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Cell Growth and Division of *Staphylococcus aureus*

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Annu. Rev. Microbiol. 2024. 78:293–310

The *Annual Review of Microbiology* is online at micro.annualreviews.org

https://doi.org/10.1146/annurev-micro-041222-125931

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Keywords

cytokinesis, cell cycle, divisome, peptidoglycan, penicillin-binding protein, antimicrobial resistance

Abstract

Bacterial cell growth and division require temporal and spatial coordination of multiple processes to ensure viability and morphogenesis. These mechanisms both determine and are determined by dynamic cellular structures and components, from within the cytoplasm to the cell envelope. The characteristic morphological changes during the cell cycle are largely driven by the architecture and mechanics of the cell wall. A constellation of proteins governs growth and division in *Staphylococcus aureus*, with counterparts also found in other organisms, alluding to underlying conserved mechanisms. Here, we review the status of knowledge regarding the cell cycle of this important pathogen and describe how this informs our understanding of the action of antibiotics and the specter of antimicrobial resistance.

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1. INTRODUCTION

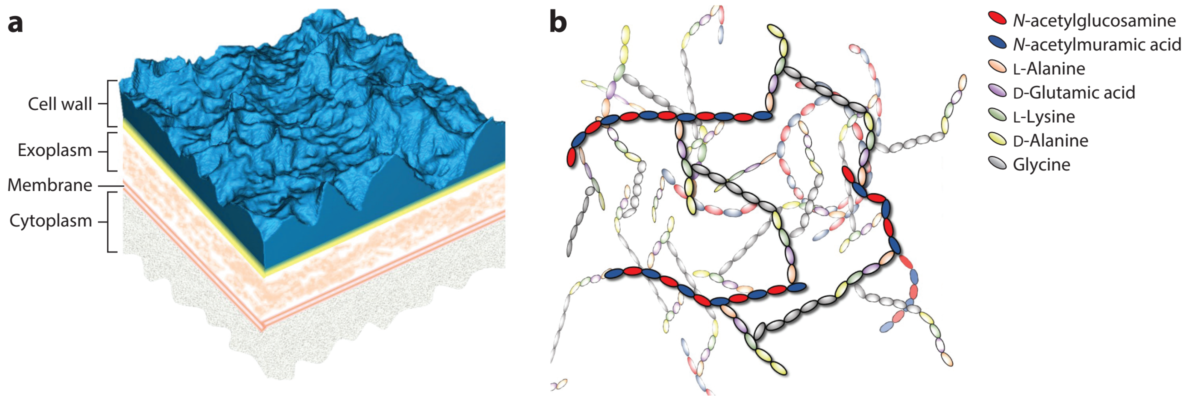
Staphylococcus aureus is an important, opportunist pathogen able to cause a wide range of different diseases. The very name *Staphylococcus* stems from early observations of the organisms as "bunches of grapes" within the characteristic disease abscess, which is a direct sequela of its cell division mode in orthogonal planes, where daughter cells remain joined after division (54, 117). The development of antibiotics to combat *S. aureus* infections has been a mainstay of human healthcare over the past 80 years, with the β-lactams and vancomycin being major contributors to saving millions of lives. These cell wall targeting antibiotics inhibit peptidoglycan biosynthesis reactions required for cell growth and division and, thus, give paramount importance to the study of this area. This has been exacerbated by the insidious spread of antibiotic resistance, as exemplified by methicillin-resistant *S. aureus* (MRSA), which is alone responsible for over 100,000 deaths per annum (71). The MRSA resistance mechanism is entirely dependent on the ability of the bacteria to alter their cell wall growth and division capabilities during the onslaught of otherwise bactericidal antibiotics (57). Here, we review the *S. aureus* cell cycle, within the context of cell structure and molecular mechanisms, exploring how these can inform and be informed by the mode of action of antibiotics and resistance mechanisms.

2. THE CELL WALL

2.1. Cell Wall Composition

As a monoderm firmicute, *S. aureus* has the archetypal gram-positive cell wall envelope (**Figure 1*a***) that determines cell shape, is essential for the maintenance of cellular integrity in the face of internal turgor pressure, and forms the interface with the environment (104). This requires a complex structure that has mechanical strength and yet maintains flexibility and dynamics to permit growth, division, and its myriad other functions. The cell envelope is made of three layers—the cytoplasmic membrane, the exoplasm, and the cell wall (**Figure 1*a***)—and is 30–40 nm thick when hydrated

(79). The exoplasm, which separates the cell membrane from the cell wall, is a region analogous to the periplasm of gram-negative bacteria, but bounded by a single membrane (65). The major structural component of the cell wall is the polymer peptidoglycan (PG), which forms a single macromolecule around the cell, called the sacculus (116). The chemistry of PG is well described and consists of glycan strands of different lengths, made of repeating disaccharide residues of *N*-acetylglucosamine and *N*-acetylmuramic acid, cross-linked by short peptides (**Figure 1*b***) (121).



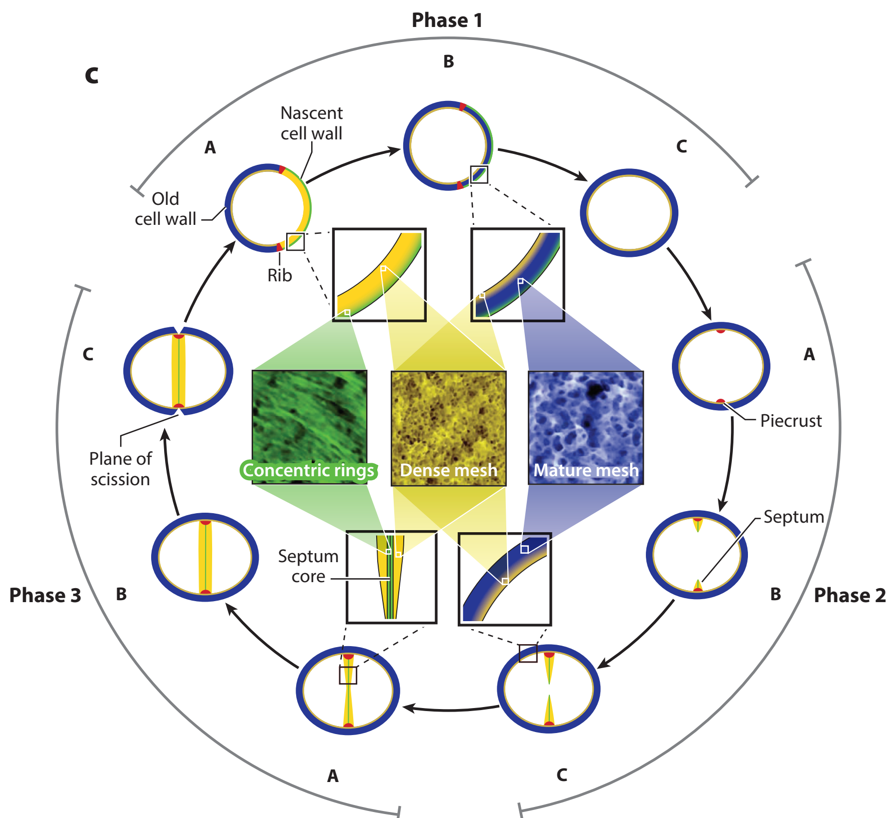


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Figure 1 (*Figure appears on preceding page*)

Staphylococcus aureus cell wall structure and dynamics during the cell cycle. (*a*) Cross-sectional representation of the *S. aureus* peripheral cell envelope. The cytoplasmic membrane has lipoteichoic acids embedded and reaching out into the exoplasm. This is surrounded by the cell wall peptidoglycan (PG) with attached wall teichoic acids and proteins forming a mature, open-mesh hydrogel of increasing pore size toward its outer surface. Relative thickness of layers is derived from Matias & Beveridge (65). (*b*) A diagrammatic representation of the basic PG chemical structure, revealing a 3D mesh derived from glycan strands and peptide cross-links, with the constituents shown. (*c*) Cell wall structure and dynamics during the cell cycle. The cell cycle is depicted in three main stages as cross sections, before (Phase 1), during (Phase 2), and after (Phase 3) septal synthesis. During growth, in Phase 1A and 1B, the cell increases in volume and slightly elongates due to PG synthesis and hydrolysis. In Phase 1C, the early divisome assembles, prior to Phase 2A, where the initial cell wall morphological feature, the PG piecrust (*red*), is synthesized. The piecrust is a thin band of PG that serves as a foundation for the initiation of the septal plate. During Phase 2B, the "V"-shaped septal plate progresses inward by synthesis of an initial, concentric ring-like-structured PG, synthesized by FtsW/PBP1 at its core (*green* in the *colored inset*). The FtsW/PBP1-derived ring structure acts as a framework for the synthesis of the ensuing fine-mesh-structured PG produced by PBP2 (*yellow* in *colored inset*). At the end of Phase 2C, the annulus closes, resulting in a bowed septum. Phase 3A is characterized by the closed septum being filled out by PG insertion, executed by PBP2, which continues until the cross wall is of uniform thickness (Phase 3B). Finally, during Phase 3C, the mother cell wall is hydrolyzed at the plane of septation, and the daughter cells separate. This reveals, in Phase 1A, the concentric rings on the outside of the newly exposed septum (*green* in *colored inset*). The cell wall of the predivisional cell (Phase 1B; *colored inset*) is a gradient of internal fine, nascent (*yellow*) to external open, mature (*blue*) mesh PG and residual concentric rings (*green*) at the site of the previous septation event. The central squares show false-colored images of PG architecture representative of atomic force microscopy images. Diagram based on Wacnik et al. 2022 (122).

In mature *S. aureus* PG, the average glycan strand length is approximately six disaccharides, which is rather short among the bacteria, as average glycans of *Escherichia coli* and *Bacillus subtilis* are over 30 and 50 disaccharides long, respectively (10, 43, 123). The macromolecular structure of the PG is maintained by peptide stems that arise from the muramic acid residues and, in *S. aureus*, have pentaglycine cross bridges that link one glycan strand to another, thus building the sacculus (116). *S. aureus* has a high cross-linking index in which 74–92% of available stem peptides are cross-linked to another muropeptide (24, 38, 105), resulting in an overall macromolecule whereby the strength and architecture are likely highly contributed by both glycans and peptides. The sacculus is decorated with other polymers such as wall teichoic acids (WTA) and proteins that are both ionically and covalently bound to its structure. WTA are polymers of repeating ribitol phosphate moieties covalently bound to the muramic acid residues of the PG (130). WTA and proteins are important in cell wall homeostasis and interaction with the environment during colonization and pathogenesis (34, 130). Another important component of the cell envelope is the polymer lipoteichoic acid (LTA), which is tethered to the cytoplasmic membrane and extends into the exoplasm. The presence of anionic teichoic acids has been suggested to bring neutralizing cations into the exoplasm, making it isosmotic with the cytoplasm (29). This would allow the exoplasm to be maintained, preventing the cytoplasmic membrane from being forced against the PG due to turgor pressure (29).

2.2. Peptidoglycan Synthesis

Synthesis of PG starts in the cytoplasm with the production of the UDP-*N*-acetylglucosamine and UDP-*N*-acetylmuramyl-pentapeptide precursors (4). The latter is linked to the membrane transporter lipid undecaprenyl pyrophosphate by the MraY enzyme, resulting in the formation of lipid I (13). This is followed by the addition of *N*-acetylglucosamine from the UDP-*N*-acetylglucosamine cytoplasmic precursor, by MurG, to produce lipid II, which in *S. aureus* is completed with the addition, by FemXAB enzymes, of a five-glycine peptide crossbridge to L-lysine, the third amino acid of the pentapeptide (92). The lipid II precursor is then flipped to the outer side of the cytoplasmic membrane by the MurJ flippase, which is conserved across species (95, 103), so that it can be used in the final stages of PG synthesis that occur in the exoplasm. Here, transglycosylase

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(aka glycosyltransferase) activity assembles glycan strands, which are cross-linked into the existing sacculus structure by transpeptidase activity. *S. aureus* only has four native transpeptidases involved in PG assembly, and these are the penicillin-binding proteins (PBPs) 1–4 (85). Of these, only PBP1 and 2 are essential for growth, representing a rather minimal system (80, 88). PBP2 is a Class A PBP and is the only bifunctional PBP, with both transpeptidase and transglycosylase activity (81, 82). *S. aureus* also has two monofunctional transglycosylases (SgtA and Mgt) but only Mgt can substitute for the PBP2 transglycosylase activity (89). PBP1 and 3 are Class B PBPs, with only transpeptidase activity, which functionally interact with cognate SEDS (shape, elongation, division, and sporulation) proteins with transglycosylase activity, called FtsW and RodA, respectively (90). The pair PBP1/FtsW is required for septal PG synthesis and to stabilize the divisome at midcell, whereas PBP3/RodA are involved in lateral PG incorporation leading to mild elongation of *S. aureus* cells (90). PBP4 is a low molecular weight Class C PBP with transpeptidase activity, responsible for the high-level secondary cross-linking characteristic of *S. aureus* (2, 129).

2.3. Cell Wall Architecture

The PG sacculus determines both cell shape and integrity, as it maintains the overall cell morphology and dimensions when purified, and cells lyse if the PG is enzymatically removed (115). To fully understand the relationship among PG synthesis, chemistry, and architecture, and their impact on function, it is necessary to analyze PG in situ. Although there is a level of local chemical order (52), morphological analysis hinted at a heterogeneous architecture (1, 113). High-resolution atomic force microscopy (AFM) on both isolated PG sacculi of staphylococci (107, 115) and live cells (79) has revealed a complex architecture that is dynamic throughout the cell cycle. Overall, the cell wall (**Figure 1*a***) is a porous, expanded hydrogel with the mature surface having an open, disordered mesh with large (up to 60 nm diameter) and deep (up to at least 23 nm) pores (79). These pores do not span the entire wall, as the inner surface of the entire sacculus, where PG is made, is characterized as a much denser disordered mesh, with a glycan spacing of less than 7 nm. Upon splitting of the division septum during cell scission, the final stage of division, a novel, external, concentric ring-like structure of long glycans is revealed (113, 115). The basic PG architectural features are also conserved in the rod-shaped *B. subtilis* (79). As *S. aureus* divides in more than one plane, the rings of one division round are bisected by the next septum and disappear over time as they mature into the open-mesh structure observed around the cell periphery. As well as being architecturally distinct from the cell periphery, the septum also has different biochemical properties, as WTA are displayed across the surface but are not present or may have an immature form at the septum (2, 100).

3. THE CELL CYCLE

The cell cycle of *S. aureus*, also reviewed in Reference 3, can be described as a series of morphological phases, as depicted in **Figure 1*c*** (68). In Phase 1, newly separated, nonseptating cells go through volume increase and slight elongation (Phase 1A–1C). The division machinery is assembled, and septation is first evidenced at the beginning of Phase 2 by the formation of a small band of PG at the division site, on the internal face of the mother cell wall, called the piecrust (Phase 2A). The piecrust forms the foundation for septal synthesis that occurs during the rest of Phase 2. The closure of the septal annulus with the fusion of the two diametrically opposed membrane sections (Phase 3A) marks the initiation of Phase 3, during which the cytoplasm of the two daughter cells is no longer shared. The septum matures, and cells undergo further elongation (Phase 3B), prior to cell scission (Phase 3C) and rapid separation, to generate two daughter cells (Phase 1A). The daughter cells will divide along a division plane perpendicular to the previous one, used by the

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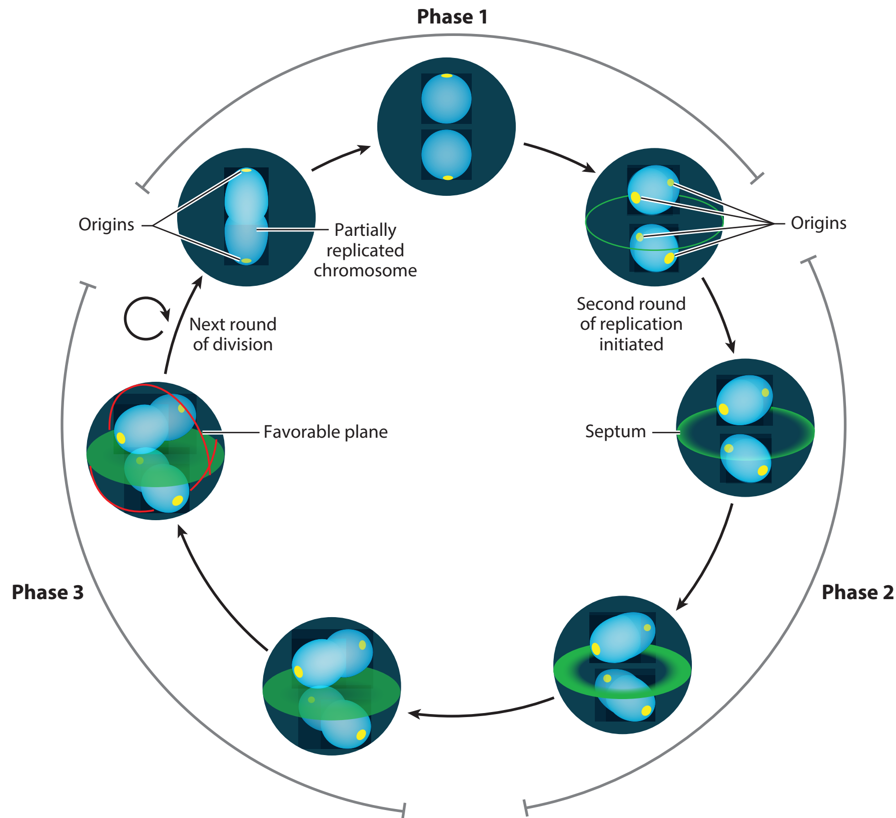

Figure 2

Staphylococcus aureus chromosome dynamics during the cell cycle. *S. aureus* cells start the cell cycle with a partially replicated chromosome (*blue*), showing two origins (*small yellow circles*). Chromosome segregation proceeds during Phase 1, and a second round of replication is initiated at the end of Phase 1 or beginning of Phase 2, resulting in cells in which four origins of replication are present. During Phase 2, while the septum (*green ring/disk*) is synthesized, and Phase 3, chromosome segregation occurs along an axis parallel to the division plane. As the nucleoid binding protein Noc (inhibitor of Z ring assembly, not shown) binds the origin-proximal region of the chromosome, cells in Phase 3 have only one favorable plane (*red ring*), which does not bisect the chromosome, to assemble the divisome in preparation for the next round of division. Notice that chromosome segregation may occur along different axes in the two hemispheres of a Phase 3 cell (although both are parallel to the division plane), resulting in different division planes in the two future daughter cells. Cell division in three consecutive orthogonal planes over three division cycles would imply that sister cells would select the same division plane, perpendicular to the two previous ones. As this is not the case (97), it follows that *S. aureus* divides in two, but not necessarily in three consecutive perpendicular planes. Figure adapted with permission from José Pedro Costa, from still images from a video created for the Pinho Lab (2020) (**https://youtu.be/2Bw-SKu7pbQ?si=WEvnqrAdUmExMvT1**).

mother cell (97), giving rise to the characteristic clumps of cells that can be observed in clinical samples.

3.1. Chromosome Replication and Segregation

S. aureus has a single circular chromosome of approximately 2.8 Mb (26, 37, 39, 56) that undergoes one round of replication during the cell cycle. Newly divided cells (**Figure 2**, Phase 1)

typically have two origins of replication (*oriC*), indicating that chromosome segregation is already under way at the start of the new cell cycle (S. Schäper & M.G. Pinho, unpublished). These origins replicate at the end of Phase 1 or beginning of Phase 2, so by the end of septum synthesis each hemisphere of the septating cell usually has two origins (16, 118). Chromosome segregation continues during Phase 3, along an axis parallel to the closed septum (**Figure 2**).

Bacterial cells lack a macromolecular machine for chromosome segregation similar to the mitotic spindle used by eukaryotes. Instead, multiple players have a role, and therefore the mechanisms underlying this process are far less understood in bacteria. Among these players are the *parABS* partitioning systems, which actively push or pull plasmids or sister chromosomes, composed of a ParA ATPase protein, a ParB CTPase DNA binding protein, and a centromere-like *parS* site (40, 44). ParB proteins load at *parS* sites, which are located near the origin of replication, forming a nucleoprotein structure known as the partitioning complex. After origin replication, the two partitioning complexes interact with a ParA gradient to segregate the origins toward the poles (40, 44). Interestingly, *S. aureus* has a ParB homologue (Spo0J), but lacks ParA. Given that ParB/Spo0J binds specific origin proximal *parS* sequences, fluorescent derivatives of this protein have been used to visualize *oriC* in living cells (16, 83, 118). The exact role of ParB/Spo0J in chromosome segregation in *S. aureus* is not clear, but it is known that it is required for the correct localization of SMC (structural maintenance of the chromosome) (16). SMCs are condensins that compact the chromosome by locally looping DNA (40). These ring-shaped proteins, with ATPase activity, are loaded near the origin by ParB proteins, and then they translocate along the left and right chromosome arms, keeping them together, and are unloaded near the terminus (50). In agreement with a role in *S. aureus* chromosome segregation, depletion of SMC increases the number of anucleate cells (16, 132). Other players in bacterial chromosome segregation are FtsK DNA translocases that move the DNA away from the division site during septum closure. *S. aureus* has two of these proteins, FtsK and SpoIIIE, and impairing the DNA translocase activity of both causes severe nucleoid segregation and morphological defects (120). Interestingly, FtsK has a second function in controlling daughter cell separation by regulating the levels of septal PG hydrolase Sle1 (119). *S. aureus* cells lacking both ParB/Spo0J and SMC (16) or both FtsK and SpoIIIE (120) are viable, indicating that additional, possibly passive, mechanisms such as DNA replication, DNA transcription, or entropic forces are likely to contribute to efficient segregation of the chromosomes in this organism.

3.2. Division Site Selection

S. aureus was characterized for many years as dividing in three alternate orthogonal planes over consecutive division cycles (54, 117), but more recently it was found that a plane of division is always perpendicular to the previous one, but not necessarily to the penultimate division plane (97). Division site selection is defined by the location of FtsZ ring assembly, which can rely on both negative and positive regulators. The two best-studied regulators in bacteria are the Min system (23) and nucleoid occlusion (126, 127), both of which act by preventing Z-ring assembly, the former at the cell poles and the latter over the nucleoid. *S. aureus* lacks a Min system or any known positive regulators to determine midcell and, therefore, relies mostly on the nucleoid occlusion effector Noc to define the division plane (85). Noc binds to the origin proximal region of the chromosome and antagonizes assembly of the FtsZ ring (128). Once chromosome segregation is under way, the origins move away from midcell, releasing this region from the presence of Noc and allowing the assembly of the Z-ring (**Figure 2**). In *S. aureus*, segregation occurring during Phases 2 and 3 of the cell cycle takes place in a hemisphere that corresponds to a future daughter cell. At this stage, segregation toward the "poles" (the points furthest away from the division septum) is

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unlikely, as it would be less favorable for chromosome demixing due to spatial constraints (48). Therefore, chromosome segregation occurs parallel to the ongoing division plane, releasing only one possible plane that does not bisect the nucleoid as the next plane of division. This plane is necessarily perpendicular to the previous one; hence, the axis of chromosome segregation could be sufficient to ensure division in two perpendicular planes (**Figure 2**). However, segregation along any axis parallel to the septum is geometrically equivalent, so sister cells can, and often do, choose different segregation axes and, as a consequence, *S. aureus* divides in two, but not necessarily in three consecutive alternating perpendicular planes as previously thought (97) (**Figure 2**).

Lack of the Noc protein in *S. aureus* leads to cells with multiple FtsZ rings, cells where the septum closes over the DNA causing double-strand breaks and anucleate cells, but its lack is not lethal (118), suggesting that other factors may contribute to placement of the division plane. Positive regulators that mark the division site and position of the FtsZ ring have been described in organisms such as *Streptococcus pneumoniae* (MapZ) (31) or *Myxococcus xanthus* (PomZ) (102, 114), but not in *S. aureus*. One possibility is that DivIVA may be an early marker of the future division plane in this organism. DivIVA is targeted to regions of high negative membrane curvature (58), and in *S. aureus* it can be found in foci localized at the division site (12, 83) but also at the cell periphery, including, in a few cells, in foci at the poles (12). Here, DivIVA may interact with SMC, pulling it away from midcell, facilitating chromosome segregation (12) and/or, with the recently identified PcdA, forming a complex that would recruit unpolymerized FtsZ to the new division site (87). However, a *divIVA* deletion mutant shows no obvious phenotype with regard to growth rates and chromosome morphology (12, 83), and only a very mild effect of orientation of the division planes (87; Pinho Lab, unpublished). Recently, a number of other components involved in optimal division have been identified, although the mechanisms underlying their roles are still not fully understood. These include FacZ, which interacts with GpsB [a protein thought to promote lateral interactions of FtsZ (30)], and is required to prevent the assembly of extra Z-rings at aberrant places (5); CozEAB, which is involved in controlling cell division, possibly through interactions with cell division proteins like EzrA (106); and SmdA, a membrane protein that interacts with several cell division proteins, including EzrA, as well as with cell wall synthesis proteins, and affects septum formation and septum splitting (72).

3.3. Divisome Assembly

The approximately spheroid cell morphology of *S. aureus* makes it an excellent model for microscopy studies of division, as it enables clear visualization of the division machinery, i.e., the divisome, in different planes, including in the microscopy imaging plane. The central cytoskeletal protein that organizes the divisome is FtsZ, a bacterial tubulin homologue that is conserved across most bacteria. FtsZ is anchored to the membrane by FtsA and possibly by the FtsZ-positive regulator SepF and FtsZ-negative regulator EzrA, although these two proteins are nonessential in *S. aureus* (47) (H. Veiga and M.G. Pinho, unpublished data).

Further divisome proteins are then recruited to the divisome, including the DNA translocase FtsK, the septal SEDS/bPBP pair of PG synthases FtsW/PBP1 and DivIB/DivIC/FtsL (69). These latter three membrane-bound proteins form a complex and are conserved across most bacteria (15, 22, 74). In *S. aureus*, evidence suggests that these proteins may be regulators of division, acting at different checkpoints to ensure effective morphogenesis. DivIB is a PG binding protein, whose loss prevents septal completion (11), whereas DivIC is a WTA binding protein with a role in the recruitment of PBP2 to the growing septum, facilitating efficient septal synthesis (111).

In the late stage of divisome assembly, the lipid II flippase MurJ is recruited, and its arrival to the divisome concentrates the flipping of lipid II specifically at midcell (69). The increased

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availability of substrate at midcell then recruits the main PG synthase, the bifunctional PBP2, which is responsible for the bulk of PG synthesis that needs to take place for septum synthesis to be completed (69).

FtsZ is a dynamic protein that undergoes treadmilling, a process by which cytoskeletal filaments grow in length at one end while shrinking at the opposite end, through the constant addition and removal of protein subunits, resulting in the movement of FtsZ filaments around the division site while individual molecules remain static (8, 69, 131). FtsZ treadmilling is essential in the very early stages of cytokinesis but becomes dispensable once MurJ is concentrated at midcell and massive PG synthesis is initiated (69). After this timepoint, PG synthesis is the process that drives cytokinesis (69) and FtsZ treadmilling is not required for its progression nor does it have a role in regulating speed of septum constriction (99).

3.4. Septum Formation

The first cell wall synthesis event during division is the laying down of the thin PG piecrust, which forms the initial foundation for the septum and the interface with the mother cell wall (**Figure 1*c***) (115). This may be the product of PBP2 activity, as loss of PBP1 protein leads to incomplete septa but does not prevent their initiation (122). The next defining feature of the septum undergoing synthesis is the concentric ring-like structure, which will become the core of the septum until scission. These rings are the proposed product of the concerted activity of FtsW/PBP1 (90, 122), with FtsW transglycosylase activity being stimulated by the presence of PBP1 (109). During septum synthesis, FtsW/PBP1 move directionally around the division site, with molecules moving processively for distances of up to 3 μm, corresponding to the entire circumference of the cell, presumably synthesizing long glycan strands that are later processed by PG hydrolases (99). Importantly, this movement does not require FtsZ treadmilling activity but rather is powered by the enzymes' activity, i.e., by PG synthesis (99). Loss of FtsW or PBP1 leads to an inability to make a septum and to a destabilization of the divisome at midcell (90). PBP1 also plays a coordinating role, as deletion of its PG binding PASTA domains alone results in an inability to divide (122).

As septum formation progresses, it takes on a "V" shape, prior to the filling of the annulus (63, 66). The cytoplasmic membrane face of the septal PG has a fine mesh architecture (**Figure 1*c***), as found around the rest of the cell periphery, which is proposed to be the product of the transpeptidase activity of PBP2 (79). Thus, PBP2 is likely responsible for the bulk of the septal PG synthesis, using the FtsW/PBP1-generated rings as a template on which to fill in the rest of the septal wall. Being V shaped gives the septum a large surface area for synthesis of this essential structure as is evidenced by localization of the PG synthesis machinery and incorporation of PG at the leading edge and across the developing septal surface (62, 63). Even after septal annulus completion, FtsZ remains until the septum has parallel sides, and PG synthesis is complete prior to scission (63). The structure of the septal PG is further modified by PBP4, which is recruited to the septum in a manner dependent on WTA synthesis and establishes the high level of cross-linking found in *S. aureus* PG (2).

3.5. Cell Scission

The final stage of cell division involves the splitting of the mother cell wall and scission of the two nascent daughter cells. Initiation of this process can be observed by AFM as pits appearing in the mother cell wall at the outer edge of the septum (79, 113), followed by an ultrafast, millisecond cracking that propagates around the cell, releasing the daughter cells (68, 133). Scission likely occurs owing to both mechanical forces associated with turgor and enzymatic activity of

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PG hydrolases at the complete septum (68). The observed pits are likely the result of the activity of PG hydrolases, including that of the major bifunctional enzyme Atl that has both amidase and glucosaminidase activity (9, 32, 73, 75). The role of Atl in scission is exacerbated by the loss of two other glucosaminidases, SagA and ScaH, demonstrating linked or partially redundant functions (124). Furthermore, two other PG hydrolases have been shown to be involved in scission, the Sle1 amidase (49) and the bifunctional amidase/endopeptidase LytN (35). The speed of the cracking process that follows suggests that it is mainly mechanical, with little enzymatic activity being required to separate the newly completed septa (68). This is supported by cryo-electron microscopy images showing a mid-zone in closed septa, alluding to a loose connection between septal cross walls (66). After scission, the two daughter cells remain attached to each other by vestiges of the mother cell wall, building up the characteristic "bunch of grapes" over several generations (115).

Cell scission is a dangerous activity for dividing bacteria as it can result in death if it occurs before completion of the septum. Thus, the PG hydrolases must be highly regulated, and this occurs at various levels. Many PG hydrolases are positively, transcriptionally regulated, during active growth, by the two-component system WalKR, and its loss results in reduction in hydrolase activity and inability to efficiently divide (25, 28, 96). Additionally, other layers of regulation ensure a tight control of specific PG hydrolases. Sle1 is proteolytically degraded by the ClpXP proteolytic complex, so loss of ClpX results in increased Sle1 levels and premature scission (45). ClpXP degradation of Sle1 is prevented by the divisome protein FtsK, which recruits Sle1 to the septal proximal region, promoting its timely export at the septal region (119). This mechanism likely ensures coordination of early (chromosome segregation) and late (septum scission) stages of the cell cycle (119). LytN is also preferentially secreted at the septum via its YSIRK motif containing signal peptide (35). The reduced levels of WTA at the septum result in increased levels of Atl, Sle1, and LytN in this crucial region, as loss of WTA leads to their redistribution across the cell and increased lysis (36, 100). The ability of LTA to bind the repeat regions of the mature Atl amidase also controls both its activity and septal localization (134). PG hydrolases are present throughout the cell cycle, so temporal and spatial regulation of their activity is critical for cell integrity, as is the coordination with septum completion to result in accurate splitting at the interface of the PG ring-like structures.

3.6. Peripheral Growth

After scission, the newly exposed septal outer layer, consisting of PG rings, constitutes ~30% of the total surface area of each daughter, leading to a transiently flatter side to each cell (1, 68, 133). As the cell cycle progresses, the rings are matured to a mesh by the activity of various PG hydrolases (79). Meanwhile, the cells grow in volume via PG synthesis and hydrolysis (68, 124). As *S. aureus* only requires PBP1 and 2, and PBP1 is seemingly septum-specific, peripheral cell wall synthesis can be carried out by PBP2 alone, but in wild-type cells it also involves PBP3 and PBP4. PG hydrolases are also important in allowing surface area expansion and controlling growth. The glucosaminidase SagB is responsible for the characteristic short glycans in the mature PG (17, 124) and acts with other glucosaminidases to permit expansion of the cell surface and relaxation of cell wall stiffness (124). SpdC is a CAAX protease-like protein, which interacts with SagB, controls its activity, and thereby has been proposed to be responsible for the release of newly synthesized material from the membrane (98, 125). LytH is an amidase that removes peptides from uncross-linked PG and prevents runaway growth at the cell periphery (27). It acts with a cognate regulator, ActH, that binds to LytH and stabilizes metals in its catalytic site (77). How all these PG hydrolases are coordinated during the cell cycle remains to be discovered.

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3.7. Cell Elongation

Besides peripheral growth due to PG synthesis and hydrolysis over the entire cell surface, *S. aureus* cells also incorporate sidewall PG at midcell, leading to mild cell elongation (68, 90). For decades *S. aureus* was described as a truly spherical cocci, incapable of undergoing elongation. This was in line with the fact that it lacks a homologue of MreB, the cytoskeletal protein involved in organizing the elongation machinery in rod-shaped bacteria (46). It was therefore intriguing why *S. aureus* retained various of the other proteins associated with rod elongation, namely MreC and MreD (110), whose specific functions are not clear but may have a role in regulation of elongasome activity across many bacteria (93); RodZ, which anchors MreB to the Rod system and alters MreB filament bending (14, 19, 70); and RodA, the SEDS protein associated with cell elongation in rods (42, 67). The activity of RodA, together with its class B PBP cognate pair PBP3, has been found responsible for the PG synthesis at the septal region required for elongation in *S. aureus* (90). Depletion of MreC and MreD or RodZ also affects cell elongation by yet unknown mechanisms, and therefore these proteins may also be part of a *S. aureus* elongasome machinery (20). Besides this machinery, *S. aureus* elongation requires a small coiled-coil protein, GpsB, that regulates the abundance, and therefore activity, of PBPs at the cell periphery versus the division septum (20, 108).

4. CELL WALL TARGETING ANTIBIOTICS

4.1. Antibiotic Action

Given the essential nature of the bacterial cell wall, it is not coincidental that its biosynthesis is the target of many different antibiotics, which have saved countless human lives over the decades. Among the most relevant cell wall targeting antibiotics are the β-lactams, which act as pseudo-substrates targeting the essential PBP transpeptidase activity, and vancomycin, which binds to D-ala-D-ala residues on the PG precursor lipid II at the cell membrane, preventing its incorporation, as well as to mature PG, directly inhibiting further cross-linking and PG hydrolase activity (96, 101). Both β-lactams and vancomycin are bactericidal and lead to the arrest of the cell cycle due to inhibition of PG synthesis (86, 96).

Considering the simple model for cell growth requiring PG synthesis and hydrolysis, it follows that loss of PBP activity in the presence of ongoing PG hydrolase activity will result in lysis over time. This is manifested by the appearance of wall-spanning holes at the inner surface of the sacculi, in antibiotic treated cells, that expand to the point of an inability to maintain turgor (96). β-lactams have a further mode of killing specifically during cell division, whereby premature scission occurs in cells with incomplete septa, resulting in catastrophic lysis, which does not occur with vancomycin as it inhibits the PG hydrolase activity of Atl in *S. aureus* (96). Importantly, loss of PG hydrolase activity reduces the rate of cell death (96). Multiple other mechanisms have also been proposed to contribute to the bactericidal activity of cell wall antibiotics including oxidative stress, delocalization of the cell wall synthesis machinery and a futile cycle of glycan synthesis/degradation that depletes the pools of cellular PG precursors, indicating that the mode of action of these well-established antibiotics is still not fully understood (18, 51, 53, 61, 84, 112).

4.2. Methicillin-Resistant *Staphylococcus aureus*

MRSA continues to be an immense problem as these strains are resistant to clinically relevant β-lactams that are commonly used to treat *S. aureus* infections (57). The most common mechanism that underpins the ability of MRSA to grow in the presence of inhibitory concentrations of antibiotics is due primarily to the acquisition of a novel PBP, from an extra species source,

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called PBP2A (encoded by the *mecA* gene), resistant to β-lactam acylation (7, 21, 41, 94). The active site of PBP2A is usually in a closed conformation that prevents binding of a β-lactam, and binding of a second substrate molecule to an allosteric site over 60 Å away from the active site causes a conformational change required to open the active site (64, 76). PBP2A alone cannot catalyze PG synthesis as it is a monofunctional transpeptidase, so it cooperates with the transglycosylase domain of native PBP2 for PG synthesis in the presence of β-lactams (60, 82). PBP2 is not the only native protein required for full expression of resistance in the presence of PBP2A. Several so-called *aux* (auxiliary) or *fem* (factors essential for the expression of methicillin resistance) genes are also required, as their inactivation causes a decrease in resistance (6, 7, 59, 91). The Aux/Fem factors fall into several broad categories including cell wall biosynthesis, the cell wall stress stimulon or alternative sigma factors, indicating that β-lactam resistance, mediated by the activity of PBP2A, creates a significant burden on cell physiology (6, 7, 59, 91). Interestingly, resistance levels are enhanced by mutations in chromosomal genes, termed *pot* (potentiators) (7). The *pot* factors are intriguing and include, for example, the proteolytic machinery ClpXP and the PG hydrolase LytH, suggesting that a realignment of PG homeostasis is required to accommodate the optimal activity of PBP2A (7). Mutations in *rpoB* and *rpoC* (encoding RNA polymerase) that increase resistance have also been found, in both experimental and clinical settings (55, 78). The MRSA-associated *rpo* mutations lead to a pleiotropic phenotype, with no clear connection to alterations in transcription, suggesting a link to wider physiological processes (7, 78). It is not surprising that many of the Pot and Aux factors are involved in cell growth, division, and cell wall homeostasis, thus providing an opportunity to inform our understanding of basic biology, as well as having translational potential. Importantly, the existence of factors required for resistance in the presence of PBP2A opens the door to the design of synergistic therapies, as targeting these factors should resensitize MRSA strains to existing β-lactams (33, 91).

5. FUTURE PERSPECTIVES

Cell growth and division are feats of biological engineering whereby cellular structures maintain their essential mechanical functions and yet are in continual flux. We now know of likely nearly all of the major components required for growth and division of *S. aureus*, but we are lacking in our understanding of how the ensemble acts to permit morphogenesis. It is by elucidating those integrated cell cycle mechanisms that we will define the emergent cell behavior during growth, division, their demise due to antibiotics, and how resistance allows them to cheat death.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We would like to thank the Wellcome Trust (S.J.F.), the European Research Council (M.G.P.), and Fundação para a Ciência e Tecnologia (M.G.P.) for funding. We are grateful to Dr. Lucia Lafage and Dr. Laia Pasquina-Lemonche for help with developing the figures. We thank members of the Pinho and Foster labs for helpful comments and discussions.

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