Regulation of Cell Division in Streptococci: Comparing with the Model Rods

Zhenting Xiang[1], Zongbo Li[1], Jumei Zeng[2], Yuqing Li[1]*, Jiyao Li[1]*

[1]State Key Laboratory of Oral Diseases, National Clinical Research Center for Oral Diseases, West China Hospital of Stomatology, Sichuan University, Chengdu, PR China.

[2]Department of Infectious Diseases, Boston Children's Hospital, Harvard Medical School, Boston, Massachusetts, USA.

*Corresponding authors: liyuqing@scu.edu.cn, jiyaoliscu@163.com

Abstract

Streptococcus is a genus of oval-shaped bacteria that act as both commensals and pathogens. Streptococcal infections are relevant to high morbidity and huge socioeconomic costs, with drug resistant strains becoming an increasing threat. Cell division plays an essential role during streptococcal colonization and infection, rendering it an ideal target for antibiotics. Substantial progress has been made to uncover the molecular biology and cellular processes of cell division, favoring the target strategies. This review discusses recent advances in

our understanding of streptococcal cell division and its regulatory mechanisms regarding the conserved proteins, by comparing with model rods. Peptidoglycan synthesis that involved in septum formation and the maintenance of the unique oval shape have been spatiotemporally controlled in concert with the pace of division. With newly available tools of genetic and cytological study, streptococci will become an additional model bacterial system for cytokinesis and novel therapeutic agents that target cell division.

Introduction

Most bacteria divide into two identical progeny cells by binary fission, which requires the initial formation of a division septum. Extensive studies have elucidated several aspects of cell division in model rod-shaped bacteria *Escherichia coli* and *Bacillus subtilis* (Adams and Errington, 2009; Haeusser and Margolin, 2016). Such studies have revealed that cytokinesis is coordinated by the divisome: a protein complex assembled at the future site of cell division. Cell division begins with the assembly of the conserved tubulin homologue FtsZ, which performs a GTP-dependent polymerization (Scheffers et al., 2002) to form a contractile ring-like structure (Z ring) at the future site of cytokinesis and recruit downstream components of the macromolecular divisome (Egan and Vollmer,

2013). After maturation, divisome constricts the cell membrane in coordination with new peptidoglycan (PG) synthesis at the division plane, leading to the separation of two daughter cells (Adams and Errington, 2009; Haeusser and Margolin, 2016). Bacterial cell division requires tight regulation and precise spatiotemporal coordination of Z ring assembly to ensure equality of the progenies and genomic integrity (Wu and Errington, 2011). The components involved are of critical importance for the propagation and viability of bacteria, and thus play essential roles for pathogenic species in the occurrence of infectious diseases.

The interest in cell division of pathogens started mainly with the intention to identify and characterize targets to develop novel broad-spectrum antimicrobials. The mounting studies on bacterial cell division not only make significant advances in the biological process, they also help us to identify effective cell division inhibitors, especially for pathogens. However, knowledge on the precise roles of cell division proteins mainly lies in model organisms, it is important to characterize and validate the function of cell division proteins in pathogenic species such as *Streptococcus*.

Streptococcus is a genus of Gram-positive bacteria that consists of 122 recognized diverse species (http://www.bacterio.net/streptococcus.html). Despite the relationship between host and streptococci varying from commensal to pathogenic, they can cause serious diseases, ranging from dental caries and pharyngitis to pneumonia and meningitis (Mitchell, 2003; Shak et al., 2013). In addition to numerous deadly infections, non-lethal streptococcal infections are associated with high morbidity and high socioeconomic costs. Drug resistant *Streptococcus pneumoniae,* a human commensal bacterium of streptococcus, was listed in Antibiotic Resistance Threats Reports-2013 to be a severe threat by the Centers for Disease Control and Prevention (CDC) (https://www.cdc.gov/drugresistance/threat-report-2013/).

Streptococcal cell division plays important roles during colonization and infection stages, but many questions regarding molecular biology and cellular processes of cell division remain to be answered (Engholm et al., 2017). Although some fundamental cytokinetic mechanisms in best studied model rods are similar with streptococci, the regulatory systems of bacterial cell division show general specificities, with many additional proteins conserved in the divisome. Herein, after a summary about recent discoveries regarding dynamics of Z-ring assembly

and PG synthesis in model rods, we discuss insights into streptococcal Z-ring and cell wall, comparing similarities and differences with model rods. We intend to review these current studies critically and compare similar or distinct views upon streptococcal cell division to provide clues on further research and novel therapeutic agents.

Cell-division pathway in model rods

Z ring dynamics in rods

FtsZ is a cytoplasmtic protein that contains two termini connected by a central core helix. The highly conserved N-terminal globular domain is responsible for polymerization, while the carboxy terminal domain consists of a structured folded part, a flexible linker of variable sequence and length, and a short alpha helix C-terminal end (the central hub) that forms binding sites for several division proteins (Buske and Levin, 2013; Lowe and Amos, 1998; Ma et al., 1996). GTP promotes the FtsZ assembly into tubulin-like protofilaments by head-to-tail monomeric association, leading to initial formation of a ring-like structure (de Boer et al., 1992; Scheffers et al., 2002). When the cell cycle repeats, GTP hydrolysis is coupled to the disassembly of FtsZ.

Although FtsZ is an essential element for membrane constriction during cytokinesis, it does not attach with direct affinity to the cytoplasmic membrane. In *E.coli,* FtsA and ZipA cooperate to tether FtsZ polymers to the inner surface of cytplasmic membrane (Du and Lutkenhaus, 2017; Hale and de Boer, 1997; Ortiz et al., 2016; Pichoff and Lutkenhaus, 2002).

FtsA, which acts as the second most conserved division protein among bacterial species, is structurally homologous to actin. However, unlike other members of the actin family, FtsA contains the four subdomains 1A, 1C, 2A and 2B, with the absence of 1B subdomain adjacent to the 2B subdomain and the addition of 1C subdomain on the opposite site (Szwedziak et al., 2012). The 2B subdomain binds to the central hub of FtsZ while a C-terminal amphipathic helix, which functions as a membrane tageting sequence (MTS), anchors to the membrane (Pichoff and Lutkenhaus, 2005). ATP binding is important for FtsA to recruit FtsZ on a lipid monolayer and to stimulate FtsZ polymerization dynamics, while on the other hand, FtsA exhibits negative regulation on FtsZ filament network organization. The two antagonistic functions of FtsA facilitates Z-ring diameter adjustment during constriction (Loose and Mitchison, 2014). Furthermore, the

increase of FtsA oligomerization or polymerization is essential for the integrity of the Z-ring (Shiomi and Margolin, 2007).

Conversely, the bitopic protein ZipA is conserved in Gammaproteobacteria and comprises three domains: a large cytoplasmic C-terminal domain, a basic arm rich in proline and glutamine residues, and an N-terminal transmembrane end (Mosyak et al., 2000). The ZipA C-terminal globular domain contains a binding site that interacts directly with the C-terminal tail of FtsZ to recruit to the assembling Z-ring (Hale and de Boer, 1997). It is thought that ZipA can recruit FtsZ monomers, producing a stronger but inflexible physical link between FtsZ and the membrane, whereas FtsA can only recruit FtsZ polymers, and is a potentially reversible membrane tether (Haeusser and Margolin, 2016; Loose and Mitchison, 2014). FtsA*, a gain-of-function mutant, can compensate for the loss of ZipA during cell division (Geissler et al., 2003). Moreover, a gain-of-functional FtsZ mutant is capable of both bypassing ZipA requirements during divisome assembly and enhancing FtsZ polymer bundling (Haeusser et al., 2015). These results support the hypothesis that ZipA can self-associate into homodimers through its N-terminal domain and could potentially facilitate FtsZ polymer bundling and stability (Skoog and Daley, 2012). In addition, the

interaction between FtsZ and ZipA protects FtsZ from degradation by the housekeeping protease complex ClpXP (Pazos et al., 2013). This activity cannot be performed by FtsA*, suggesting additional roles of ZipA other than a Z-ring tether.

In other Gram-positive species that lack ZipA, such as *B. subtilis*, it is speculated that the FtsZ-interacting protein SepF (previously known as YlmF) (Duman et al., 2013) and the FtsZ assembly inhibitor EzrA (Cleverley et al., 2014), which share the same membrane topology with ZipA, may provide alternatives to the role of ZipA. SepF is located in a broadly conserved gene cluster in most Gram-positive bacteria (Hamoen et al., 2006) and serves as an alternative membrane anchor of FtsZ in *B. subtilis* (Duman et al., 2013). In other cases, SepF was reported as an essential tether for FtsZ in mycobacteria (Gupta et al., 2015). Another potential tether in low-GC-content Gram-positive species is EzrA, whose inactivation in *B. subtilis* results in extra Z rings. Containing a single N-terminal transmembrane domain followed by a cytoplasmic domain, EzrA inhibits Z-ring assembly at cell poles and forms an interface between Z-ring and cell wall biosynthesis through an indirect role in recruitment of PBPs (Cleverley et al., 2014; Land et al., 2014).

Spatial regulation in cell division of rods

The Z-ring must tether to the membrane and position precisely at the future division site, usually at the mid-cell. There are two main negative regulatory mechanisms for this in rods: the nucleoid occlusion (NO) and the Min system (Monahan et al., 2014; Rowlett and Margolin, 2015).

The NO system prevents Z ring assembly proximal to the nucleoid or chromosome (Wu and Errington, 2011). SlmA, a DNA-binding protein that serves as an NO system effector in *E. coli,* binds to specific DNA sequences as an orientated dimer that interacts with the FtsZ C-terminal central hub, preventing FtsZ protofilament propagation and bundling (Du and Lutkenhaus, 2014; Schumacher and Zeng, 2016; Tonthat et al., 2013). However, the FtsZ C-terminal domain binds with SlmA as an extended conformation in a narrow, surface-exposed pocket. This conformation occurs rather than as a helix only in the SlmA DNA-bound state, indicating a wide range of conformations are held by the FtsZ C-terminal domain (Schumacher and Zeng, 2016). The GTPase activity of FtsZ is unaffected by the effect of SlmA on accelerating FtsZ fragmentation (Cabre et al., 2015). However, the molecular mechanism by which SlmA inhibits Z-ring assembly is yet to be fully revealed. In *B. subtilis*, Noc, similarly to SlmA,

binds to specific DNA sequences, but their modes of action are different. Noc binds to the cell membrane via its N-teminal amphipathic helix, where it simultaneously recruits DNA sequences to function as a division inhibitor (Adams et al., 2015).

The Min system inhibits FtsZ polymerization, blocking Z ring assembly at the cell poles to produce nonviable anucleated minicells. MinD is an ATPase that forms a dimer upon ATP binding in order to associate with the cytoplasmic membrane. Upon binding to MinD, MinC activates and directly interacts with FtsZ to antagonize the longitudinal interactions of FtsZ subunits within a protofilament, the lateral interactions between FtsZ protofilaments, and also the interactions with FtsA and ZipA (Arumugam et al., 2014).

Specifically, to prevent FtsZ assembly at the cell poles, MinE, a topological factor that spatially restricts the inhibitory MinCD complex is needed. In *E. coli*, MinE harbors an amphipathic helix at its N-terminus that serves as an MTS. This helix can stimulate MinD ATPase when sensing a MinD dimer at the membrane. which leads to MinD monomerization and its removal from the membrane (Bonny et al., 2013; Park et al., 2011). The soluble MinCD complex rebinds to the

membrane distal to its most recent complex, which is the opposite pole where MinE is absent, through ADP-ATP exchange. The movement of MinCD results in a pole-to-pole oscillation of the Min system governed by MinE conformational changes (Park et al., 2011). More recently, hydrogen-deuterium exchange coupled to mass spectrometry (HDX-MS) was used to examine the conformational dynamics of MinE, suggesting the MTS renders MinE interaction with the membrane dynamic and reversible (Park et al., 2017). Moreover, proteins recruited during early stages of divisome assembly, including FtsZ, ZipA, ZapA, and ZapB, counter-oscillate with the Min system (Bisicchia et al., 2013).

The min system in *B. subtilis* differs from that of *E. coli* because of the lack of MinE. Instead, *B. subtilis* contains DivIVA, which undergoes a curvature-based localization mechanism to localize MinCD to the poles of the cell via the bridging protein MinJ (Bramkamp et al., 2008; Eswaramoorthy et al., 2011). The Min system putatively formed a static bipolar gradient of MinC highest at the cell poles and lowest at the mid-cell to inhibit cell pole Z-ring assembly. However, evidence shows that Min proteins are recruited to the mid-cell prior to septation and prevent polar FtsZ rings forming adjacent to new cell poles, and also promote

cytokinetic ring disassembly to establish a new bipolar gradient in daughter cells (Gregory et al., 2008; van Baarle and Bramkamp, 2010).

Recent data show that NO and Min systems are not alone at play in *E. coli* and *B. subtilis*. Other mechanisms, not well identified yet, likely contribute to determining the mid-cell very early during the cell cycle (Rodrigues and Harry, 2012). In bacteria that lack NO and/or Min systems, negative and positive regulators of Z ring formation have been identified, highlighting a diversity of division site selection mechanisms. In *Caulobacter crescentus*, which lack Min and NO proteins, MipZ forms a bipolar gradient that acts as an FtsZ inhibitor, providing spatiotemporal cues for Z ring formation (Kiekebusch et al., 2012). Positive spatial regulators include SsgAB proteins in *Streptomyces coelicolor,* which localize to future sites of multiple septation (Willemse et al., 2011), and PomZ that recruits FtsZ to the division site in *Myxococcus xanthus* (Treuner-Lange et al., 2013).

Peptidoglycan synthesis regulation in rods

Rod-shaped models require robust mechanisms to maintain and pass on their characteristic shape; as a result, cell growth and cell division are closely linked

during the cell cycle (Willis and Huang, 2017). First, dispersed elongation is achieved with the cooperation of MreB, an actin-like rod-shape-determining protein. In the process, PG inserts into multiple sites in the lateral wall of newly divided cells to elongate the cell along the longitudinal axis. Soon after, FtsZ localizes to the mid-cell to guide a 'preseptal' phase of cell elongation, followed by 'constrictive' septum synthesis, which enables cell division and daughter cell separation (Typas et al., 2011).

Cell elongation requires a set of proteins to assemble into elongation machinery called the 'elongasome' (Egan et al., 2017; Errington, 2015). The elongasome is a membrane-spanning complex comprising of MreC, MreD, RodA, RodZ and PG cell-wall biosynthetic enzymes PBP1A and PBP2. MreB (and orthologues Mbl and MreBH in *B. subtilis*) localize to the lateral wall of the bacterial cell and spatiotemporally organize the elongasome (Jones et al., 2001). Its crucial role in directional PG insertion was further verified in *E. coli*, where MreB point mutations can tune cell size over a broad range (Monds et al., 2014). Until recently, a generally accepted model of cell wall synthesis proposed MreB guides class A PBPs, which in turn produce PG strands via transglycosylation domains. Class A PBPs (aPBPs) and class B PBPs (bPBPs) then crosslink these strands

into a tight PG mesh, fitting new material into cell wall gaps provided by the cleavage activity of autolysins (Gopalani et al., 2016). MreB interacts closely with RodA and RodZ in *E. coli* (Morgenstein et al., 2015) and the latter mediates indirect interaction between MreB and a bPBP. The rotation of MreB patches around the cell depending on bPBP transpeptidation activity and the presence of RodA. The established “Rod complex” (MreB-RodAZ-bPBP) has been suggested by recent works to mediate cell wall synthesis primarily during cell elongation (Zhao et al., 2017).

The relationships between elongation and division machineries are not well-understood, however, there is evidence that a direct interaction between MreB and FtsZ is required for Z-ring contraction, owing to its role in transfer of cell-wall biosynthetic enzymes (Fenton and Gerdes, 2013).

Cell division pathway in *Streptococcus*

Z-ring dynamics in streptococci

FtsA was found to colocalize with FtsZ during exponential growth in *Streptococcus*, consistent with the findings in model rods. The first FtsA protein isolated from *S. pneumoniae* exhibited *in vitro* activity whereby FtsA filaments

assembled into large helical structures in the presence of ATP, but did not hydrolyze ATP or depolymerize (Lara et al., 2005). Polymerization and membrane attachment of FtsA is favored by ATP binding through a molecular mechanism by which binding triggers a conformational switch of the amphipathic helix located at the C-terminus. This conformational change accompanies the membrane attachment and facilitates its polymerization (Krupka et al., 2014; Mura et al., 2016). (Table 1)

The FtsZ inhibitor EzrA was hypothesized to function in division site selection in a previous review (Pinho et al., 2013). EzrA is an early cell division protein with a partially redundant role in *B. subtilis.* In *B. subtilis*, EzrA depletion results in a strong phenotype only when combined with other cell division gene mutations, such as *noc* or *gpsB* (a homologue of *divIVA* that is involved in controlling the cell-elongation division cycle, see below). Interestingly, EzrA is essential in *S. pneumoniae* and certain *S. aureus* strains, suggesting it is not as functionally redundant in cocci as *B. subtilis* (Steele et al., 2011; Thanassi et al., 2002). A conflicting result suggests EzrA is non-essential for *S. aureus* viability, but is required for cell size homeostasis through the coordination of proper FtsZ dynamics at mid-cell. EzrA might prevent Z-ring formation near the cell poles of

Table 1. Proteins that involved in cell division in streptococci.

Protein	Function	Localization	References
FtsZ	Tubulin homologue that forms a contractile ring structure (Z ring) at the future cell division site.	Septa	(van Raaphorst et al., 2017)
FtsA	Actin-structural homologue serve as a membrane anchor for the Z ring.	Septa	(Krupka et al., 2014; Mura et al., 2016)
MapZ	Forms ring structures at mid-cell that move apart as peptidoglycan synthesis elongates the cell to mark the future cell division site.	Septa	(Fleurie et al., 2014a; Li et al., 2018)
ZapA, B	Mediate additional stabilization of the Z-ring.	Not determined	(Maggi et al., 2008)
SepF (YlmF)	Cell division protein that is part of the divisome complex and is recruited early to the Z-ring. Its function overlaps with FtsA.	Septa	(Mura et al., 2016)
EzrA	A negative regulator of FtsZ ring formation that modulates the frequency and position of FtsZ ring formation.	Septa	(Rued et al., 2017)
FtsE, X	Cell division ABC transporter ATP-binding protein	Septa	(Sham et al., 2013)
FtsK(SpoIIIE)	SpoE family protein that coordinates cell division and chromosome segregation.	Septa	(Massidda et al., 2013)
DivIB(FtsQ)	Cell division protein that may be involved in stabilizing or promoting the assembly of the division complex	Septa	(Le Gouellec et al., 2008; Masson et al., 2009; Noirclerc-Savoye et al., 2013)
DivIC(FtsB)	Cell division protein that may be involved in stabilizing or promoting the assembly of the division complex	Septa	(Masson et al., 2009; Noirclerc-Savoye et al., 2013)
FtsL	Cell division protein that may play a role in septal PG synthesis.	Septa	(Masson et al., 2009; Noirclerc-Savoye et al., 2013)
FtsW	Septal PG synthesis	Septa	(Maggi et al., 2008; Morlot et al., 2004)
PBP1a	PG glycosyltransferase/transpeptidase for septal and peripheral PG synthesis	Septa	(Land and Winkler, 2011; Wen et al., 2015)
PBP2a	PG glycosyltransferase/transpeptidase, septal PG synthesis?	Septa	(Fenton et al., 2018; Rued et al., 2017)
PBP2b	PG transpeptidases for peripheral PG synthesis.	Septa	(Sharifzadeh et al., 2017; Straume et al., 2017)
PBP2x(FtsI)	PG transpeptidase for septal PG synthesis and remodeling.	Septa	(Sharifzadeh et al., 2017; Tsui et al., 2014)

Table 1. continued.

Protein	Function	Localization	References
PBP3 (DacA)	A D, D-carboxypeptidase that cleaves the ultimate D-Ala from PG peptides.	Cell surface	(Kocaoglu et al., 2015; Morlot et al., 2004; Morlot et al., 2005)
GpsB	A molecular switche that balances septal and peripheral PG synthesis.	Septa	(Rued et al., 2017)
DivIVA	A protein of divisome that is required to correctly localize the elongasome between the septal and lateral cell wall.	Septa and poles	(Ni et al., 2018; Straume et al., 2017)
PcsB (GbpB in *S. mutans*)	PG hydrolase that is required for septa splitting.	Septa	(Bartual et al., 2014; Duque et al., 2011; Sham et al., 2011)
LytB	PG hydrolase that is required for final separation of the daughter cells.	Septa	(Arrigucci and Pozzi, 2017; Zucchini et al., 2017)
MreC, D	Regulator that directs peripheral PG synthesis and control PBP1a localization or activity.	Septa	(Land and Winkler, 2011; Stamsas et al., 2017)
RodZ	Peripheral PG synthesis.	Not determined	(Stamsas et al., 2017; Straume et al., 2017)
RodA	A lipid II flippase and PG polymerase that related to peripheral PG synthesis.	Septa	(Stamsas et al., 2017)
StkP	Serine/threonine kinase; control of cell division	Septa	(Grangeasse, 2016; Zucchini et al., 2017)
PhpP	Phosphatase; control of cell division	Septa	(Martin et al., 2017)
Pmp23	A cell wall hydrolase that is important for proper localization of the Z-ring and MapZ.	Septa	(Jacq et al., 2018; Zucchini et al., 2017)
MacP	A substrate for the kinase StkP that functions as a membrane-anchored cofactor of PBP2a.	Septa	(Fenton et al., 2018)

ovococci, as has been suggested for *B. subtilis.* The model of EzrA with FtsA and FtsZ trapped inside its arch structure was largely based on a recent crystal structure of a homologous protein from *Bacillus subtilis* solved by Cleverley *et al,* who also suggested FtsA and FtsZ filaments position on the side of the EzrA bridge (Cleverley et al., 2014) (Figure 2).

Spatial regulation in cell division of Streptococci

Genomes of *Streptococcus* show an absence of the Min system and nucleoid occlusion effector homologs (Pinho et al., 2013). The lack of NO is further supported by observations that Z-rings in *S. pneumoniae* frequently form over nucleoids and cell constriction occurs concurrently with the separation of the DNA (Land et al., 2013). It was previously speculated that DivIVA functions with ParB to anchor the chromosome and provide a polar gradient of DNA in *S. pneumoniae* (Fadda et al., 2007), but no precise evidence has shown this. This suggests that the septation site selection in *Streptococcus* might be unique, unlike mechanisms present in the rod-shaped *E. coli* and *B. subtilis,* which are based on inhibition of Z-ring assembly.

Recently, studies in *S. pneumoniae*, have demonstrated that a positive mechanism occurs by which FtsZ becomes attracted towards the septation site (Fleurie et al., 2014a; Holeckova et al., 2014). MapZ, also known as LocZ is a transmembrane protein that acts as a molecular beacon to permanently signal the future division site, and was described as an “all-in-one” positive regulatory system (Garcia et al., 2016). Cells lacking MapZ are viable but severely deformed, indicating MapZ is not absolutely required for Z-ring formation, but is essential for its correct placement. MapZ localizes as rings to new division sites prior to FtsZ and FtsA (Holeckova et al., 2014). The MapZ extracellular domain then recognizes and tethers to the nascent PG synthesized at the mid-cell, and its cytoplasmic domain provides a physical anchor for FtsZ. Once the mid-cell Z ring forms, the MapZ-ring splits into two new rings which gradually move apart as the cell elongates from mid-cell to the cell quarters: the sites of the next division. As the two MapZ-rings move, a third MapZ-ring positions at the constricting division site and possibly regulates the cell constriction until the division is complete (Fleurie et al., 2014a). It was thus proposed that MapZ firmly attached to cell wall and shuttled toward the cell equator by the PG elongating the cell (Figure 1). In support of this, further structure-function analysis was performed for molecular characterization of MapZ (Manuse et al., 2016). The extracellular domain of

MapZ undergoes a new bi-modular structure composed of two subdomains: a C-terminal subdomain with conserved patch of amino acids that play a crucial function in binding PG and positioning MapZ at the cell equator, and an N-terminal subdomain that serves as an indispensable pedestal for the C-terminal subdomain. The two subdomains are separated by a flexible serine-rich linker.

Conserved in the *Streptococcaceae* and most other *Lactobacillales*, MapZ is regulated through phosphorylation by the serine/threonine protein kinase StkP, which is involved in the stabilization of the Z-ring and does not affect FtsZ polymerization or GTPase activity (Fleurie et al., 2014a; Li et al., 2018) (Figure 2). Moreover, PG synthesis colocalized with irregular FtsZ structures in *mapZ* mutant, suggesting that cell wall synthesis in *S. pneumoniae* is constantly associated with the divisome (Bramkamp, 2015).

Chromosome segregation in Streptococci

The pneumococcal ParB protein is a homologue of the Noc protein that mediates nucleoid occlusion in *B. subtilis* (Wu and Errington, 2011). However, pneumococcal *ΔparB* mutants are viable and accumulate only a low number (<

5%) of anucleate cells (Minnen et al., 2011). Likewise, pneumococcal Δ*smc* mutants (which lack a nucleoid condensin homologue) and double Δ*smc* Δ*parB* mutants show mild nucleoid segregation defects (Minnen et al., 2011). Another candidate that likely plays an important role in nucleoid segregation in *S. pneumoniae* is FtsK (SpoIIIE), which is a divisome component belonging to a DNA pump superfamily (Demarre *et al*., 2013). The pneumococcal *ftsK* (*spoIIIE*) gene (*sp0878*) is essential in serotype 4 strain TIGR4, but the role of FtsK (SpoIIIE) in pneumococcal nucleoid separation remains to be determined (Massidda et al., 2013; Pinho et al., 2013; van Raaphorst et al., 2017).

Peptidoglycan synthesis regulation in Streptococci

Cell division requires the coordination of synthesis and remodeling of the cell wall PG. *Streptococcus* are ovoid cocci (named 'ovococci') (Pinho et al., 2013; Zapun et al., 2008) and models for their cell wall synthesis have been well-reported during recent decades (Higgins and Shockman, 1970; Massidda et al., 2013; Morlot et al., 2003; Philippe et al., 2014; Zapun et al., 2008). The two-site model for streptococcal growth and division is based on early ultrastructure reconstruction (Higgins and Shockman, 1976) where two systems, septal and peripheral PG synthesis machineries, both localize to the septum. Unlike the

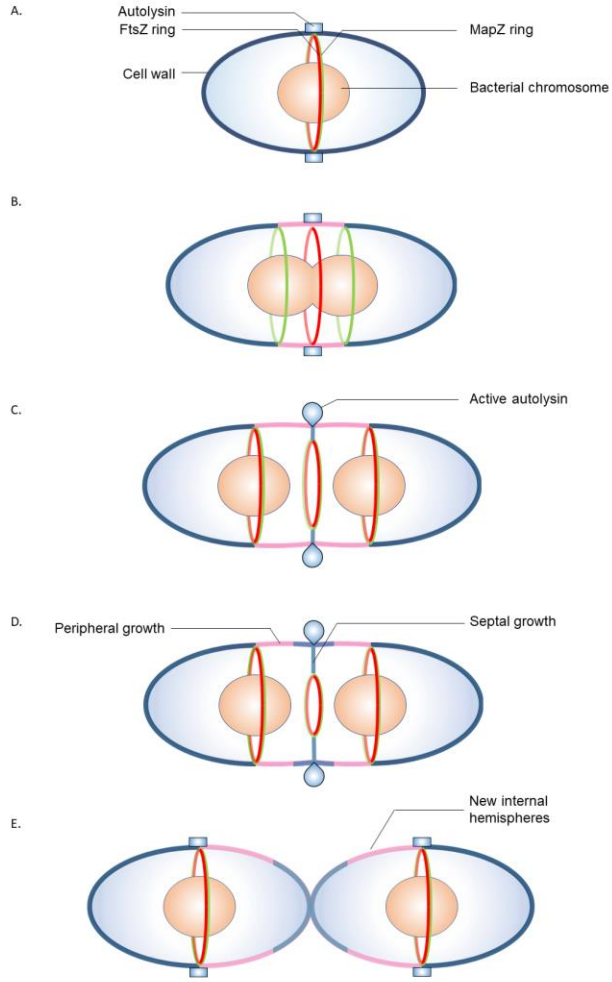

Figure 1. Division site selection and cell wall synthesis during streptococcal cell cycle. (A) Rings formed by FtsZ (red) and MapZ (green) are localized at divisional site. (B) MapZ ring splits into two rings and move apart as cell elongation achieved by PG synthesis. (C) The two MapZ rings migrate to the future division sites of daughter cells while a third MapZ appears at mid-cell. FtsZ rings then relocalize at the beacon of MapZ rings at the future division sites. Peripheral PG synthesis occurs in close proximity to the division site to insert new PG insertion between the present and the future division sites to achieve cell elongation while septal PG synthesis to achieve cell invagination. (D) Rings (FtsZ and MapZ) at mid-cell constrict in concert with new PG synthesis. (E) New internal hemispheres are formed and the resulted daughter cells with FtsZ and MapZ rings localized at mid-cell.

more spherical cocci such as *S. aureus*, streptococcal elongation mode is intimately linked with cell division, whereby cell growth proceeds and overlaps cell division, resulting in the characteristic prolate cell poles.

After an initial inward growth of the cross wall, the ovococcal cell forms an equatorial ring at the mid-cell where cell division begins. Two new equatorial rings are formed on both sides of the original ring, defining the new mid-cell growth zone of the two daughter cells. New PG is inserted in-between the two new equatorial rings, resulting in peripheral growth characterized by simultaneous elongation and constriction. This occurs until the newly formed hemispheres have the same length as the old ones, which is followed by septal PG synthesis to close the septum and PG hydrolyzes to separate the daughter cells (Massidda et al., 2013) (Figure 1). A more recent study confirms this model using super-resolution three-dimensional structured illumination fluorescence microscopy of cells with labeled PG synthesis sites (Wheeler et al., 2011).

The PG synthesis machines produced by *S. pneumoniae* are divided into 3 different types: three class A bifunctional PBPs (PBP1a, PBP1b and PBP2a) that have both transglycosylase (TG) and transpeptidase (TP) activities; two class B

monofunctional transpeptidases (TPs) (PBP2x and PBP2b); and one D, D-carboxypeptidase PBP (PBP3 or DacA) (Kocaoglu et al., 2012; Massidda et al., 2013). Both the mid-cell located pneumococcal PBP2x and PBP2b that correspond to PBP3 and PBP2 in *E.coli,* respectively (Egan and Vollmer, 2013), are individually essential, and PBP2x is putatively involved specifically in septum formation, while PBP2b is dedicated to peripheral cell wall synthesis (Berg et al., 2013; Land et al., 2013).

Septal growth

Evidence that PBP2x functions in septal PG synthesis in ovococci was demonstrated by inhibition of PBP2x by the β-lactam antibiotic methicillin, which led to the formation of elongated cells in *Lactococcus lactis* (Sham et al., 2012).

PBP2x inhibition of *S. pneumoniae* with specific antibiotics results in cell elongation, consistent with inhibition of septal PG synthesis (Land et al., 2013; Perez-Nunez et al., 2011). Unexpectedly, depletion of PBP2x in *S. pneumoniae* laboratory strain R6 reportedly led to a mixture of round enlarged, sometimes elongated cells with oddly pointed ends, which was indicative of a complicated division defect (Berg et al., 2013; Peters et al., 2014). This notable difference in

appearance suggests that inhibition of PBP2x activity inhibits septal ring closure, whereas the absence of PBP2x may result in a more severe disruption of the division apparatus.

As a key component of divisome, PBP2x employs its extracellular C-terminal PASTA domains for normal localization at the septum (Peters et al., 2014). Fluorescent D-amino acid (FDAA) probes and 3-dimensional Structured Illumination Microscopy (3D-SIM) have been used to demonstrate that PBP2x locates separately from adjacent closing rings including PBP1a, PBP2b, MreC, and StkP to the centers of septa in mid-to-late stages but not in early stages during cell division of pneumococcus D39 cells (Tsui et al., 2014). However, this phenomenon contradicts with the idea discussed by Fleurie *et al* (Fleurie et al., 2014b) of widely separated PG synthesis machines at different mid-cell locations, probably due to limited resolution of approaches. These studies together suggest a localization pattern that, in pre-divisional cells, PBP2x functions for cross-link formation in septal PG synthesis that probably requires PBP2x-StkP interactions (Morlot et al., 2013). At later stages after PBP2x separates to septal centers, adjacent PBP1a and other class A PBPs may continue to synthesize septal PG by glycan strands, indicating that the PG transpeptidase cross-linking activity of

PBP2x may play other roles in PG remodeling rather than septal PG synthesis alone (Tsui et al., 2014).

PcsB is required for septum synthesis and cell separation, although the precise mechanism is yet to be elucidated. PcsB binds to the extracellular part of the division protein complex FtsX and FtsE (Bartual et al., 2014; Sham et al., 2011). FtsEX was placed within the septum membrane in a recent study to illustrate how it bridges the septum width by binding PcsB (Bartual et al., 2014).

PG is not only built, it is also remodeled at the septum by hydrolytic enzymes such as LytB, a process responsible for separating the two daughter cells (Bai et al., 2016). A recent study identified a gene in *Streptococcus gordonii* that putatively encodes LytB (Arrigucci and Pozzi, 2017).

Peripheral growth

In the case of cell wall synthesis, ovococci seem to be similar to rod-shaped bacteria. However, the absence of MreB-like cytoskeletal proteins, which the side-wall PG synthesis of rods are dependent on, indicates peripheral growth in

ovococci is mechanistically different from rods (Pinho et al., 2013; Typas et al., 2011).

Studies on *B. subtilis* have revealed that PBP2a, which shares the same subclass with pneumococcal PBP2b, is involved in cell elongation (Sauvage et al., 2008). Further evidence has demonstrated in *Lactococcus lactis*, an ovoid bacterium, that the deletion of PBP2b was not lethal but led cells to the adoption of spherical morphology (Perez-Nunez et al., 2011). A more recent study aimed to uncover the role of PBP2b using a titratable gene depletion method of *pbp2b* in *S. pneumoniae* (Berg et al., 2013). The mutant exhibited a dramatic change in cell morphology whereby individual cells acquired lentil-like appearances in long chains. PBP2b-depleted pneumococci are dependent on a larger proportion of branched muropeptides to fulfil normal growth comparing to wide type (Berg et al., 2013; Straume et al., 2017). In contrast to *S. pneumoniae*, the essentiality of PBP2b homologues in the closely related species *S. sanguinis* and *S. thermophilus* remains to be further investigated (Massidda et al., 2013; Thibessard et al., 2002; Xu et al., 2011).

Depletion of the essential MreC or MreD protein from the *S. pneumoniae* D39 genetic background results in formation of chains of spherical cells remarkably similar to those formed by blocking peripheral PG synthesis by PBP2b depletion (Berg et al., 2013; Land and Winkler, 2011). Taken together, these results suggest that MreCD regulates peripheral PG synthesis in *S. pneumoniae*.

Pneumococcal class A PBPs have been considered functionally redundant. Each of the genes encoding these PBPs can be deleted individually, demonstrating that none are essential for laboratory growth. It is also possible to isolate *pbp1b pbp2a* and *pbp1a pbp1b* double mutants, whereas *pbp1a pbp2a* double mutants are inviable. However, PBP1a and PBP2a are not entirely functionally redundant, because the essentiality of MreCD in *S. pneumoniae* is suppressed in *Δpbp1a* but not *Δpbp2a* mutants from the serotype 2 D39 genetic background (Land and Winkler, 2011). Notably, *Δpbp1a* mutants showed reduced cell length and width compared with their parent strain, an unencapsulated derivative of strain D39 used to reduce masking of cell-shape phenotypes by the capsule (Land and Winkler, 2011). It has been strongly implicated that PBP1a functions in peripheral PG synthesis, although a second role in septal PG synthesis was not ruled out (Land and Winkler, 2011).

Regulation of cell division in *Streptococcus*

DivIVA, GpsB regulatory protein and StkP protein kinase have been proposed as molecular switches that balance septal and peripheral (side-wall like) PG synthesis by the divisome and elongasome in *S. pneumoniae* (Fleurie et al., 2014b).

DivIVA, a switch in peptidoglycan synthesis

In addition to its role in positioning the PBPs, DivIVA may act as a switch to change between septal and peripheral PG synthesis. Although not shown in Figure 2, DivIVA can be connected to the Z ring through EzrA, and it is phosphorylated by the Ser/Thr kinase StkP (Fleurie et al., 2014b).

In bacterial two-hybrid assays *S. pneumoniae* DivIVA interacts with itself, cell division proteins FtsZ, FtsA, ZapA, FtsK and FtsL, PG hydrolase PcsB, and chromosome segregation protein Spo0J. The same study did not detect an interaction of DivIVA with either PBP2X or PBP1a (Le Gouellec et al., 2008). The shorter and rounder appearance of Spn Δ*divIVA* mutant cells in chains suggests a defect in peripheral or in polar PG biosynthesis, as proposed in several papers

(Boersma et al., 2015; Fadda et al., 2007; Fleurie et al., 2014b; Massidda et al., 2013; Straume et al., 2017), but the exact function of DivIVA in Spn remains to be determined.

GpsB, a major regulator of peptidoglycan biosynthesis

GpsB is a cytoplasmic protein conserved in low-GC Gram-positive bacteria. High-resolution crystal structures have further demonstrated that the hexameric GpsB contains two isolated domains, an N-terminal domain (which is highly homologous to DivIVA that interacts with the cytoplasmic face of the cell membrane), and an α-helical C-terminal domain (Cleverley et al., 2016; Rismondo et al., 2016). GpsB is dispensable for growth in *Bacillus subtilis*, where it acts as a guide in PBPs shuttling between septal and lateral sites of PG synthesis. Recent studies have confirmed GpsB plays a role as a switch protein to regulate PBPA1 function and localization in the rod-shaped human pathogen *Listeria monocytogenes* (Rismondo et al., 2016). Being a switch between septal and peripheral PG biosynthesis, however, GpsB in ovococci acts differently to that of rod-shaped bacteria (Figure 2).

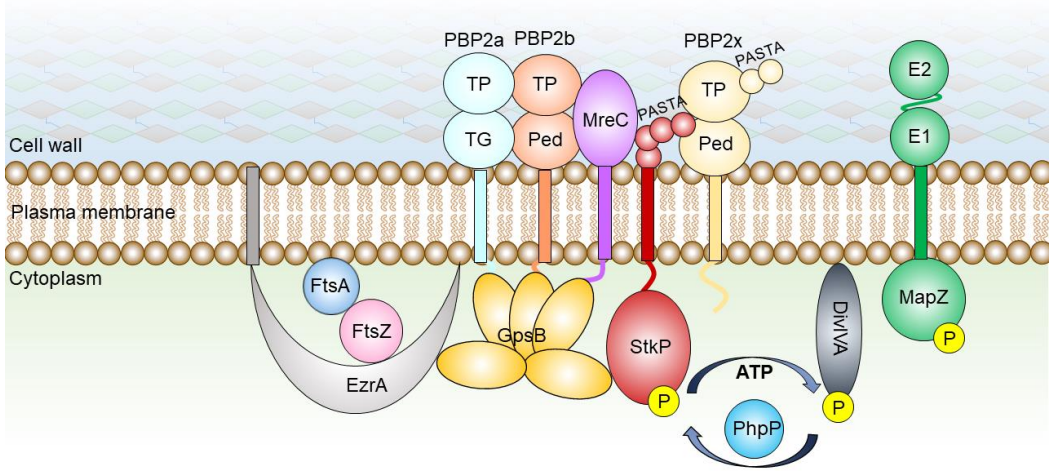

Figure 2. A schematic of the interactions of GpsB and regulatory networks for PG biosynthesis in *Streptococcus* (Rued et al., 2017). GpsB and EzrA form complexes that link the dynamics of divisome to septal and peripheral PG biosynthesis. The level of GpsB can also mediate the phosphorylation cycle of protein including DivIVA and MapZ by StkP kinase and PhpP phosphatase. TP: transpeptidase; TG: transglycosylase; ped: pedestal domains; E1 and E2: extracellular domains of MapZ; P: phosphorylation.

The role of GpsB in cell wall synthesis in *Streptococcus* is crucial, demonstrated by the elongated *S. pneumoniae* D39 growth phenotypes from *gpsB* deletion mutants (Land et al., 2013). The enlarged cell has a defect in cell division septum closure, producing multiple, un-constricted Z-rings along the length of the elongated cell, and is eventually sensitive to lysis (Land et al., 2013). This suggests that GpsB may be a crucial connector in controlling septal ring closure in *S. pneumoniae*. Further studies have investigated the connector mechanisms of

GpsB. PBP2x transpeptidase activity is barely detectable at the centers of division septa upon the depletion of GpsB, suggesting a regulatory role of GpsB in PBP2x function (Rued et al., 2017). Considering the previous hypothesis that extracellular PASTA domains of StkP interact with PBP2x (Morlot et al., 2013), GpsB might positively modulate division ring closure via its interaction with StkP to activate PBP2x in pre-divisional cells. GpsB could also be required continuously during later stages of septal ring closure by associating with other components such as SepF, FtsL and DivIC, but more evidence is needed to support this hypothesis.

In *B. subtilis*, the absence of GpsB causes no change in phenotype under normal conditions, but *gpsB* mutant cells form polar bulges, which is a result of PBP1 delocalization in high-salt media or in combination with the deletion of *ezrA* (Claessen et al., 2008). In *S. pneumoniae*, PBP1a rings are of normal appearance in aseptate cells depleted for GpsB (Land et al., 2013). However, co-immunoprecipitation results indicate a direct interaction between GpsB and proteins involved in peripheral PG synthesis (Rued et al., 2017). The interaction of GpsB directly activates PBP2a (whose exact function in pneumococcal PG synthesis is still unknown) (Tsui et al., 2016), whereas the complexes formed by

GpsB and PBP2b, as well as GpsB and MreC negatively regulate peripheral PG synthesis (Berg et al., 2013; Straume et al., 2017; Tsui et al., 2016). By regulating both septal and peripheral PG synthesis, GpsB is considered to balance the PG synthesis and link PBP regulation to a dynamic divisome through its interaction with EzrA (Fleurie et al., 2014b; Rued et al., 2017).

Regarding localization pattern, GpsB localizes at the lateral walls during cell elongation and moves to the divisional septa before cell constriction in *B. subtilis* (Claessen et al., 2008). Land *et al*. showed that the location patterns of GpsB overlapped with that of FtsZ, but GpsB had a dynamic localization to areas unoccupied by FtsZ in intracellular regions during different stages of cell division (Land et al., 2013).

GpsB is speculated to positively regulate protein phosphorylation levels in several Gram-positive species (Fleurie et al., 2014b; Grangeasse, 2016; Pompeo et al., 2015; Rued et al., 2017). In *B. subtilis,* GpsB is required for PrkC kinase activity, and phosphorylation of GpsB may provide a negative feedback loop by inhibiting PrkC autophosphorylation (Pompeo et al., 2015). In addition, GpsB is phosphorylated *in vivo* by Stp1in *S. agalactiae* (Burnside et al., 2011). However,

there is no evidence that GpsB is phosphorylated by StkP, even though GpsB and StkP show overlapping localization during pneumococcal cell division (Rued et al., 2017), in which GpsB is also required for the localization and optimal activity of StkP (Fleurie et al., 2014b). These results may explain epistasis of Δ*divIVA* and Δ*gpsB* in R800 and support the hypothesis that GpsB acts as a regulator through phosphorylation of DivIVA by the StkP kinase to modulate cell elongation (Fleurie et al., 2014b; Grangeasse, 2016; Lewis, 2017; Rued et al., 2017).

It should be noted that, unlike in D39, GpsB was not essential in R800 or R6 background pneumococcal cells (Fleurie et al., 2014b). A similar phenomenon has been found in the specificity of epistasis of *divIVA* mutations to *gpsB* mutations in R800 genetic background cells. Likewise, a requirement for GpsB in localization and kinase activity of StkP toward septal rings is also confined to the R800 strain (Fleurie et al., 2014b; Rued et al., 2017). According to Rued *et al.,* these discrepancies may result from the suppressor accumulation effects on laboratory strains, which was demonstrated in mutant strains using phenotypic and genotypic methods (Lewis, 2017; Rued et al., 2017). This phenomenon suggests an optimal choice for clinical virulent progenitor strains in characterizing cell division mechanisms.

Serine/Threonine kinases and phosphatases

In order to adapt to changes in their environment, bacteria often use phosphorylation/dephosphorylation to transmit cell cycle signals and to respond to external changes, such as nutrients, oxygen, light and osmotic pressure. Two-component signal transduction (TCS) systems are based on transient phosphorylation of a response regulator by a membrane anchored histidine kinase. TCS systems are the most abundant systems in prokaryotes that allow communication between the cell envelope and cytoplasm (Stock et al., 2000). Serine/threonine protein kinases (STPKs) and their cognate Ser/Thr phosphatases represent another major mechanism of transmembrane signaling. Since the early 1990s many eukaryotic-type STPKs have been identified in bacterial genomes. This includes a broad spectrum of pathogens, where STPKs regulate various cellular functions, including cell wall synthesis and cell division through phosphorylating key proteins on Ser/Thr residues to elicit specific downstream effects (Dworkin, 2015; Zhang et al., 2017).

Target cell division-bacterial cell-division inhibitors

With the recent campaign “World Antibiotics Awareness Week” held by the World Health Organization:

(http://www.who.int/campaigns/world-antibiotic-awareness-week/en/), there is little doubt that pathogens resistant to conventional antibiotics are an increasing threat to global health. The emergence of antimicrobial resistance has forced the development of new antimicrobials to combat this global issue and therapeutic agents with novel targets, rather than established synthesis pathways are desperately required (Cheng et al., 2016; Kohanski et al., 2010; Oldfield and Feng, 2014). Among all the targeting process, cell division is a fundamental and essential bacterial process multiply and colonize their niche: proteins underlying this could prove ideal candidates as novel antibiotic targets (Lock and Harry, 2008). Moreover, since the cell-division proteins conserved among many bacterial species are mostly absent from eukaryotic cells, the specific inhibitors will be safe without perturbing the human homologue (den Blaauwen et al., 2014; Sass and Brotz-Oesterhelt, 2013; Vollmer, 2008). In addition, the external location of cell division proteins and protein-protein interaction that is required for divisome assemble are more accessible for inhibitory compounds (Lock and Harry, 2008).

Novel agents that target bacterial cell division proteins has been accepted as promising strategies for antimicrobial attack, which have currently become a

research hotspot (Sass and Brotz-Oesterhelt, 2013). For *Streptococcus*, which mainly consist of opportunistic pathogens that gain predominance in the host, targeting cell division is an effective and efficient strategy to prevent their proliferation and colonization, thus also raising interests of researchers (Sham et al., 2012).

Strategies concerning inhibition of cell division mainly target FtsZ, the central mediator of divisome. The first antibacterial agent that targets cell division was reported in 2008 by Haydon *et al.*, who identified PC190723 as an FtsZ assembly inhibitor against *Staphylococcus aureus* (Haydon et al., 2008). PC190723 has potent and selective bactericidal activity against drug resistant S. *aureus* with minimal inhibitory concentrations (MICs) in the range of 0.5 to 1.0 μg/ml (Haydon et al., 2008). However, PC190723 showed species-selectivity with no effect against *S. pneumoniae*. With the development of research on its structural, biochemical and biological properties, multiple antibacterial approaches that interfere with FtsZ assembly and disassembly dynamics, GTPase activity, degradation of FtsZ protein, and expression of *ftsZ,* have been validated as effective (Hurley et al., 2016). Natural products such as phenylacrylamide and 5-methylphenanthridium derivatives have also been

shown to inhibit FtsZ activity in *Streptococcus pyogenes* (Liu et al., 2017). Other approaches focus on FtsZ regulators that modulate the stability of FtsZ. Acyldepsipeptides convert the ClpP peptidase to an uncontrolled protease that degrades FtsZ, resulting in cell division inhibition in *B. subtilis*, *S. aureus* and *S. pneumoniae* (Sass et al., 2011).

The contrast between the huge amount of FtsZ targeting strategies in model rods and *S. aureus* and the few in streptococcus may have resulted from the limited knowledge of the cell division biological process in *Streptococcus*. Nevertheless, the development of several strategies including fragment discovery approaches and phage display has been made to discover small-molecule inhibitors of protein–protein interactions in cell division. Moreover, the emergence of antisense nucleic acids and high-throughput screening, along with further research to uncover the mysteries of cell division will lead us towards the discovery of new approaches (Hurley et al., 2016; Zhang et al., 2018).

Future Perspectives

In this review we look into the progress made in cell division of *Streptococcus* over recent years, detailing major events that are similar to those observed in

model rods. However, studies also highlighted the specificities and differences in streptococcal cell division compared to the model organisms. Although the precise mechanism of nucleoid occlusion and some functional proteins still remain unclear, the wealth of knowledge available has aided researchers towards the discovery of novel therapies targeting cell division. With the development of new available genetic and cytological tools, *Streptococcus* will rapidly become another model bacterial system for the study in cell division.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (31200985, 81371135, and 31870065).

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