

The results on the replisome and Z-ring localization point to the crucial localization of the origin of replication during the cell cycle. Indeed, in chapter 4, we noticed that the localization of the chromosomal origin is robust. While the replication fork and the terminus are dynamically localized during the largest part of the cell cycle, the origin of replication gradually moves from mid-cell to the $\frac{1}{4}$ positions of the cell. The localization of the origin is undisturbed by perturbation of cell division by deleting cell cycle protein *mapZ*, but it is disturbed by deleting *parB* or *smc* or the addition of antibiotics that disrupt the organization of the chromosome (ciprofloxacin or HPUra). Note that to visualize the origin-proximal region, we have labeled the position at 3 kb from the actual DnaA-trio (Richardson *et al.*, Nature 2016) with a *parS* site/*tetO* array, so the exact origin position and origin dynamics might be slightly different from our observations (chapter 4).

Rather than completely mislocalizing the origin of replication, the splitting of the origin is delayed in these cells. Cells with delayed origin localization have anucleate cells, but also mislocalized MapZ and – to a much lesser extent – FtsZ. This mild defect in cell division could possibly be connected to the mislocalization of the replication origin at the point of replication firing in a small subset of the cells. Alternatively, origin localization is crucial for the correct placement of the septal ring. Again, analysis with higher spatial- and time resolution, using single-cell information can help separate these two hypotheses.

What places the chromosomal origin?

Whether chromosomal origin placement is crucial for septal formation directly, as hypothesized in chapter 4, or through replication firing, as hypothesized in Gallay *et al.*, 2019, the question remains what segregates and moves the origin of replication to the right spot so robustly. Perturbing SMC, ParB, or both only leads to relatively mild defects. The membrane protein RocS was recently discovered as an interactor with ParB. *rocS* deletion leads to similar, though slightly more severe, phenotypes as *parB* or *smc* deletions (Mercy *et al.*, 2019). In time-lapse registrations of these deletion mutants, chromosomes failing to segregate stay in the middle of the cell or on one side of the closing septum. In some cases, this leads to anucleate cells or chromosome guillotining, but the chromosome often segregates at the very last moment. These observations suggest that even when segregation of the chromosomal origin is perturbed, there are mechanisms in place ensuring bulk chromosome segregation before septation.

One protein that might be responsible for the rescue of chromosome segregation in these mutants is FtsK, a topic we started to explore in chapter 5. FtsK is a highly conserved cell cycle protein that acts as a motor, by translocating DNA (Jean, Rutherford & Löwe, 2019). In *E. coli*, FtsK is an essential protein that localizes at the Z-ring early during division. Its deletion leads to mislocalized chromosomes and cell filamentation. However, its deletion phenotype can be partially rescued by overexpression of FtsQAZ or introduction of a gain-of-function mutant of FtsA (Dubarry, Possoz & Barre, 2010; Geissler & Margolin, 2005). In *B. subtilis*, FtsK (or SpoIIIE) is mainly localized at the cell membrane, but single-molecule studies suggest FtsK hexamers stabilize at the septum upon DNA damage (El Najjar *et al.*, 2018). However, *B. subtilis* FtsK is primarily involved in sporulation and not essential (Errington, Bath & Wu, 2001; Koo *et al.*, 2017), while it also harbors another unessential DNA translocase – a cytoplasmic FtsK homolog called SftA (El Najjar *et al.*, 2018).

On the contrary, FtsK is essential in the pneumococcus and cells slowly die upon depletion of FtsK by CRISPRi. This effect is strongly exacerbated when SMC, CcrZ and HU are also depleted (Julien Denereaz and Jan-Willem Veening, unpublished). In chapter 5, we show that FtsK localizes at the membrane and moves to the closing septum late during the cell cycle, at the moment in time when the chromosome is nearly segregated. Until now, several attempts by different members of our laboratory to make deletions and depletions failed, but using alternative approaches such as combining CRISPR interference with the degron system described in chapter 5 could possibly help investigating the role of FtsK further. The recent description of the mechanism of action of *E. coli* FtsK (Jean *et al.*, 2019) can also aid in the design of functional mutants in the pneumococcus. It would also be interesting to follow the movement of FtsK in the membrane during cell division in both wild type as in segregation mutants to see if there are changes in FtsK recruitment to the septum.

6.

Chromosome shape and composition as a segregation factor

Interestingly, mutations disrupting chromosome segregation, *ccrZ* depletion, *hu* degradation and blocking gyrase with ciprofloxacin all lead to similar events as observed by microscopy: guillotining of chromosomes, anucleate cell formation and last-minute segregation of chromosomes (chapter 5, Gally *et al.*, 2019). The underlying mechanisms behind these defects are very different. This suggests that overall chromosome integrity and DNA replication have a significant role in chromosome segregation. This is a good reminder that as DNA replication, chromosome segregation and compaction, cell growth and cell division all happen at the same time; these processes all influence each other.

Theoretical studies on the conformation of the chromosome in bacteria combined with recent *in vivo* studies are pointing in the direction that molecular crowding and cell morphology are important drivers for chromosome segregation and compaction. The simplest model leading chromosomes to segregate consists of DNA and crowders, confined in a tube. Theoretical models are supported by the observation that L-form bacteria can segregate their chromosome accordingly when they are confined in microfluidic channels (L. J. Wu *et al.*, 2019). Similarly, *E. coli* cells forming large cell filaments still segregate their chromosomes at the $\frac{1}{4}$ positions of the cell (F. Wu *et al.*, 2019). The nucleoids of these filamentous *E. coli* cells also scale up when the cells get larger.

The work of (Gray *et al.*, 2019) showed that in a large range of bacteria, the mobility and localization of the ribosomes is correlated with the cell-to-nucleoid size scaling. In other words, in cells where the nucleoid is highly compacted, the ribosome localization is anti-correlated

with the nucleoid and the ribosomes are very mobile. Ribosomes are thought to be important molecular crowders. When they are caught in the meshwork of the nucleoids, as in *C. crescentus*, it can be interesting to find out what keeps the chromosome decondensed in this bacterium.

As for *Streptococcus pneumoniae*, which is not a rod-shaped bacterium but oval-shaped, many of the geometrical principles laid out in these studies (Gray *et al.*, 2019, L. J. Wu *et al.*, 2019) can still count. Since the cell is nearly round when starting to grow, the segregation forces from the cell shape might only come to play late during cell division. This might explain the essentiality of FtsK in the pneumococcus, as well as the importance of DNA flexibility and compaction in the pneumococcus for fast segregation during late cell division.

Unlike *C. crescentus*, the nucleoid-to-cell size ratio of the pneumococcus is rather small (between 0.25-0.5 depending on the way of measurement vs. 0.75 for *C. crescentus*). Interestingly, addition of sublethal concentrations of ciprofloxacin makes the cell population lose the nucleoid-cell size scaling, though this experiment would have to be repeated using SIM images combined with a membrane marker to be sure of the cell sizes. If ciprofloxacin indeed disrupts nucleoid size scaling of the pneumococcus, it would be interesting to see what the role of topoisomerase IV is in the maintenance of the nucleoid-to-cell size ratio. This would again lead to the investigation of the latest point in chromosome segregation, something we have not focused on to a great extent in the chapters of this thesis.

It is important to note that the nucleoid-to-cell ratio measurements could be influenced by more than the segmentation of phase contrast images. A drawback of using HU-FP fusions to measure nucleoid sizes in cells with disrupted chromosome organization is that HU might be differently localized, over- or underexpressed. Even though syto-13 and HU localization show a good overlap and ChIP-seq did not show any high localization peaks (chapter 5), it is difficult to rule this out completely. However, as long as DNA staining always has some effect on cell viability, using HU-FP fusions is the most trustworthy DNA localization method at hand. When drawing conclusions on nucleoid sizes, it might be wise to use two different DNA visualization methods or check HU levels inside the cell.

6.

The anatomy of the pneumococcal chromosome

This thesis presents a detailed description of the organization of the pneumococcal chromosome during the cell cycle. To conclude, I will go through the chromosome cycle, highlighting the order of events. The pneumococcal cell cycle is also summarized in Figure 1 on the previous page.

While the septum closes and two daughter cells are born, DNA replication starts bidirectionally from the origin of replication, which localizes at the new mid-cell position (or $\frac{1}{4}$ position of the mother cell). Whether a new round of replication is fired before or after septum closure is an open question. Imaging the replisome and the septum together with high time- and spatial resolution could answer this, if it is possible to overcome the technical challenges of fluorescence bleaching and phototoxicity.

After replication has started, the origin of replication migrates steadily to the $\frac{1}{4}$ positions of the cell, as does cell cycle protein MapZ (Figure 1). As the chromosome is replicated further at mid-cell, where the replisomes are dynamically localized, the chromosome arms move away from mid-cell. During the cell cycle, the terminus is not specifically localized inside the cell, until the final stage of chromosome segregation, where the terminus is localized at the septum. FtsK localizes here as well. FtsZ assembles at the $\frac{1}{4}$ positions of the cell. The replisomes dissociate from mid-cell, associate near FtsZ and the septum closes. A new round of replication begins when CcrZ is brought to midcell by a direct interaction with FtsZ and then stimulates DnaA to trigger replication (Gallay *et al.*, Figure 1).

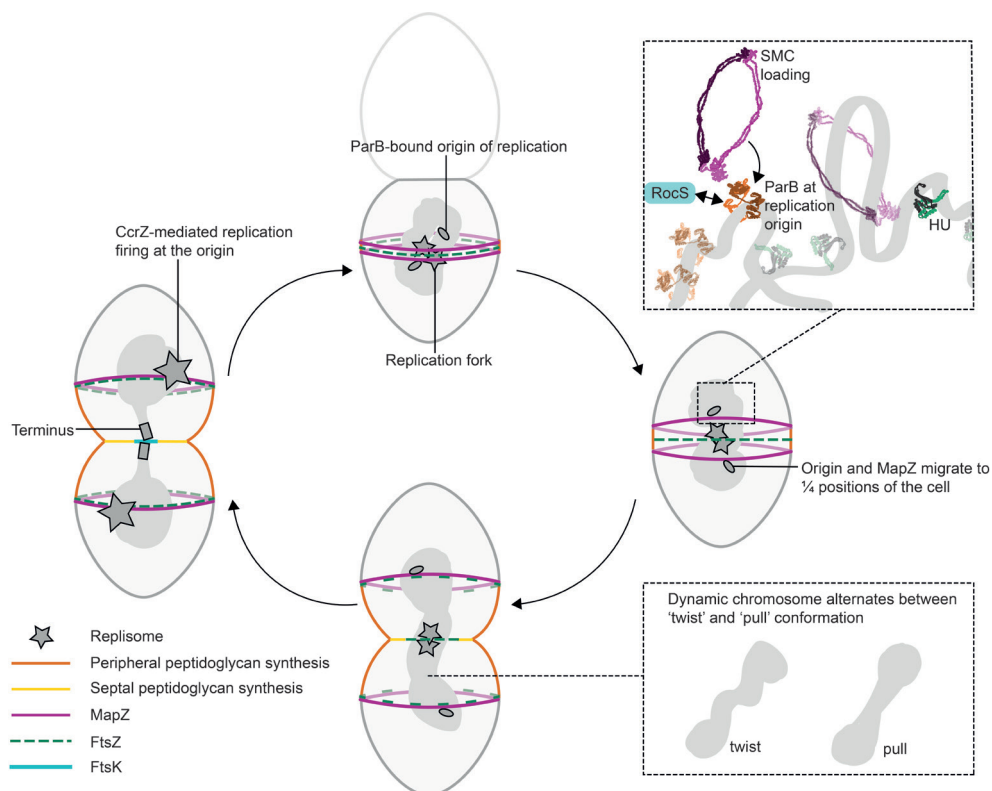


Figure 1. Summary of the pneumococcal cell cycle. **Main panel:** From top, clockwise: in a new-born cell, DNA is replicated at the septum, while the ParB-bound origin and MapZ are migrating towards the $\frac{1}{4}$ positions of the cell. FtsZ filaments are moving towards the $\frac{1}{4}$ positions of the cell afterwards, while the old septum is closing. When a full FtsZ-ring is visible at the $\frac{1}{4}$ positions of the cell, a new round of replication is fired close to the Z-ring. This is mediated by CcrZ, that colocalizes with FtsZ. The replication termini are close to the closing septa, where FtsK is localized, possibly to translocate the last parts of the chromosome towards both daughter cells. **Top-right panel:** Proteins important for chromosome segregation and chromosome conformation: ParB (orange) is bound to the origin of replication and aids the loading of SMC (pink) to the chromosome. Both SMC and HU (green) are important for the global conformation of the chromosome. RocS (blue) is a membrane-bound protein that interacts with ParB. **Bottom-right panel:** the chromosome is a dynamic structure, of which the conformation switches from a twisted, wet-towel like structure, to a straight structure where the two newly formed chromosomes oppose each other.

The segregation of the chromosomes is mediated by SMC, ParB, RocS, HU and possibly FtsK. Both HU and SMC influence chromosome condensation, shaping and flexibility, which is important for the proper segregation of the chromosome. Hi-C will be a next step in the mapping of the chromosome organization of the pneumococcus to understand the differences in the influence of the different proteins on chromosome organization. Combining this with high resolution microscopy of the chromosome in different conditions, if possible using dual-color SIM of HU-FP fusions with markers for chromosomal loci, could complete the anatomy of the pneumococcal chromosome as laid out in this thesis. In order to do this, there are some technical challenges to overcome, as mentioned above and in chapter 5 of this study.

With its low number of (known) proteins involved in chromosome segregation and robust chromosome cycle, *S. pneumoniae* could be a good model for investigating the role of other, passive processes on chromosome segregation. Where *E. coli* has many different NAPs, the pneumococcus only has one. The known chromosome segregation mediators are not essential.

Of course, there could be more unknown proteins interacting with the pneumococcal chromosome. Synthetic lethal screens and/or localization studies could help identify novel players for chromosome segregation in the pneumococcus.

Where we could follow the movement of the chromosomal origins using conventional fluorescence microscopy, there are steps to be taken in mapping the final stage of chromosome segregation. Getting a more complete description of late chromosome segregation events could help understand the forces behind the robust segregation of the chromosomes in the pneumococcus, even when early chromosome segregation fails. Coming to the end of this chapter, the most exciting unknown of the pneumococcal chromosome cycle might lie in its tail.

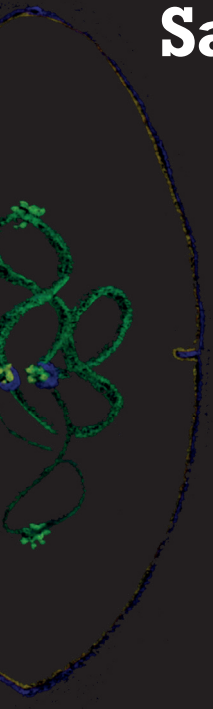
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Scientific Summary

**Wetenschappelijke
Samenvatting**



Scientific Summary

Pneumococcal pathogenicity

The pneumococcus resides in the nasopharynx of over half of the population of healthy children under four and about 10% of the adult population (Regev-Yochay et al., 2004; Southern et al., 2018). While it lives there as a commensal bacterium, causing no harm, it can move from the nasopharynx into the inner ear and respiratory tract and cause pneumonia, inner ear infections, meningitis or sepsis (Weiser, 2010).

Since the introduction of pneumococcal vaccines, global mortality of pneumococcal infections has dropped by 7% (Troeger et al., 2018). However, the pneumococcus changes its **serotype** – the **polysaccharide capsule** composition that is the target of the immune system and therefore also the vaccines – rapidly, both through genetic and epigenetic alterations. A shift to different, untargeted serotypes can explain the relatively low decline in pneumococcal carriage and infections after vaccine introduction.

At the same time, pneumococcal resistance to antibiotics is on the rise (CDC Antibiotic Threats Report, CS298822-2, 2019). To combat pneumococcal infections, a better understanding of the mechanisms of immune system evasion and antibiotic resistance as well as a continued search for new antibiotics are vital.

A better look at the pneumococcal cell

Already in the beginning of the last century, the pneumococcus (then also called diplococcus) was known to be a cause of pneumoniae. There is a rich history of infection research with the pneumococcus at the center, and the pneumococcus has one of the most well researched systems for DNA-uptake and incorporation into its genome (Avery, MacLeod & McCarty, 1944), a process called **transformation**. However, for a long time, bacterial cell biology has mostly been limited to model organisms as *Escherichia coli*, *Bacillus subtilis* and *Caulobacter crescentus*. This was partly due to the lack of available techniques, but also because the focus of pneumococcal research has been largely on the eradication of the bug rather than to understand it.

Since the development of **fluorescence microscopy** the field of bacterial cell biology flourished. For the pneumococcus specifically, many tools & techniques, from genetic tools to fluorescent proteins to CRISPR-i, are being developed and deployed in recent years. This leads to a better understanding of the pneumococcal cell cycle, which in turn can lead to new targets for antibiotics and a better understanding of mechanisms underlying antibiotic resistance in the pneumococcus.

The pneumococcal cell cycle

In **eukaryotic** cells, the **mitotic** cell cycle consists of processes strictly separated in time by checkpoints: DNA replication, chromosome segregation, cytokinesis and cell growth. In bacteria, this is generally not the case: all these processes happen simultaneously. The lack of temporal separation between processes does not mean there is no order of events or tight regulation in pneumococcal (or bacterial, for that matter) cell division.

Cell wall growth in the pneumococcus can be divided in **lateral and septal peptidoglycan synthesis**, similar to *B. subtilis* (see for instance Pinho, Kjos, and Veening 2013). Both septal and lateral peptidoglycan synthesis happen at mid-cell. A central player in regulating these processes is **FtsZ**. This protein forms a ring of rapidly turning (treadmilling) filaments at mid-cell (Perez et al., 2019). FtsZ recruits the proteins necessary for cell wall formation.

FtsZ is positioned at the future mid-cell of daughter cells before the pneumococcal cell is dividing. How FtsZ positions here correctly is unknown. The pneumococcus lacks known systems for FtsZ positioning. A recently discovered protein called **MapZ** localizes at these quarter positions of the cell before FtsZ and was proposed to be responsible for the correct positioning of FtsZ (Fleurie et al., 2014).

The pneumococcal chromosome cycle

There are few proteins known to be involved in chromosome segregation. The **SMC complex** is important for the overall condensation of the chromosome as well as for chromosome segregation. SMC is loading on the chromosome at the origin of replication, facilitated by ParB (Minnen, Attaiach, Thon, Gruber & Veening, 2011). **ParB** localizes at *parS* sites close to the chromosomal origins of replication. In other bacteria, ParB is a driving force of chromosome segregation, as it binds to ParA, which is localized close to the cell poles (Badrinarayanan, Le & Laub, 2015). *S. pneumoniae* lacks ParA homologs. Neither SMC, nor ParB are **essential**, however, deletion of either or both genes leads to 5-10% of anucleate cells.

In the past year, two new chromosome-related proteins were discovered in *S. pneumoniae*: RocS and CcrZ. RocS is a membrane-bound protein that interacts with ParB (Mercy et al., 2019). Similar to SMC and ParB, deletion of *rocS* leads to a fraction of anucleate cells. CcrZ is proposed to fire DNA replication initiation at the septum (Gallay et al., 2019). Deletion mutants of *ccrZ* have a large fraction of anucleate cells and severely impaired cell growth.

Other proteins important in maintaining the compaction and segregation of the chromosome are so-called **NAPs** (Nucleoid Associated Proteins, Badrinarayanan, Le, and Laub 2015; Zaritsky and Woldringh 2015). These small, DNA-binding proteins have roles in DNA folding and unfolding, interactions between chromosomal loci and **DNA supercoiling**.



Anatomy of the pneumococcal nucleoid

This thesis focuses on the central ‘organelle’ of the pneumococcus: the **nucleoid**. To map chromosome segregation and the nucleoid’s shaping and dynamics during the cell cycle, we developed and benchmarked several tools. This went from bench (chapter 2, 4 and 5) to desktop (chapter 3). In the following paragraphs, I summarize the tools, benchmarks, and the major findings of each chapter.

Chapter 2:

Red fluorescent proteins for gene expression and protein localization studies

The pneumococcus is a **microaerophilic** organism with a low oxygen tolerance. Folding and maturation of fluorescent proteins often require sufficient concentrations of oxygen. In this chapter, we benchmarked four Red Fluorescent Proteins in the pneumococcus: two orange-red variants, mOrange2 and TagRFP, and two far-red, mKate2 and mCherry.

To identify which of the **RFPs** is the brightest and most rapidly maturing *in vivo*, the four RFP genes were placed under control of inducible promoters P_{zn} and P_{subB} . When *tagRFP*, *mKate2* and *mCherry* were expressed, the fluorescent signal was well detectable using microscopy and plate reader assays. Expressing *mOrange2* yielded no fluorescent signal in the pneumococcus.

To assess the suitability of the fluorescent proteins as protein fusions, mCherry, mKate2 and TagRFP were fused C-terminally to **HU (HlpA)** and FtsZ. As the pneumococcus can take up

linear fragments of DNA by natural transformation, we tested out whether we could make the fusions of FtsZ to the RFPs using linear **Gibson Assembly** products. In this way, multiple PCR products could be assembled in one step and the product was successfully transformed into *S. pneumoniae*.

While it was possible to make a functional fusion of mCherry to HU, this was not possible for TagRFP or mKate2. **Western Blots** showed that a of the HU-mCherry proteins were degraded, probably leaving a fraction of untagged and fully functional HU. To be able to compare the three RFPs, the fusions were placed into the genome as an extra copy on the original locus. Where the HU-mCherry signal was fuzzy and not very high, both HU-mKate2 and HU-tagRFP resulted in bright, clear localizations. RFP fusions to FtsZ lead to similar results. We conclude that for measuring gene expression, mCherry is the most suitable RFP with the fastest folding time and the highest fluorescent signal. With a good signal, no protein degradation and a spectrum easily combined with GFPs, mKate2 is the most suitable for protein fusions in the pneumococcus.

Chapter 3:

BactMAP: an R package for integrating, analyzing and visualizing bacterial microscopy data

With the emergence of single-cell fluorescence microscopy, high-throughput microscopy analysis has rapidly become a critical tool in the field of bacterial cell biology. While most of the current popular cell **segmentation** programs include tools for exploring and visualizing the data, there are limitations to analyzing the data within the detection program. Often, multiple datasets describing different conditions or replicates need to be combined before analyzing them altogether. It can also be useful to combine outputs from different programs.

For these purposes we created BactMAP (Bacterial toolbox for Microscopy Analysis and Plotting), an **R package** that can be used to convert segmentation and fluorescence detection data to a standard format, allowing a user to view and combine datasets from different programs and different experimental conditions. BactMAP contains a number of plotting functions, making it easy to explore the data in different ways. Lastly, the vast number of statistical tools already available in R combined with BactMAP's import functions are a good starting point for custom analysis.

We used BactMAP to analyze the localization of the origin of replication of three **Gram-positive** bacteria: *Bacillus subtilis*, *Streptococcus pneumoniae* and *Staphylococcus aureus*. First, we tested the efficacy of five popular segmentation programs on phase-contrast images of these three bacteria (Cass, Stylianidou, Kuwada, Traxler & Wiggins, 2017; Ducret et al., 2016; Paintdakhi et al., 2016; Ursell et al., 2017; Vischer et al., 2015). Using BactMAP, we found that the measured cell numbers and sizes of the same microscopy image differed greatly depending on the segmentation software as well as the end user. Subsequently, we used BactMAP to plot the global localization of the origin of replication in the three different bacteria.

To demonstrate the power of BactMAP as a starting point for custom analysis, we analyzed a time-lapse microscopy dataset of pneumococcal cells carrying FtsZ-RFP and **DnaX-GFP**. Using cluster analysis of our time-lapse data, we could show that in 38% of the cells DnaX dissociates at the old septum and associates at the new septum slightly after a new FtsZ-ring is formed. In the remaining cells, association and dissociation could not be observed.

Chapter 4:

Chromosome segregation drives division site selection

In this chapter, we map the main known players of the pneumococcal cell cycle in detail to gain insight in the interplay between DNA replication, chromosome segregation and cell division. As mentioned above, the pneumococcus lacks known systems for FtsZ positioning, but the protein MapZ was proposed to be responsible for this (Fleurie et al., 2014). We show that while MapZ is important for placing the FtsZ ring perpendicular to the length axis of the cell (the division plane), deleting the *mapZ* gene has no effect on the distance of FtsZ to mid-cell (the division site).

Using a combination of a fluorescent repressor operator system (**FROS**) and a ParB/*parS* pair originating from a Gram-positive plasmid, we followed different chromosomal loci simultaneously. We also tracked fluorescent protein fusions with different members of the replication fork. The pneumococcal chromosome is oriented in an origin-terminus-origin fashion: the chromosome is replicated at the origin at mid-cell, after which the origins segregate towards the two future mid-cells (the $\frac{1}{4}$ -positions of the cell). While the two replication forks are mobile, they stay close to mid-cell.

The most striking about the chromosome orientation in the pneumococcus, is that the origin of replication moves to the $\frac{1}{4}$ -positions of the cell before an FtsZ ring is formed. When deleting *mapZ*, this pattern remained the same. However, when deleting the *smc* gene, the origin of replication placement at the new septa is delayed. Cells lacking *smc* are longer and irregularly shaped. Moreover, in these cells FtsZ and MapZ localization is irregular, although FtsZ placement at the new septum is not delayed in the majority of the cells.

We observe similar phenotypes when cutting the chromosome using an inducible CRISPR-CAS as well as when adding sub-MIC concentrations of antibiotics targeting DNA replication (HPUra) and chromosome integrity (ciprofloxacin). The similar effects of different ways of perturbing chromosome segregation and integrity shows that the irregular localization of FtsZ and MapZ is linked to delayed chromosome segregation and origin placement rather than to SMC specifically. The mechanism linking origin placement to correct Z-ring and the force behind the robust and precise segregation of the chromosome remain to be elucidated.



Chapter 5:

Investigations into the condensation and segregation dynamics of the pneumococcal nucleoid

There are two proteins involved in chromosome segregation and integrity that are essential for pneumococcal growth: **FtsK** and HU (HlpA). Besides being found as essential in synthetic lethal screens, FtsK has not been studied in *S. pneumoniae*. In *E. coli* FtsK acts as a motor to pull through the last bit of replicated DNA while the septum is closing. FtsK localizes at the septum in *E. coli* (Jean, Rutherford & Löwe, 2019). HU, the only known nucleoid associated protein in the pneumococcus, is important for DNA supercoiling (Ferrándiz, Carreño, Ayora & de la Campa, 2018).

We investigated the role of pneumococcal HU in maintaining the chromosome shape and chromosome segregation. For this, we performed **ChIP-seq**, showing that HU binds chromosomal DNA uniformly with a slight preference for GC-rich DNA. To avoid repressor mutations caused by a deletion mutant of an essential protein, we decided to degrade HU on the protein level using an inducible **ClpX/SspB degradation system**. Degrading HU led to irregular shaped and a small fraction of anucleate cells, but the overall growth rate was affected

only marginally.

We decided to use fluorescent protein fusions to HU as a chromosome label for microscopy. Using HU-mKate2 as a marker, we determined the global nucleoid shape and nucleoid to cell size (N/C) ratios in wild type cells, cells lacking the *smc* gene and cells treated with sub-MIC concentrations of ciprofloxacin and HPURa. Interestingly, the N/C ratio, which is shown to be a robust, species-specific feature (Gray *et al.*, 2019), is disrupted after ciprofloxacin treatment.

In many model bacteria, the nucleoid has a dynamic, helical shape (Berlitzky, Rouvinski & Ben-Yehuda, 2008; Fisher *et al.*, 2013a; Gorle *et al.*, 2017). To gain detailed insight in the pneumococcal nucleoid shape as well as HU dynamics, we used the superresolution microscopy techniques **SIM** and **PALM**. Using PALM, we could image HU-mEos2 localization in 160 cells with a Nyquist resolution of 18 nm, allowing us to distinguish different nucleoid shapes throughout the cell. In 20% of the cells, we observed a helical nucleoid shape, while in the remaining 80% of the cells the replicating nucleoid was seen as two blobs being pulled apart. Time-lapse 3D-SIM of HU-GFP confirmed that the helical shape is a transient state. PALM and SIM images also showed a thin line of HU-FP signal between the segregating chromosomes in the final stage of segregation. Using SIM and time-lapse fluorescence microscopy of a strain carrying HU-mKate2 and FtsK-mNeon, we observed that this thin line of HU-mKate2 colocalizes with FtsK-mNeon.

This study shows that the pneumococcal nucleoid is a dynamic structure, similar to previously observed in other bacteria. HU is important for maintaining the chromosome structure. To study the cell in absence of HU while being sure that there is absolutely no HU around, a combination of induced HU degradation (protein level) and depletion (transcription level) could give a clearer view of the function of HU in the cell. The same strategy on FtsK might help gain insight in its role in the later stage of chromosome segregation.

From bench to desktop

Together, the four studies described in this thesis present a detailed mapping of the pneumococcal chromosome cycle, starting with an exploration and development of tools for fluorescence microscopy and analysis. From bench to desktop, there are several recommendations for the future.

Firstly, while the benchmarking of GFPs and RFPs presents a great and useful starting point for fluorescence microscopy in the pneumococcus, these studies are currently being overtaken by the fast development of novel fluorescent protein variants. Therefore, it is important that laboratories keep testing these new variants, not only on **brightness**, but also maturation time and possible degradation.

It is similarly necessary to stay critical when looking at microscopy data. While automatic segmentation and fluorescence detection might seem free of human bias, this is rarely the case since researchers still check their data and set the parameters. Finding more anchor points helping to determine the point in the cell cycle, such as membrane or septum markers, can improve the uniformity of segmentation data from researcher to researcher.

BactMAP employs visualization strategies that show as much raw data as possible, so the user has a feeling for the noisiness of the data. Still, in some cases, I made the choice to show secondary, lower-noise data, as in for instance density-kymographs. This is easier on the eye and sometimes necessary to see patterns, but sometimes equally problematic because they are also easier to misinterpret. Plans for new version of BactMAP include interactive, GUI-based plotting, and more options for taking single cell information into account.

The last experimental chapter of this thesis also discusses the use of PALM and SIM in the pneumococcus. Both techniques are challenging and prone to artefacts. In both cases, it only works when the abundance of fluorescent proteins is high enough. While SIM microscopy demands very stable fluorescent proteins, both GFP and mNeonGreen fusions in the pneumococcus turn out to be very suitable. Because of this, SIM has the potential to become a routine complementation to wide-field fluorescence microscopy.

From replication to segregation

The major biological conclusions range from the start to the end of the chromosome cycle. Starting at the beginning, DNA replication in the pneumococcus is localized close to the septum. In several cases, two replication forks are visible, which speaks against the **factory model** of replication (Dingman, 1974). The origin of replication travels to the new septum immediately after replication, ready for a new round of replication. When chromosome organization is perturbed by antibiotics or deletion of *smc*, not only the origin arrives late at the septum, but also the replication fork is not localized at the septum any more.

It is unclear what makes the origin of replication robustly arrive at the new septum. Most likely, it is a combination of known proteins involved in chromosome integrity and segregation (SMC/ParB, HU, RocS), physical and chemical factors as molecular crowding that keep chromosome shape intact. The protein FtsK might be important for the rescue of slowly segregating chromosomes in for instance *smc* mutants.

While the origin of replication moves to the $\frac{1}{4}$ position of the cell, MapZ is moving to the $\frac{1}{4}$ position as well. The rest of the chromosome follows gradually after being replicated. At this point, FtsZ moves to the $\frac{1}{4}$ positions of the cell. Whether the cell closes before or after initiation of a new round of replication at the septum, is unknown. The future deciphering of the order of events in the final stage of chromosome segregation will be an exciting addition to the current mapping of the pneumococcal nucleoid.



Glossary

Brightness	Property of fluorescent proteins; the product of the molecular extinction-coefficient and the quantum yield, as measured <i>in vitro</i> . In practice, the brightness says something about the expected quality of the fluorescent signal.
Cell biology	The field within biology that describes the growth, buildup and division of cells.
ChIP-Seq	ChIP-Seq stands for <i>Chromatin ImmunoPrecipitation Sequencing</i> . This technique is used to determine the locations on the chromosome where a specific protein binds.
ClpX/SspB degradation	When a specific protein sequence is located at the terminus of a protein, SspB will bring the protein to ClpX to be degraded. In the pneumococcus, we used SspB from <i>E.coli</i> together with a specific degradation sequence to be able to degrade proteins when desired.
DNA- en RNA-sequencing	Techniques that read DNA and RNA sequences.
DNA-supercoiling	The winding and unwinding of DNA.
DnaX	Part of the <i>beta sliding clamp loading complex</i> of the replication fork.
Epigenetic	Inheritable traits that are not encoded in the genome, for instance DNA-methylation.
Essential proteins	Proteins of which the genes can not be deleted without causing severe growth defects or cell dead. Can be condition-dependent.
Eukaryotes	Organisms with a nucleus: fungi, plants and animals.
Factory-model	As proposed by Dingman, 1974. A theory proposing that DNA-replication could be the driving force of chromosome segregation, if the replisomes would be anchored at mid-cell.
Fluorescence microscopy	Optical microscopy where fluorescent objects are excited with a specific wavelength and the emission is observed through a lens and/or imaged using a camera. Used frequently to localize organelles or specific proteins labelled with fluorescent markers.
FROS-system	A combination of a repressor protein with a fluorescent tag and an operator sequence that is inserted on a specific place of the genome makes it possible to localize genomic locations using microscopy.
FtsK	<i>Filamenting temperature-sensitive mutant K</i> . FtsK is a motor protein, translocating DNA through the closing septum in <i>E. coli</i> .
FtsZ	<i>Filamenting temperature-sensitive mutant Z</i> . FtsZ is a central player in cell division of almost all bacteria, and recruits the proteins necessary for cell division.
Gibson Assembly	A cloning method developed by Daniel Gibson, where different DNA-fragments with overlapping ends are assembled using one isothermic reaction.
Gram-positive	Bacteria detectable with Gram-stain, having a single membrane and thick outer cell wall layer.

HU (HlpA)	HU stands for <i>Heat Unstable protein</i> , <i>HlpA</i> for <i>Histon-like protein A</i> . Both are names for the same small, DNA-binding protein that is a member of the NAPs. In the pneumococcus it is the only known NAP.
MapZ	<i>Mid-cell anchored protein Z</i> . The membrane-bound cell cycle protein is important for the orientation of the septum perpendicular to the cell length axis of the pneumococcus.
Micro-aerophile	An organism that needs oxygen, but only tolerates low oxygen levels.
Mitosis	Cell cycle of eukaryotic cells during growth where one mother cell forms two identical daughter cells.
NAP	<i>Nucleoid Associated Protein</i> . Small proteins having a role in the maintenance of the structure of the chromosome.
Nucleoid	The bacterial version of the nucleus. The bacterial chromosome, compacted and bound to numerous proteins forms one entity called the nucleoid.
OriC/origin of replication	<i>oriC</i> or the origin of replication is the location on the chromosome where DNA replication is initiated.
PALM	<i>Photoactivated Localization Microscopy</i> . This superresolution technique uses fluorescent proteins that stochastically switch color. Because of this, every protein can be imaged separately. The final result – the PALM reconstruction – is a combination of all localized proteins.
ParB/<i>parS</i>	<i>Partitioning system B</i> . Par-systems are important in bacteria for the segregation of chromosomes and plasmids. In the pneumococcus, ParB binds to <i>parS</i> -sites on the chromosome close to the origin of replication.
Peptidoglycan synthesis	The build-up of peptidoglycan, an important building block of the cell wall. Lateral synthesis makes up the elongation of the cell, while septal synthesis closes the cell wall during cell division.
Plate reader	A machine that measures optical density, light intensity and fluorescence.
Polysaccharide capsule	The most outer layer of the pneumococcus made of polysaccharides has a role in the invasion of the immune system.
RFPs	Red fluorescent proteins.
R-package	A collection of functions written in the language R.
Segmentation	The automatic detection of the dimensions of bacterial cells based on microscopy images.
Serotype	Classification of pneumococcal strains based on the composition of the polysaccharide capsule.
SIM	<i>Structured Illumination Microscopy</i> . For this superresolution microscopy technique, no special fluorescent proteins are needed.
Single-cell	A term used to notify that an experimental method does read out individual cells instead of populations.



SUMMARY

SMC-complex

Structural Maintenance of Chromosomes. These protein complexes are important for the structure of chromosomes from humans to bacteria.

Transformation

The process where a bacterium takes up DNA from its environment and incorporates DNA into its genome.

Western Blots

A biochemical method where specific proteins can be detected using antibodies.



Wetenschappelijke Samenvatting

Een longpathogeen

De pneumokok (*Streptococcus pneumoniae*) komt voor in de neuskeelholte van meer dan 50% van de gezonde kinderen onder de vier jaar en 10% van de gezonde volwassenen (Regev-Yochay *et al.*, Southern *et al.*, 2018). Onder normale omstandigheden is de pneumokok een onschadelijke commensaal, maar het kan zich plotseling verplaatsen van de keelholte naar het binnenoor en de luchtwegen. Hierdoor kan het pneumonie, otitis media, meningitis en sepsis veroorzaken (Weiser, 2010).

Sinds de introductie van vaccinatieprogramma's tegen de pneumokok is de globale mortaliteit van pneumokokinfecties gedaald met 7% (Troeger *et al.*, 2018). De pneumokok kan echter snel veranderen van **serotype** – de compositie van de **polysacharide capsule** aan de buitenkant van de cel die vaak het doelwit is van het immuunsysteem en daarom ook vaccins. Die veranderingen in serotype, door genetische en **epigenetische** aanpassingen, kunnen mogelijk verklaren hoe de hoeveelheid mensen die de pneumokok bij zich draagt maar mondjesmaat is verlaagd na de introductie van vaccinatieprogramma's.

De resistentie van pneumokokken tegen antibiotica stijgt (CDC Antibiotic Threats Report, CS298822-2). Om pneumokokinfecties beter te kunnen bestrijden, is het nodig om te leren hoe de pneumokok resistent wordt tegen antibiotica. Tegelijkertijd is het belangrijk om beter te begrijpen hoe de pneumokok het immuunsysteem kan omzeilen en om alternatieven voor de huidige beschikbare antibiotica te ontwikkelen.

De celbiologie van de pneumokok

Al in het begin van de vorige eeuw wist men dat de pneumokok longontstekingen kon veroorzaken. Er is een rijke historie aan infectieonderzoek geweid aan de pneumokok. Ook heeft de pneumokok een van de best onderzochte systemen voor DNA-opname en incorporatie in het eigen genoom, een proces dat **transformatie** wordt genoemd en al werd aangetoond in 1944 (Avery, MacLeod en McCarthy, 1944). De **celbiologie** van bacteriën is echter voor lange tijd voornamelijk geweid aan modelorganismen zoals *E. coli*, *B. subtilis* en *C. crescentus*. De celbiologie van de pneumokok is deels zo lang van de radar gebleven door een gebrek aan geschikte onderzoekstechnieken voor bacteriële celbiologie, maar ook doordat de focus van onderzoek naar pneumokokken voor lange tijd heeft gelegen op het bestrijden van de bacterie in plaats van op het begrijpen ervan.

De laatste decennia is daar verandering in gekomen: het onderzoeksveld van bacteriële celbiologie is aan een ware opmars bezig. Met de komst van nieuwe technieken voor genetische manipulatie, **DNA- en RNA-sequencing** en **fluorescentiemicroscopie** is er veel meer mogelijk. Hierdoor kunnen we de celcyclus van de pneumokok steeds beter begrijpen, dat weer kan leiden tot nieuwe aanknopingspunten voor de ontwikkeling van antibiotica en beter begrip van de mechanismen achter antibioticaresistentie.

De celcyclus van de pneumokok

In **eukaryoten** is de **mitose** opgedeeld in strikt gescheiden onderdelen: DNA-replicatie, chromosoomsegregatie, cytokinese en celgroei. Deze celdelingsfases gebeuren na elkaar en zogenoemde checkpoint-eiwitten controleren een goed verloop voor de volgende fase ingaat. In bacteriën is dit niet het geval: al deze processen gebeuren gelijktijdig. Desondanks is het geen ongeordende chaos in de bacterie. Er is wel degelijk een volgorde waarop de celdeling plaatsvindt en de celdeling van bacteriën (en dus de pneumokok) wordt gereguleerd om te zorgen dat

niks fout gaat.

De groei van de celwand van de pneumokok kan worden onderverdeeld in laterale en septale **peptidoglycaansynthese**, net als in *Bacillus subtilis* (zie bijvoorbeeld Pinho, Kjos en Veening, 2013). Zowel septale als laterale peptidoglycaansynthese gebeurt over het midden van de lengteas van de cel (het mid-cel punt). Een belangrijke speler in de regulatie van dit proces is het eiwit **FtsZ**. FtsZ vormt een ring van snel roterende filamenten op het mid-cel punt (Perez *et al.*, 2019). FtsZ trekt de eiwitten aan die nodig zijn voor het vormen van de celwand.

Als de celwand bijna is gesloten, verplaatst FtsZ zich naar de toekomstige mid-celpunten van de twee dochtercellen: de kwartposities van de cel. Hoe FtsZ dit punt kan vinden is onbekend. De pneumokok heeft geen van de systemen voor FtsZ-positionering die bekend zijn in andere bacteriën. Het eiwit **MapZ** positioneert zich nog eerder op de kwartposities dan FtsZ en wordt dan ook als kandidaat-verantwoordelijke genoemd voor het positioneren van FtsZ (Fleurie *et al.*, 2014).

Tegelijkertijd met de opbouw van de celwand vindt DNA-replicatie en chromosoomsegregatie plaats. De pneumokok heeft één cirkelvormig chromosoom van iets meer dan twee miljoen **baseparen**. Het chromosoom is te zien in de microscoop als een compact gevouwen geheel: de **nucleoïde**. Het **SMC-complex** is belangrijk voor het compact houden van het chromosoom en voor chromosoomsegregatie. SMC is een ringvormig eiwitcomplex dat zich om het chromosoom van de pneumokok laadt, geholpen door **ParB** (Minnen, Attaiech, Thon, Gruber en Veening, 2011). ParB is een eiwit dat bindt aan *parS*-sites die zich bevinden dichtbij *ori*, de plek in het genoom waar DNA-replicatie start. In andere bacteriën werkt ParB samen met ParA, waar ParB de chromosomen aan de *ori* uit elkaar trekt in de richting van ParA, dat zich in de uiteindes van de bacterie bevindt. In de pneumokok is dit niet het geval: ParB is alleen. Noch SMC, noch ParB zijn **essentiële eiwitten**. Het verwijderen van de genen *parB* of *SMC* resulteert in een klein groeidefect en 5 tot 10% cellen zonder DNA.

In het afgelopen jaar zijn twee nieuwe eiwitten ontdekt die een rol spelen in de chromosoomcyclus van de pneumokok: RocS en CcrZ. RocS is een membraangebonden eiwit dat interacties aangaat met ParB (Mercy *et al.*, 2019). Net als SMC en ParB leidt de verwijdering van het *rocS*-gen tot een fractie cellen zonder DNA. CcrZ lijkt een rol te hebben in de initiatie van DNA-replicatie (Gallay *et al.*, 2019). Ook het verwijderen van *ccrZ* zorgt voor een fractie cellen zonder DNA, maar daarnaast heeft het ook een grote impact op de verdubbelingstijd van de pneumokok.

Andere eiwitten die belangrijk zijn voor de vorm, condensatie en segregatie van het chromosoom zijn de zogenoemde **NAPs** (nucleoïde-geassocieerde-proteïnen, zie bijvoorbeeld Badrinarayanan, Le en Laub, 2015; Zaritsky en Woldhringh, 2015). Deze kleine, DNA-bindende eiwitten spelen een rol in het vouwen en ontvouwen van DNA, DNA-DNA-interacties en DNA-supercoiling.

De anatomie van de nucleoïde van de pneumokok

In dit proefschrift ligt de focus op het centrale ‘organel’ van de pneumokok: de nucleoïde. Om de segregatie van het chromosoom en de dynamiek en vorm van de nucleoïde in kaart te brengen ontwikkelden en testten we verschillende methodologieën, van het laboratorium (**hoofdstuk 2, 4, en 5**) tot de computer (**hoofdstuk 3**). In de volgende paragrafen zal ik deze methodes en de belangrijkste bevindingen per hoofdstuk beschrijven.



Hoofdstuk 2:

Rood-fluorescente eiwitten voor genexpressie- en eiwitlokalisatiestudies

De pneumokok is een **micro-aerofiel** organisme met een lage tolerantie voor zuurstof. De processen van vouwing en maturatie van fluorescente eiwitten hebben vaak voldoende zuurstofconcentraties nodig. In dit hoofdstuk brengen we daarom de werking van vier rood-fluorescente eiwitten (**RFPs**) in de pneumokok in kaart: twee varianten in het oranje-rode spectrum, mOrange2 en TagRFP, en twee diep-rode varianten, mKate2 en mCherry.

Om erachter te komen welke van deze vier RFPs de helderste is en welke *in vivo* de snelste maturatietijd heeft, werden de vier genen die de RFPs beschrijven onder invloed van de induceerbare promotors P_{zn} en P_{shp} geplaatst. Wanneer *tagRFP*, *mKate2* en *mCherry* tot expressie werden gebracht resulteerde dat in fluorescentie detecteerbaar met zowel de microscoop als met een **plate reader**. *mOrange2* werd wel tot expressie gebracht, maar resulteerde niet in een detecteerbaar fluorescent signaal.

Om de geschiktheid van de fluorescente eiwitten voor eiwitfusies te testen werden mCherry, mKate2 en TagRFP C-terminaal gelinkt aan de eiwitten **HU (HlpA)** en FtsZ. Omdat de van nature pneumokok lineaire DNA-fragmenten kan opnemen via transformatie, testten we of we de FtsZ-RFP-fusies konden maken met behulp van **Gibson Assembly**. Zo konden we meerdere PCR-producten aan elkaar voegen in één stap. Het product werd daarna succesvol in de pneumokok gebracht door middel van transformatie.

Hoewel het mogelijk bleek om functionele fusies te maken van mCherry met HU, bleek dit niet te kunnen met TagRFP of mKate2. **Western Blots** lieten zien dat een deel van de HU-mCherry-eiwitten gedegradeerd waren. Daardoor was er waarschijnlijk een fractie van de HU-eiwitten in de cel ongelinkt en daarmee volledig functioneel, in tegenstelling tot de gelinkte eiwitten. Om toch de drie RFPs als fusie te kunnen vergelijken werden ze als extra kopie in het genoom ingebracht. Terwijl het HU-mCherry-sig-naal niet erg sterk was, resulteerden zowel HU-mKate2 als HU-tagRFP in heldere, duidelijke lokalisaties. RFP-fusies met FtsZ leidden tot vergelijkbare resultaten.

Wij concluderen dat mCherry het meest geschikte rood-fluorescente eiwit voor genexpressiestudies is, met de snelste eiwitvouwing en het hoogste fluorescente signaal. Voor eiwitlokalisatiestudies is mKate2 het meest geschikt, met een helder signaal, geen eiwitdegradatie en een spectrum dat goed te combineren is met groen-fluorescente eiwitten.

Hoofdstuk 3:

BactMAP: een R-package voor het integreren, analyseren en visualiseren van microscopie van bacteriën

Sinds de ontwikkeling van **single-cell**-fluorescentiemicroscopie een vlucht heeft genomen, is high-throughput microscopieanalyse een belangrijk gereedschap binnen de bacteriële celbiologie geworden. De meeste populaire computerprogramma's gemaakt voor het **segmenteren** van cellen hebben ook functies voor het onderzoeken en visualiseren van data. Toch kan het soms beter zijn om datasets te analyseren in een ander programma dan het segmentatieprogramma. Vaak moeten meerdere datasets die verschillende condities of replicaties beschrijven gecombineerd worden voordat de analyse gestart kan worden. Het kan ook zinvol zijn om de outputs van verschillende segmentatie- en detectieprogramma's te combineren.

Daarom presenteren wij BactMAP (Bacteriële toolbox voor Microscopieanalyse en Plots), een

R-package dat gebruikt kan worden om segmentatie- en fluorescentiedetectiegegevens naar een standaard format te converteren. Hierdoor kan een gebruiker datasets van verschillende programma's en experimentele condities vergelijken en bekijken. BactMAP bestaat onder andere uit een set plot-functies, wat het gemakkelijk maakt om de data op verschillende manieren te bekijken. Uiteindelijk is BactMAP, vooral wanneer gecombineerd met de grote set aan statistische tools beschikbaar in R, een goede basis voor het ontwikkelen van eigen analyse op maat.

Als eerste testten we de effectiviteit van vijf populaire segmentatieprogramma's voor de analyse van fasecontrastmicroscopie van drie **Gram-positieve** bacteriën, namelijk in *Bacillus subtilis*, *Streptococcus pneumoniae* en *Staphylococcus aureus* (Cass, Stylianidou, Kuwada, Traxler en Wiggins, 2017; Ducret *et al.*, 2016, Paintdakhi *et al.*, 2016, Ursell *et al.*, 2017; Vischer *et al.*, 2015). Met behulp van BactMAP vergeleken we de output. De hoeveelheid gedetecteerde cellen en de gemeten celgrootte bleek erg te verschillen afhankelijk van welk segmentatieprogramma werd gebruikt, maar ook afhankelijk van de gebruiker zelf. Hierna gebruikten we BactMAP om de globale lokalisatie van *ori* in de drie verschillende bacteriën te plotten.

Om te laten zien hoe BactMAP een basis kan zijn voor de ontwikkeling van meer gecompliceerde analyses, onderzochten we een dataset van een time-lapse-fluorescentiemicroscopisch experiment. Hier werden groeiende pneumokokken die FtsZ-RFP en DnaX-GFP in zich droegen elke twintig seconden vastgelegd. Deze time-lapse film werd geanalyseerd met segmentatie- en fluorescentiedetectiesoftware en met door middel van BactMAP geïmporteerd in R. Door clusteranalyse toe te passen op onze data konden we laten zien dat DnaX dissocieert van het oude septum en associeert bij het nieuwe septum na de formatie van een nieuwe FtsZ-ring in 38% van de cellen. In de rest van de cellen was het niet mogelijk om associatie en dissociatie waar te nemen.

Hoofdstuk 4:

Chromosoomsegregatie is de kracht achter de positionering van het septum

In dit hoofdstuk brengen we de belangrijkste factoren van de celcyclus van de pneumokok in kaart, zodat we een gedetailleerd beeld kunnen krijgen van de relatie tussen DNA-replicatie, chromosoomsegregatie en celdeling. Zoals hiervoor genoemd heeft de pneumokok geen bekende systemen die ervoor zorgen dat de FtsZ-ring zich op de kwartposities van de cel kan plaatsen, maar was MapZ genoemd als een kandidaat voor deze rol (Fleurie *et al.*, 2014). Wij laten zien dat MapZ belangrijk is voor de hoek die de FtsZ-ring maakt ten opzichte van de lengteas van de cel. Het verwijderen van het *mapZ* gen had echter geen effect op de afstand van FtsZ tot mid-cel punt.

Met behulp van een **FROS-systeem** (Fluorescent Repressor-Operator System) en een ParB/*parS*-paar uit een Gram-positieve plasmide konden we verschillende locaties op het chromosoom tegelijkertijd volgen. We volgden ook fluorescente-eiwitfusies met verschillende eiwitten van de replicatievork en brachten zo de chromosoomcyclus van de pneumokok in kaart. Het chromosoom heeft een *ori-terminus-ori*-oriëntatie: het chromosoom wordt gerepliceerd vanaf *ori* op het mid-cel punt. Daarna segregeren beide *ori*'s naar de kwartposities van de cel. De twee replicatievorken zijn beweeglijk, maar blijven dicht bij het mid-cel punt gedurende de celcyclus.

Wat het meest verrassend is aan de chromosoomcyclus van de pneumokok, is dat *ori* al naar de kwartposities van de cel beweegt voordat daar een nieuwe FtsZ ring wordt gevormd. Als het *mapZ*-gen ontbreekt is dat patroon hetzelfde, maar als het *smc*-gen wordt verwijderd is de beweging van *ori* naar de kwartposities vertraagd. Cellen zonder *smc* zijn langer en onregelmatig van vorm. Daarbovenop komt dat FtsZ en MapZ onregelmatiger lokaliseren. FtsZ blijft wel in het



grootste deel van de cellen op het goede moment op de kwartposities aankomen.

Door het chromosoom door te knippen met een induceerbaar CRISPR-CAS-systeem konden we dezelfde fenotypes waarnemen als wanneer *smc* verwijderd was. Ook het toevoegen van lage doseringen antibiotica die DNA-replicatie vertragen (HPUra) of de chromosoomintegriteit aantasten (ciprofloxacin) leidde tot dezelfde vertraagde positionering van *ori* en onregelmatige lokalisatie van MapZ en FtsZ. Dit laat zien dat niet SMC, maar dat de chromosoomintegriteit en positionering van *ori* belangrijk zijn voor het correct plaatsen van het septum. Het mechanisme voor de correcte plaatsing en voor de robuuste segregatie van het chromosoom moet nog worden achterhaald.

Hoofdstuk 5:

Een blik op de condensatie- en segregatiedynamiek van de nucleoïde van de pneumokok

Twee eiwitten die een rol spelen in de vorm en segregatie van het chromosoom zijn essentieel voor de groei van de pneumokok: **FtsK** en HU (HlpA). Over de rol van FtsK in de pneumokok is weinig bekend. In *Escherichia coli* is FtsK een motor-eiwit dat ervoor zorgt dat het laatste beetje gerepliceerde DNA gesegregeerd wordt terwijl het septum van de cel sluit. FtsK bevindt zich op het septum in *E. coli* (Jean, Rutherford en Löwe, 2019). HU is het enige nucleoïde-geassocieerde eiwit (NAP) in de pneumokok en het is belangrijk voor **DNA supercoiling** (Ferrándiz, Carreño, Ayora en de la Campa, 2018).

Wij onderzochten de rol van HU in het onderhoud van de vorm van het chromosoom en chromosoomsegregatie. We gebruikten **ChIP-Seq** om aan te tonen dat HU uniform bindt aan chromosomaal DNA, met een kleine voorkeur voor GC-rijk DNA. Om de rol van HU te kunnen onderzoeken maar tegelijkertijd repressormutaties te kunnen voorkomen, besloten we het HU-eiwit te degraderen met behulp van een induceerbaar **ClpX/SspB degradatiesysteem** in plaats van het *hns*-gen te verwijderen. De degradatie van HU leidde tot onregelmatig gevormde cellen en een klein percentage cellen zonder DNA, maar de gemiddelde groeisnelheid van de cellen was nauwelijks verstoord.

Omdat HU al het chromosomale DNA bindt, besloten we fluorescente-eiwitfusies met HU te gebruiken als een marker voor het chromosoom. Met gebruik van HU-mKate2 konden we de globale vorm van de nucleoïde en de verhouding van de grootte van de nucleoïde tot de cell (de N/C-ratio) in gezonde cellen, cellen zonder het *smc*-gen en cellen behandeld met lage concentraties ciprofloxacin en HPUra meten. Hoewel vorig jaar werd aangetoond dat de N/C-ratio een robuuste, soortspecifieke constante is (Gray *et al.*, 2019), is de N/C-ratio in de pneumokok verstoord na behandeling met ciprofloxacin.

In veel bacteriën is de nucleoïde een beweeglijke helix (Berlitzky, Rouvinski en Ben-Yehuda, 2008; Fischer *et al.*, 2013; Gorle *et al.*, 2017). Om inzicht te krijgen in de vorm van de nucleoïde van de pneumokok en in de dynamiek van HU, gebruikten we de superresolutiemicroscopietechnieken **PALM** en **SIM**. Met behulp van PALM konden we de lokalisatie van HU-mEos2 in 160 cellen weergeven met een resolutie van 18 nm. Daardoor konden we zien dat de nucleoïde in 20% van de cellen een helix vormde, terwijl dat in 80% van de nucleoïdes niet het geval was. Time-lapse registraties van HU-GFP met behulp van 3D-SIM bevestigden de helixvorm en liet zien dat dit een kortstondige staat is. PALM- en SIM-reconstructies lieten ook zien dat er zich een dunne lijn van HU-GFP of HU-mEos2-sigitaal bevindt tussen de segregerende chromosomen juist voor het einde van de celdelingscyclus. Met behulp van SIM en time-lapse-fluorescentiemicroscopie van pneumokokken die FtsK-mNeon en HU-mKate2 tot expressie brachten, observeerden we dat deze dunne lijn colocaliseert met FtsK-mNeon.

Deze studie laat zien dat de nucleoïde van de pneumokok een dynamische structuur is, gelijkend met voorgaande observaties in ander bacteriën. HU is belangrijk om die structuur te behouden. Om te rol van HU beter te kunnen bestuderen, zal een combinatiestrategie van zowel degradatie en depletie kunnen helpen. Op deze manier kunnen we de concentratie van HU in de cel nog verder naar beneden brengen in korte tijd. Een soortgelijke strategie met FtsK kan helpen om inzicht te verkrijgen in de rol van FtsK in het late stadium van de chromosoomcyclus.

Van het lab tot de computer

Samen beschrijven de vier experimentele hoofdstukken in dit proefschrift een gedetailleerde inkaartbrenging van de chromosoomcyclus van de pneumokok, beginnend met een verkenning en de ontwikkeling van tools voor microscopie en analyse. Van laboratorium tot de computer zijn er verschillende aanbevelingen voor de toekomst.

Allereerst, hoewel het benchmarken van GFP's en RFP's een goed startpunt is voor fluorescentiemicroscopie in de pneumokok, worden deze studies ingehaald door de snelle ontwikkeling van nieuwe fluorescente eiwitten. Daarom is het belangrijk dat laboratoria deze nieuwe varianten blijven vergelijken, niet alleen kijkend naar de **brightness** van de eiwitten, maar ook maturatie, toxiciteit en mogelijke eiwitdegradatie.

Het is net zo belangrijk om kritisch te blijven wanneer men naar microscopiedata kijkt. Hoewel automatische segmentatie en fluorescentiedetectie vrij van menselijke fouten lijkt te zijn is dit zelden het geval; onderzoekers kijken naar hun data en bepalen vaak de parameters. Daarom is het belangrijk om meer ijkpunten te vinden die helpen om het punt in de celcyclus te bepalen, bijvoorbeeld septum of membraanmarkers. Dit kan de uniformiteit van segmentatiedata van onderzoeker tot onderzoeker ten goede komen.

BactMAP gebruikt visualisatiestrategieën die zo veel mogelijk onbewerkte data laten zien om de gebruiker te helpen de variabiliteit van de data te begrijpen. Toch is het in sommige gevallen beter om secundaire, schonere data te laten zien. Dit maakt het mogelijk om patronen te onderscheiden, maar kan ook problematisch zijn omdat het gemakkelijker is om een visualisatie verkeerd te interpreteren. In een nieuwe versie van BactMAP gaan we interactieve plotfuncties en functies waar individuele cellen meer in beeld komen toevoegen.

In hoofdstuk 5 komt het gebruik van SIM en PALM in de pneumokok ook aan bod. Beide technieken zijn lastig uit te voeren en werken alleen als er genoeg fluorescente eiwitten zijn met een sterk signaal. Beide microscopietechnieken hebben ondanks deze gebreken de potentie om een goede toevoeging aan reguliere fluorescentiemicroscopie te zijn in ons laboratorium. Vooral SIM, waarvoor GFP-fusies in de pneumokok zeer geschikt blijken, is relatief gemakkelijk te implementeren.

Van replicatie naar segregatie

De belangrijkste biologische conclusies van dit proefschrift gaan van de start tot het einde van de chromosoomcyclus. Om bij het begin te beginnen, DNA-replicatie vindt in de pneumokok plaats dichtbij het septum. In veel gevallen kunnen we twee replicatievorken waarnemen, wat in tegenspraak is met het **factory-model** voor replicatie (Dingman, 1974). De *ori* beweegt zich direct nadat het is gerepliceerd naar het nieuwe septum, klaar voor een nieuwe replicatieronde. Wanneer de organisatie van het chromosoom wordt gestoord door antibiotica, het doorknippen van het chromosoom of de verwijdering van *smc*, arriveert *ori* later bij het nieuwe septum. Daarbovenop is de replicatievork dan niet meer gelokaliseerd bij het septum.



Het is onduidelijk wat ervoor zorgt dat *ori* zelfs bij sterke verstoring uiteindelijk toch arriveert op het nieuwe septum. De meest waarschijnlijke verklaring is dat een combinatie van eiwitten die we al kennen die belangrijk zijn voor chromosoomsegregatie (SMC, ParB, HU, RocS) samen met natuurkundige en chemische factoren, zoals molecular crowding, ervoor zorgen dat de vorm van de nucleoïde intact blijft. Daarnaast zou FtsK belangrijk kunnen zijn voor het redden van de te langzaam segregerende chromosomen in bijvoorbeeld cellen zonder het *smc*-gen.

Terwijl *ori* naar de kwartposities van de cel beweegt, beweegt MapZ naar dezelfde positie. De rest van het chromosoom volgt stap voor stap na te zijn gerepliceerd. Op dit punt beweegt FtsZ naar de kwarposities van de cel. Of de cel zich al sluit voordat een nieuwe replicatieronde start of dat dat in omgekeerde volgorde gebeurt, is nog niet bekend. Het verder ontcijferen van de volgorde van gebeurtenissen in het laatste stadium van de chromosoomcyclus zal een spannende nieuwe toevoeging zijn aan de huidige beschrijving van de nucleoïde van de pneumokok.

Woordenlijst

Baseparen	De bouwstenen van DNA bevinden zich altijd in een paar: Adenine-Thymine (AT) en Guanine-Cytosine (GC). Afhankelijk van de oriëntatie van het paar zijn ze af te lezen als A (AT), T (TA), C (CG) en G (GC). Samen vormen ze de genetische code.
Brightness	Eigenschap van een fluorescent eiwit: het product van de moleculaire extinctie-coëfficiënt en de quantum yield, zoals gemeten <i>in vitro</i> . In de praktijk wordt de brightness gebruikt als een maatstaf voor de kwaliteit van het te verwachten fluorescente signaal.
Celbiologie	Het veld binnen de biologie dat gaat over de samenstelling, groei en deling van de cel.
ChIP-Seq	De afkorting ChIP-Seq staat voor <i>Chromatin ImmunoPrecipitation Sequencing</i> . ChIP-Seq wordt gebruikt om de locaties op het chromosoom te achterhalen waar specifieke eiwitten binden.
ClpX/SspB degradatie	Wanneer er een specifiek uiteinde aan een eiwit zit brengt SspB dit eiwit naar ClpX, dat het eiwit in stukken knipt. In de pneumokok hebben we dit specifieke uiteinde en SspB van <i>E. coli</i> gebruikt om zo kunstmatig eiwitten te kunnen laten degraderen.
DNA- en RNA-sequencing	Verzamelterm voor de technieken waarbij de DNA- of RNA-code wordt uitgelezen.
DNA-supercoiling	Het in- en uit elkaar draaien van DNA-strengen, belangrijk voor DNA-compactie, -repletie en genexpressie.
DnaX	DnaX is onderdeel van de replicatievork van de pneumokok, de eiwitten die ervoor zorgen dat het DNA gerepliceerd wordt. Het is deel van de <i>beta sliding clamp loading complex</i> .
Epigenetisch	Overerfbare eigenschappen die niet in de DNA-code zijn vastgelegd, zoals DNA-methylatie.
Essentiële eiwitten	Eiwitten waarvan het gen niet verwijderd kan worden uit een bacterie zonder dat het leidt tot extreme groeideficiëntie of celdood.
Eukaryoten	Organismen met een celkern, waaronder planten, dieren en schimmels.
Factory-model	Geponeerd door Dingman, 1974. Als DNA-replicatie zich geankerd in het midden van de cel plaatsvindt, kan de beweging van het DNA door de replicatievorken een kracht zijn die de chromosomen uit elkaar beweegt.
Fluorescentiemicroscopie	Optische microscopie waar fluorescente objecten worden geëxciteerd met een specifieke golflengte en de emissie wordt bekeken door een lens en/of vastgelegd met een camera. Wordt in de celbiologie veel gebruikt om organellen of specifieke eiwitten gelabeld met fluorescente markers te lokaliseren.
FROS-systeem	Een combinatie van een repressoreiwit met een fluorescente tag en een operator-sequence die op een specifieke plaats in het genoom wordt geplaatst zorgt ervoor dat deze plaats onder de microscoop zichtbaar wordt.
FtsK	FtsK staat voor <i>Filamentous Temperature-Sensitive protein K</i> . FtsK is



	een motor-eiwit dat DNA door het sluitende septum transporteert in <i>E. coli</i> .
FtsZ	De afkorting van FtsZ staat voor <i>Filamenting temperature-sensitive mutant Z</i> . FtsZ is een centrale speler in de celdeling van bijna alle bacteriën en rekruteert de eiwitten die nodig zijn voor de celdeling.
Gibson Assembly	Ontwikkeld door Daniel Gibson, een kloonmethode waarbij verschillende DNA-fragmenten met aan de uiteindes overlappende DNA-codes door middel van één isothermische reactie aan elkaar gevoegd kunnen worden.
Gram-positief	Bacteriën met een enkele membraan en een dikke celwand aan de buitenkant.
HU (HlpA)	De afkorting HU staat voor <i>Heat Unstable protein</i> , de afkorting <i>HlpA</i> voor <i>Histon-like protein A</i> . Beide afkortingen beschrijven hetzelfde eiwit: een klein DNA-bindend eiwit behorende tot de NAPs, waarvan in veel bacteriën een versie voorkomt. In de pneumokok is het de enige bekende NAP.
MapZ	De afkorting van MapZ staat voor <i>Mid-cell anchored protein Z</i> . Het membraangebonden celdelings-eiwit is belangrijk voor de oriëntatie van het septum ten opzichte van de lengteas van de pneumokok.
Micro-aerofiel	Een organisme dat zuurstof nodig heeft om te overleven, maar een omgeving nodig heeft waar de concentraties zuurstof lager zijn dan in de atmosfeer.
Mitose	Celdeling van eukaryoten gedurende de groei, waarbij twee gelijke dochtercellen ontstaan.
NAPs	De afkorting NAP staat voor <i>Nucleoid Associated Protein</i> . Dit zijn in het algemeen kleine eiwitten die een algemene functie hebben in het onderhoud van de structuur van het chromosoom.
Nucleoïde	De bacteriële tegenhanger van de nucleus. Het bacteriële chromosoom en alle gebonden eiwitten vormen samen één geheel: de nucleoïde.
Ori	<i>ori</i> is de locatie op het chromosoom waar DNA-replicatie start.
PALM	PALM staat voor <i>Photoactivated Localization Microscopy</i> . Deze vorm van superresolutiemicroscopie werkt met behulp van fluorescente eiwitten die stochastisch van kleur verschieten. Daardoor kan elk eiwit los van elkaar gefotografeerd worden. Het uiteindelijke resultaat – de PALM-reconstructie – is een samenvoeging van alle gelokaliseerde eiwitten.
ParB/<i>parS</i>	De afkorting van ParB staat voor <i>Partitioning system B</i> . Par-systemen zijn belangrijk in bacteriën voor het segregeren van chromosomen en plasmides. In de pneumokok bindt ParB aan <i>parS</i> -sites op het chromosoom en heeft ParB vooral een faciliterende functie voor SMC.

Peptidoglycaansynthese	De opbouw van peptidoglycaan, een belangrijke bouwsteen van de bacteriële celwand. Laterale synthese zorgt voor de verlenging (groei) van de cel, terwijl septale synthese de celwand sluit bij celdeling.
Plate reader	Een machine die zowel optische dichtheid, lichtintensiteit als fluorescentie kan meten.
Polysaccharide capsule	De buitenste laag van de pneumokok gemaakt van suikerverbindingen, heeft een rol in het vermijden van het immuunsysteem.
RFPs	Rood-fluorescerende eiwitten.
R-package	Een verzameling functies die geschreven zijn in de taal R.
Segmentatie	Het automatisch onderscheiden en vastleggen van de dimensies van bacteriecellen op basis van microscopische foto's.
Serotype	Classificatie van pneumokokstammen gebaseerd op de compositie van de polysaccharide capsule.
SIM	SIM staat voor <i>Structured Illumination Microscopy</i> . Voor deze vorm van superresolutiemicroscopie zijn geen speciale fluorescente eiwitten nodig.
Single-cell	Een term die in de celbiologie wordt gebruikt om aan te geven dat een meetmethode niet een algemene beschrijving van een populatie is, maar elke cel afzonderlijk meet.
SMC-complex	De afkorting SMC staat voor <i>Structural Maintenance of Chromosomes</i> . Deze eiwitcomplexen zijn van humane cellen tot bacteriën belangrijk voor het bij elkaar houden van chromosomen.
Transformatie	Het proces waarbij een bacterie DNA opneemt en incorporeert in het genoom.
Western Blots	Een biochemische methode waarbij specifieke eiwitten door middel van antilichamen gedetecteerd kunnen worden.

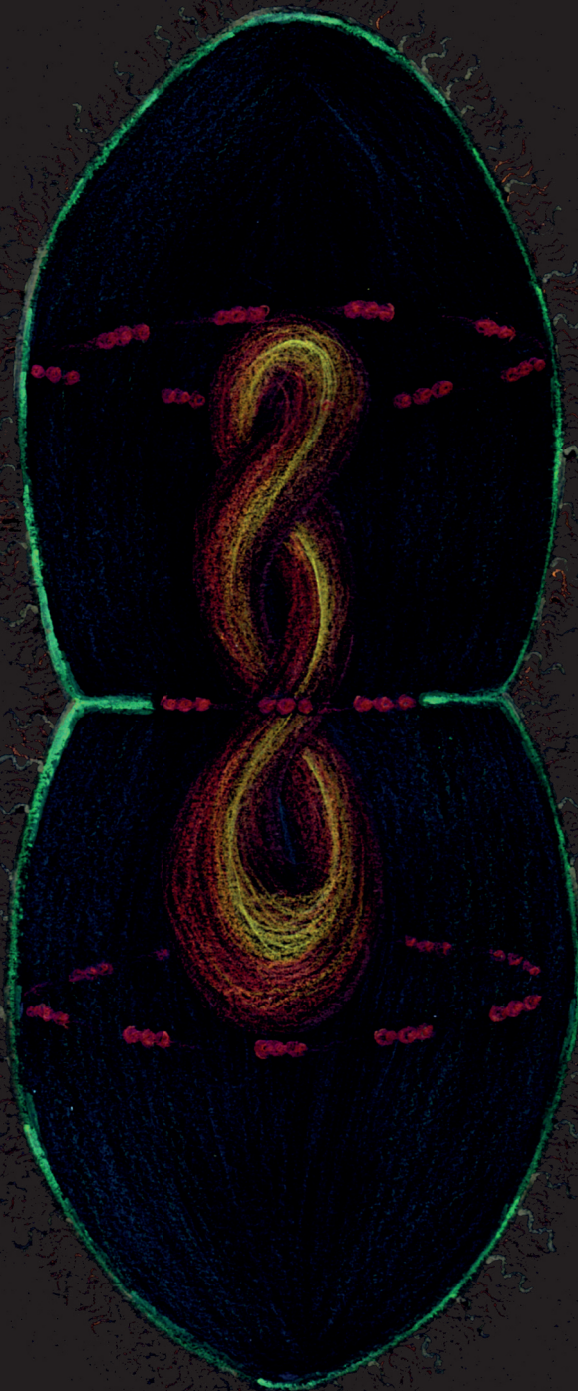


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Popular Summary

Samenvatting voor het brede publiek

Anatomy of the pneumococcal nucleoid

Popular introduction, summary and discussion

Introduction

The shape-shifting bug

Bacteria have been amongst us for as long as we exist. They were there before us and they will most probably outlive us. Still, their existence has been somewhat of a mysterious, philosophical nature for centuries, until researchers started to learn how to grow and study bacteria in the end of the nineteenth century. Many of the things we take for granted now were proven then. For instance, that bacteria cannot appear out of thin air (a claim against the then popular theory of spontaneous generation), that specific bacterial species cause specific diseases (germ theory) and that you can kill many bacteria by heat (pasteurization).

As scientists were learning to understand that bacteria could cause disease, it is not surprising that many studies of that time were focused on medical topics. One of the early-discovered bacteria was *Streptococcus pneumoniae*, also known as the pneumococcus. This bacterium lives in the upper part of the throat behind the nose of many small children and a smaller fraction of adults without causing trouble. However, in young children, elderly or immunocompromised people the pneumococcus can suddenly turn maleficent and cause pneumonia, inner ear infections, meningitis or blood poisoning.

Why this generally harmless bacterium makes the decision to become a dangerous pathogen is one of the biggest mysteries surrounding the pneumococcus. But there is more. When the pneumococcus causes disease, it is notoriously hard to combat. The pneumococcus' outer layer is made of different sugars. This layer acts as an invisibility cloak for the immune system. When the immune system finally figured out what sugars the layer is made of, the battle is not over. The pneumococcus has many different ways changing its outer layer: the pneumococcus can change how many sugars it makes, or even mutate into new variant. Because of this shape-shifting behavior, vaccines against the pneumococcus have to remain updated.

Except vaccines, humans also use antibiotics to fight pneumococcal infections. However, more and more pneumococcal variants are resistant to often-used antibiotics. Many researchers are trying to figure out how the pneumococcus is becoming resistant to antibiotics. One of the important mechanisms seems to be the ability of the pneumococcus to pick up DNA from its surroundings, sometimes encoding for traits that help the pneumococcus to become resistant. This ability, also called *competence*, is the so-many-th reason you can call the pneumococcus a true shape-shifter.

Invisible, taking up DNA from others, sudden killer – there is a whole, but largely incomplete patchwork of information available about this intriguing bacterium. Why does the pneumococcus cause disease? What causes antibiotic resistance? How can we find new antibiotics or vaccines against the pneumococcus?

Instead of looking further at the role of the pneumococcus as a pathogen, this thesis takes a different approach and focuses on gaps in the very basis of the patchwork. What you would almost forget amidst all these pressing questions is the question: how does the pneumococcus grow and divide? Or in other words, how does it work?

How does it work?

What do you do if you want to know how something works? In many cases, you look inside to find out the inner works. For bacteria, the field trying to understand the way bacteria are built

up is called *bacterial cell biology*. Already since the beginning of the previous century, people have tried to Figure out what happens inside bacteria, but there is one very big problem: they are extremely small. The pneumococcus is around $0.5\ \mu\text{m}^3$ in volume – that means it fits more than 50 million times in one grain of table salt! Even compared to human cells, bacteria are very small – hundreds of pneumococci fit into one human cell.

When you think about that, it is no wonder that it is not that straightforward to have a look inside a bacterium. It took a full century and a lot of new biological and technical knowledge before all the tools were there, but now we are truly ready to see the inner works of the pneumococcus.

Goal of this thesis

Because it is impossible to find out the complete inner works of the pneumococcus in one thesis, this thesis focuses on the most important “organ” of the bacterium: the nucleoid. The nucleoid consists of the single chromosome the pneumococcus has, combined with a set of proteins that shape and fold the chromosome into a compact structure. How is the nucleoid positioned in the cell while it is shaped, replicated into two identical copies and separated into two new daughter cells? What is the anatomy of the pneumococcal nucleoid?

Summary

One of the most important methods to map the inside of the bacterial cell is fluorescence microscopy. Because the pneumococcus is so small, you do not see much more than small, rugby-ball shaped black cells when you use regular light microscopy. Using a fluorescent microscope, we can see specific parts of the pneumococcal cells if we label them with fluorescent proteins or fluorescent dyes. Just as your bike light is very visible when it is dark outside, these small lights inside the cells can reveal the inner structures of the cells.

While fluorescent dyes are often toxic to the cells, the fluorescent proteins are made by the pneumococcus itself. Naturally, fluorescent proteins exist in jellyfish and corals, but geneticists have manipulated them into a range of colorful variants. If we want to see where a component of the cell is localized inside the pneumococcus, we alter the genome of the pneumococcus such, that the pneumococcus will make our component of interest with a fluorescent protein attached to it. When this works, we can follow our marked components live in the microscope while the pneumococcal cells are growing.

This is the theory. In practice, not all fluorescent proteins are accepted by the pneumococcus. Others are not working properly inside the cell; fluorescent proteins need oxygen, while the pneumococcus thrives in low-oxygen environments. In **chapter 2**, we tested four Red Fluorescent Proteins in the pneumococcus to see which one is the best suited for genetic studies and cell biological studies. We used the tested Red Fluorescent Proteins along the rest of the chapters.

When you have a working fluorescent protein fusion, the next challenge comes along. You can make microscopy images of millions of cells, but how can you quantify what you see to come to conclusions? For this purpose, there are many *segmentation* programs available. They automatically detect bacterial cells and give back the cell outline coordinates, cell dimensions and for instance the distance of a fluorescent signal to the middle of the cell. To help researchers make sense of this type of data, we developed BactMAP, a software package that works in the statistical language R (**chapter 3**). BactMAP mainly focuses on the automatic import of segmentation data and data visualization.

Now we have the experimental and analysis tools to map the pneumococcal chromosome during cell division. In **chapter 4**, we aimed to understand how chromosome segregation and cell division influence each other. We looked at the localization of the important cell cycle protein FtsZ,

the early cell cycle protein MapZ and the protein DnaX that is involved in the replication of the chromosome. We also tracked the chromosome during the cell cycle by adding fluorescent markers to different locations along the chromosome. By following all these proteins, we could figure out the order of events during the cell cycle.

DNA replication in the pneumococcus happens at the division location in the middle of the cell. The part of the chromosome that is replicated first moves to the future location of division, at the quarter and three-quarter positions of the cell, as does the cell cycle protein MapZ. While the rest of the chromosome is being replicated, the chromosome segregates towards the future division location. The cell cycle protein FtsZ moves to the future division location. Then, DNA-replication is finished. A new round of replication is started at the future division locations and the cell is split into two daughter cells.

To understand more about how this happens, we deleted different genes we knew to be important for either cell division or chromosome segregation. It turned out that when chromosome segregation is perturbed by making mutants, cell division is in trouble too. We conclude that the correct localization of the part of the chromosome that is replicated first is important for cell division at the correct location.

In **chapter 5**, we continue the mapping of the chromosome by focusing on a small protein of which there are thousands of copies inside each pneumococcal cell called HU. We found that HU is binding the complete pneumococcal chromosome, and that removing HU from the cell leads to chromosomes with a fuzzier shape and cells with trouble segregating their chromosomes.

Because HU binds the complete chromosome, we used HU attached to a fluorescent protein as a marker for following the nucleoid as a whole. Using superresolution microscopy, we could make images of the nucleoid shape and dynamics, showing that the nucleoid is changing shape very quickly over time, moving from a “wet towel”-structure to a more straight “pull”-structure. This flexibility might be important for the chromosome to be able to segregate and replicate quickly during the cell cycle.

Discussion

Together, this thesis gives a first view of the anatomy of the pneumococcal nucleoid. Many things still have to be figured out now: now we know a little bit about the inner components of the chromosome cycle, the next question would be: how do these components know where they should go? While there are few candidate proteins that might be important for chromosome organization, we should not forget that the chromosome is a chemical structure too. Chemical and physical forces are influencing the shaping of the chromosome together with the specific proteins. Finding those proteins and understanding how all these forces work together will be the next challenge in creating a map of the inner works of the pneumococcus.



De anatomie van het chromosoom van de pneumokok

Introductie, samenvatting en discussie voor het brede publiek

Introductie

Een verraderlijke veranderaar

Bacteriën leven al met ons samen zolang als we bestaan. Toch was hun bestaan voor lange tijd mysterieus en voer voor filosofen – tot het einde van de negentiende eeuw. In die tijd kwamen onderzoekers erachter hoe ze bacteriën konden kweken en onderzoeken. Veel dingen die we nu als vanzelfsprekend beschouwen werden toen bewezen, bijvoorbeeld dat bacteriën niet uit het niets kunnen ontstaan (een idee dat lang populair was onder wetenschappers en ‘spontane generatie’ werd genoemd). Onderzoekers kwamen er ook achter dat er verschillende soorten bacteriën bestonden die specifieke ziektes konden veroorzaken, en dat veel bacteriën gedood konden worden door middel van verhitting (pasteurisatie).

Wetenschappers kwamen er langzaam achter dat micro-organismen de oorzaak waren van veel ziektes. Het is dan ook niet zo verrassend dat veel microbiologisch onderzoek in die tijd gefocust was op medische onderwerpen. Een van de bacteriën die al snel ontdekt werden was *Streptococcus pneumoniae*, ook wel bekend als de pneumokok. Deze bacterie leeft in het bovenste deel van de keel, achter de neus van veel kleine kinderen en een kleinere groep volwassenen. Daar veroorzaakt de pneumokok over het algemeen geen problemen. Maar soms, in jonge kinderen, ouderen of mensen met een verzwakt immuunsysteem kan de pneumokok ineens veranderen in een gevaarlijke ziekteverwekker die longontsteking, oorontsteking, hersenvliesontsteking en bloedvergiftiging kan veroorzaken.

Waarom deze over het algemeen onschadelijke bacterie ineens gevaarlijk wordt is een van de grootste mysteries rondom de pneumokok. Maar er is meer. Wanneer de pneumokok ziektes veroorzaakt is de bacterie maar lastig te verslaan. De buitenste laag van de pneumokok is van verschillende suikerverbindingen gemaakt. Deze laag werkt als een soort onzichtbaarheidsmantel tegen het immuunsysteem. Wanneer het immuunsysteem uiteindelijk heeft uitgevonden hoe deze buitenste laag eruit ziet is het nog niet uitgespeeld, de pneumokok heeft verschillende manieren om de buitenste laag te veranderen. Dat kan door de dikte te veranderen of zelfs door genetisch te veranderen. Door deze gedaanteverwisselingen moeten vaccins tegen de pneumokok ook vernieuwd blijven.

Behalve vaccins gebruiken we ook antibiotica om pneumokokinfecties te bestrijden. Helaas zijn steeds meer varianten van de pneumokok resistent tegen veelgebruikte antibiotica. Daarom zijn onderzoekers op zoek naar de manieren waarop de pneumokok resistent kan worden tegen antibiotica. Eén van de belangrijke mechanismen is het vermogen van de pneumokok om DNA uit zijn omgeving op te nemen. Soms staan er eigenschappen op dat DNA gecodeerd die de pneumokok helpen om resistent te worden. Het kunnen opnemen van DNA wordt ook wel ‘competentie’ genoemd en is de zoveelste reden dat je de pneumokok met recht een verraderlijke veranderaar kunt noemen.

Onzichtbaar voor het immuunsysteem, DNA van anderen in zich opnemend, een plotselinge ziekteverwekker – er is een flinke, maar voor een groot deel incomplete lappendeken aan informatie beschikbaar over deze intrigerende bacterie. Waarom veroorzaakt de pneumokok ziektes? Wat zorgt ervoor dat hij resistent wordt? En hoe vinden we nieuwe antibiotica of vaccins tegen de pneumokok?

Dit proefschrift kijkt niet verder naar de rol van de pneumokok als ziekteverwekker. In plaats daarvan nemen we een andere benadering en leggen we de focus op de gaten in de naden van de

lappendeken. Wat je bijna zou vergeten tussen alle belangrijke vragen is: hoe groeit en deelt de pneumokok eigenlijk? In andere woorden, hoe werkt de pneumokok?

Hoe werkt het?

Wat doe je als je erachter wilt komen hoe iets werkt? In veel gevallen kijk je er dan in om uit te zoeken hoe het is gebouwd. Voor bacteriën heet het onderzoeksgebied dat erachter probeert te komen hoe bacteriën zijn opgebouwd ‘bacteriële celbiologie’. Al sinds het begin van de vorige eeuw proberen onderzoekers uit te vogelen wat er aan de binnenkant van bacteriën gebeurt, maar er is één groot probleem: ze zijn ontzettend klein. De pneumokok is ongeveer 0.5 kubieke micrometer groot – dat is 50 miljoen keer zo klein als een korrel zout! Zelfs vergeleken met menselijke cellen zijn bacteriën erg klein. Honderden bacteriën passen er in één menselijke cel.

Als je daaraan denkt zie je dat is het niet zo eenvoudig is om in een bacterie te kijken. Het heeft een complete eeuw en veel nieuwe biologische en technische kennis gekost voordat alle benodigdheden er waren, maar nu zijn we er echt klaar voor om de binnenkant van de pneumokok te bekijken.

Doel van dit proefschrift

Omdat het nu eenmaal onmogelijk is om de complete pneumokok in één proefschrift in kaart te brengen, focust dit proefschrift op het meest belangrijke orgaan van de pneumokok: de nucleoïde. De nucleoïde bestaat uit het enkele, cirkelvormige chromosoom van de bacterie, dat opgevouwen is door een hele rits aan eiwitten. De pneumokok groeit door zichzelf in twee identieke dochtercellen te delen. Om te zorgen dat dit goed verloopt, moeten alle onderdelen van de bacteriecel gekopieerd en verplaatst worden naar twee helften van de cel. Tijdens de celgroei wordt het zo ook het chromosoom gekopieerd en de nucleoïde in tweeën gesplitst voordat de cel in tweeën splijt. Hoe werkt dat op zo’n manier dat er geen fouten worden gemaakt? Welke onderdelen zitten waar terwijl dit gebeurt? Ofwel, wat is de anatomie van de nucleoïde van de pneumokok?

Samenvatting

Eén van de belangrijkste methodes om de binnenkant van een bacteriële cel in kaart te brengen is fluorescentiemicroscopie. Omdat de pneumokok zo klein is, zie je niet veel meer dan kleine, rugby-bal-achtige vormen als je gewone lichtmicroscopie gebruikt. Met een fluorescentiemicroscopie kunnen we specifieke delen van de pneumokok zien als we ze labelen met fluorescente eiwitten of stoffen. Net zoals je fietslampje goed zichtbaar is in het donker kunnen deze kleine lichtjes in de cellen de innerlijke structuren van de bacteriecellen blootleggen.

Hoewel fluorescente stoffen vaak ongezond zijn voor de bacteriën, worden de fluorescente eiwitten door de bacteriën zelf gemaakt. Van oorsprong komen fluorescente eiwitten uit kwallen en koralen, maar genetici hebben de fluorescente eiwitten gemanipuleerd tot een heel scala aan kleurrijke varianten. Als we nu een onderdeel van de cel willen zien, veranderen we het genoom van de pneumokok. Hierdoor maakt hij niet langer de gewone variant van dit onderdeel, maar een variant met een fluorescent eiwit eraan vastgeplakt. Als dit werkt, kunnen we de gemarkeerde onderdelen live in de microscoop volgen terwijl de pneumokokcellen aan het groeien zijn.

Dit is de theorie. In de praktijk accepteert de pneumokok niet alle veranderingen in het genoom. Soms werken de fluorescente eiwitten niet goed in de pneumokok: fluorescente eiwitten hebben zuurstof nodig, terwijl de pneumokok het best groeit in een omgeving met lage zuurstofconcentraties. In **hoofdstuk 2** testten we daarom vier verschillende Rood-Fluorescente Eiwitten (RFP's) uit om erachter te komen welke het meest geschikt is voor genetisch en voor celbiologisch onderzoek in de pneumokok. De RFP's die het beste uit de test kwamen gebruiken we in de volgende hoofdstukken.

Als je een werkende fusie van een onderdeel van de cel hebt met een fluorescent eiwit komt de volgende uitdaging om de hoek kijken. Je kunt microscopiefoto's maken van miljoenen cellen, maar hoe kan je op basis van foto's wetenschappelijke conclusies trekken? Een eerste belangrijke stap is het omzetten van de foto's in meetbare data. Hiervoor bestaan er verschillende computerprogramma's die 'segmentatie' uitvoeren. Deze programma's detecteren bacteriële cellen en geven de coördinaten van de celomtrek, celdimensies en bijvoorbeeld de afstand van een fluorescent signaal tot het midden van de cel weer. Om onderzoekers te helpen deze uitgevoerde gegevens op hun beurt te verwerken hebben wij BactMAP ontwikkeld, een softwarepakket dat in de statistische programmeertaal R geschreven is (**hoofdstuk 3**). BactMAP verzorgt de automatische import van segmentatiedata en visualiseert deze data.

Nu hebben we het experimentele gereedschap en de analysetools in handen om het chromosoom van de pneumokok in kaart te brengen tijdens de celdeling. In **hoofdstuk 4** is ons doel een beter begrip te krijgen van hoe chromosoomsegregatie – het uit elkaar trekken van het chromosoom – en celdeling elkaar beïnvloeden. We keken naar de locatie van belangrijke onderdelen: de celdelings eiwitten FtsZ en MapZ en het eiwit DnaX, dat een rol speelt in het kopiëren van het DNA tijdens de celdeling. We volgden ook het chromosoom tijdens de celcyclus door fluorescente labels op verschillende locaties op het chromosoom te plaatsen. Door de eiwitten en chromosoomlocaties te volgen konden we het verloop van de celcyclus in kaart brengen.

DNA-replicatie, het kopiëren van het chromosoom in twee identieke exemplaren, gebeurt in de pneumokok in het midden van de cel. Dit is ook het punt waar de cel uiteindelijk in tweeën deelt: het celdelingspunt of het septum. Het deel van het chromosoom dat als eerste werd gekopieerd beweegt zich weg van het midden. De kopie beweegt naar de ene kant, het origineel naar de andere kant van de cel. Beiden bewegen zich naar de plaatsen waar de dochtercellen hun celdelingspunt zullen maken. Het celdelings eiwit MapZ, dat als een ring om het celdelingspunt zit, beweegt zich in gelijke richting: de ring split in twee ringen naar de nieuwe celdelingspunten. Terwijl de rest van het chromosoom gekopieerd wordt, blijven de kopie en het origineel naar de beide kanten van de cel bewegen. Uiteindelijk zal het celdelings eiwit FtsZ, dat als een ring op het celdelingspunt zit, plotseling dezelfde beweging maken als MapZ. Dan is DNA-replicatie klaar en begint de pneumokok een nieuwe ronde van DNA-replicatie op de nieuwe celdelingspunten, en wordt de cel in twee dochtercellen opgesplitst.

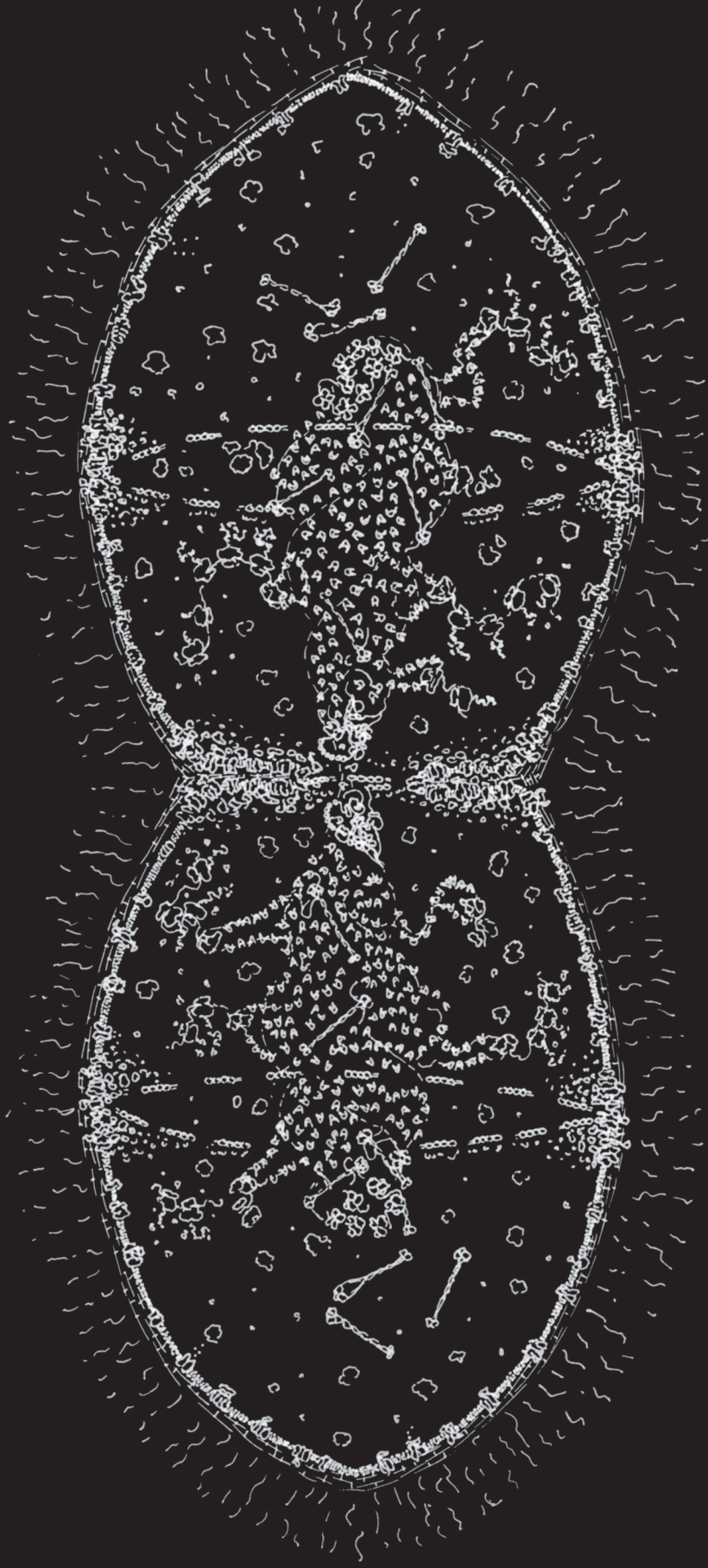
Om meer te begrijpen over hoe dit gebeurt, hebben we verschillende genen verwijderd waarvan we weten dat ze belangrijk zijn voor oftewel celdeling of chromosoomsplitsing. Het bleek dat wanneer de splitsing van het chromosoom niet meer goed gaat, celdeling ook niet meer goed werkt. Daarom concluderen we dat de correcte plaatsing van het eerst-gekopieerde deel van het chromosoom op de nieuwe celdelingspunten cruciaal is voor een goede celdeling.

In **hoofdstuk 5** gaan we verder met het in kaart brengen van de nucleoïde door in te zoomen op een klein eiwit waarvan er zich duizenden in elke pneumokok bevinden: HU. We zagen dat HU zich bindt aan het gehele chromosoom. Het verwijderen van HU zorgt voor minder compact gevouwen chromosomen die problemen hebben met chromosoomsegregatie.

Omdat HU zich op het gehele chromosoom bevindt, gebruikten we aan een fluorescent eiwit gekoppelde HU als een label voor de nucleoïde. Met gebruik van superresolutiemicroscopie, speciale technieken waarmee je nog kleinere dingen kunt onderscheiden dan met normale microscopie, konden we de vorm van de nucleoïde beter onderscheiden dan ooit. We konden de nucleoïde tijdens de celcyclus volgen, wat duidelijk maakte dat de nucleoïde een sterk veranderlijke vorm heeft, die switcht tussen een soort helix (een verwrongen handdoek) en een rechte structuur. Dat de nucleoïde flexibel is kan belangrijk zijn voor het chromosoom om snel gekopieerd te kunnen worden en daarna snel naar twee kanten te kunnen bewegen.

Discussie

Alles bij elkaar geeft dit proefschrift een eerste blik op de anatomie van de nucleoïde van de pneumokok. Veel dingen zijn nog onbekend: hoe weten al deze onderdelen van de celcyclus wanneer ze waar heen moeten? Hoewel er een aantal eiwitten belangrijk zijn voor het splitsen van het chromosoom, moeten we ook niet vergeten dat het chromosoom ook chemische eigenschappen heeft. Chemische en natuurkundige krachten spelen hoogstwaarschijnlijk een rol in het vormen van het chromosoom, al dan niet samen met nog onbekende eiwitten. De zoektocht naar deze nieuwe eiwitten, samen met het leren begrijpen hoe deze krachten samenwerken zal de volgende stap zijn in het beschrijven de anatomie van de nucleoïde van de pneumokok.



Tot slot

Acknowledgements

About the author

Publications

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About the author

Renske van Raaphorst was born on the island Texel in the north of the Netherlands. In 2007 moved to Groningen to study Life Science & Technology with a Major on Molecular Life Sciences and a Minor in Greek Mythology and Rhetorics. Interested in computational biology, she decided to do her bachelor thesis on computational tools for systems biology and synthetic biology with Marnix Medema, Eriko Takano and Rainer Breitling. Renske continued her studies in Groningen with a master in Molecular Biology and Biotechnology. She did one six-month internship with Matthias Heinemann in Groningen on the enzymatic activity of hexokinase, and one with Gürol Suel in San Diego on heterogeneity in *B. subtilis* biofilms. In between these two internships Renske joined the 2012 iGEM team of Groningen. This is how she came in contact with Jan-Willem Veening, whos group she joined as a PhD student in the end of 2013. After three years Jan-Willem's group moved to the University of Lausanne in the beginning of 2017. In August Renske will start as a postdoctoral fellow in the group of Géraldine Laloux at the University of Louvain.

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