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The Elongation of Ovococci

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The morphogenesis of ovococci has been reviewed extensively. Recent results have provided new insights concerning the mechanisms of elongation in ovoid bacteria. We present here the proteins involved in the elongation (firmly established and more or less hypothetical) and discuss the relationship between elongation and division of ovococci.

Introduction

Bacterial shape has been studied for many years for several reasons. Nature has generated bacteria with a wide variety of appearances, differing in size and morphologies, two features directly observable by optical microscopy. Specific shapes have been shown to confer advantages for survival under various environmental challenges. For example, shape plays a role in nutrient access, predation avoidance, diffusion and motility, and defense against stress (reviewed in Young[34]). The fact that cells retain their specific shape through generations, but are sometimes able to alter it upon environmental changes are strong indications of the adaptive importance of morphology.

The bacterial peptidoglycan (PG), one of the main constituent of the cell wall, is a giant molecule consisting of glycan chains reticulated by peptide links.[4] This structure totally encloses the cell and resists the internal osmotic pressure. The different morphologies observed in bacteria result from distinct mechanisms of PG insertion in the cell wall.

Among a vast diversity,[34] three main morphological types have been investigated in some details in bacteria: bacilli that are cylindrical (*e.g.*, *Bacillus subtilis*, *Escherichia coli*), cocci that are spherical (*e.g.*, *Staphylococcus aureus*), and ovococci that are ellipsoid (*e.g.*, *Streptococcus pneumoniae*, *Lactococcus lactis*, and *Enterococcus faecalis*). During growth, bacilli elongate and periodically divide, which contrasts with cocci that only divide. Ovococci exhibit an intermediate behavior with a short peripheral growth phase (or elongation) for each division round. Compared with bacilli, there are few data on the morphogenesis of ovococci, especially concerning their elongation. However, recent studies reviewed herein have brought new insights on this mechanism.

The Two Morphogenetic Machineries Model

Early work by Higgins and Shockman described in detail the growth of ellipsoid bacteria.[9] Observation by electronic microscopy of thin sections of *Enterococcus hirae* ATCC 9790 and careful reconstitution of the cell cycle showed that the cell wall is primarily assembled at midcell, also called equator, where a small amount of crosswall is first incorporated toward the septum. Then, new material is incorporated in the cell wall; while the septum is split at the periphery, the crosswall thus remaining constant in size for some time. Finally, the septal crosswall is completed and the two daughter cells are separated. At this point, PG incorporation at the equators of daughter cells has already begun when cells are growing exponentially.[9] The observation that the peripheral wall on each side of the septum is thicker than the crosswall itself ruled out the possibility that the elongation is only due to splitting of the septum. Therefore, they proposed the currently admitted model, including both the septal and peripheral phases of PG assembly.[10]

This model is supported by a number of functional observations. Inhibition of division by antibiotic treatment or mutations resulted in unchecked elongation in several ovococcal species,[14] indicating that peripheral wall incorporation is independent of septum formation. Interestingly, *Streptococcus mutans* NCTC 10449S was shown to adopt a rod-like or an ellipsoid conformation depending on the salt composition of the medium.[24,25] Similarly, planktonic cells of *L. lactis* IL1403 underwent ovococcus to rod transition in a particular medium. Biofilms grown in this medium had elongated cells at their surface, while bacteria not directly exposed to the medium were only ovococci.[19] Two types of cell wall growth, a peripheral elongation in addition to the septation, are thus present in ovococci, the balance between the activities of each being finely tuned by mechanisms that remain unknown.

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Although the model ovococcal species *S. pneumoniae*, *L. lactis*, and *E. faecalis* have similar cell cycles, subtle differences were recently described in these bacteria.[33] Structured illumination super-resolution microscopy on cells treated with fluorescent vancomycin, which labels the PG precursor and nascent unmatured PG, allowed measurement of the dimensions of PG insertion sites throughout the cell cycle. Briefly, as in *E. hirae*, *S. pneumoniae* shows overlapping rounds of cell wall synthesis, with the insertion of new wall material at the equators of daughter cells that are not separated yet, whereas *E. faecalis* and *L. lactis* show discrete rounds of division. Whereas crosswall synthesis and splitting are concomitant in *S. pneumoniae* and *L. lactis*, *E. faecalis* cells initiate splitting after the septum is completed. In *L. lactis*, cell elongation is almost completed when septation begins, in contrast with *S. pneumoniae* and *E. faecalis* where septation and elongation are mostly simultaneous.

These observations support a model with two cell wall assembly machineries, although the regulation of these machineries slightly differs among ovococcal species.

Proteins Involved in the Elongation of Ovococci

Several proteins of different functions have been assigned to the elongation machinery of rod-like bacteria, such as the Mre proteins, RodA, RodZ, GpsB, bi- and monofunctional class A and class B penicillin-binding proteins (PBPs).[5,22,27,38] We will review here those proteins that have also been assigned to the elongation, or peripheral growth, of ovococci (Fig. 1).

The PBPs assemble the PG from its lipid II precursor, a disaccharide pentapeptide linked to the membrane by a lipid pyrophosphate. Class A PBPs have both the glycosyltransferase (GT) activity, which allows polymerization of the glycan chains, and the transpeptidase (TP) activity that

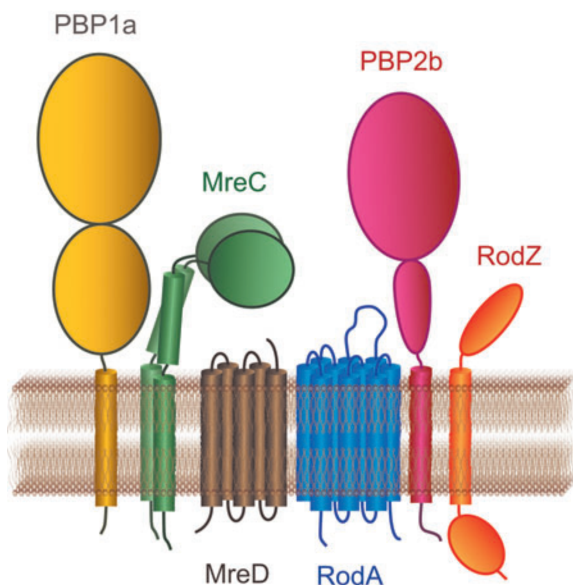

FIG. 1. Topology of the elongation proteins in ovococci. The implication of MreC,[13] MreD,[13] PBP2b,[3,26] and RodA[26] in elongation was experimentally shown in ovococci. The implication of PBP1a[13] and RodZ[1] is hypothetical.

catalyzes the crosslinking of these chains through peptide bonds.[36] Class B PBPs are monofunctional and only have the TP activity. Ovococci have three class A PBPs (PBP1a, 1b, and 2a) and two class B PBPs (PBP2b and 2x). Two exceptions are *Streptococcus pyogenes* that lacks PBP2b (Table 1), but is less elongated than other ovococcal species, and enterococci that have an additional class B PBP, PBP5, with a low affinity for β-lactams.

PBP2b of ovococci is the orthologue of *B. subtilis* PBP2 that is involved in the elongation of this rod-shaped species.[21] In an analysis of oxidative stress-resistant mutants of *Streptococcus thermophilus*, Thibessard *et al.* identified the depletion of PBP2b.[26] *S. thermophilus* cells depleted of PBP2b grew twice slower than the wild type, and they reduced their ovoid shape to be more spherical. The role of PBP2b or the shape in oxidative stress remains mysterious. A similar morphological effect of PBP2b depletion was also observed in *L. lactis*.[19] More recently, the role of PBP2b has also been described in *S. pneumoniae*, where it is essential.[3] In the absence of an inducer, conditional knocked out pneumococci took a lentil-like shape, confirming a role in peripheral growth. The TP activity of PBP2b from *S. pneumoniae* has recently been observed *in vitro*.[37]

PBP1a has both the GT and TP activities, as demonstrated *in vitro* with the recombinant enzyme from *S. pneumoniae*.[37] Unencapsulated D39 pneumococci artificially depleted of PBP1a have a smaller diameter, whereas depletion of the other bifunctional PBP2a or PBP1b has no effect on the cell diameter.[13] In rod-like bacteria, the elongation machinery was shown to affect the cell diameter,[35] supporting a role of PBP1a in the elongation of *S. pneumoniae*. Another clue that PBP1a participates in the elongation is its genetic relationship with the MreC and MreD proteins. Indeed, the presence of PBP1a, or its sequence, affects the essentiality of these two elongation proteins in *S. pneumoniae*.[13] In the unencapsulated variant of D39, MreC and MreD are essential, but deletion of *pbp1a*, or a point mutation likely affecting the GT activity of PBP1a, suppresses this essentiality. In R6 *S. pneumoniae*, the *mreCD* operon can be deleted, but this appears to depend partly on the specific sequence of PBP1a, which differs at two positions from that in the D39 strain. In *B. subtilis*, PBP1 (the orthologue of PBP1a) has been proposed to act both in elongation and division,[5] in agreement with the possibility that PBP1a plays a role in the elongation of ovococci.

The Mre proteins (for the murein region e) were first discovered in *E. coli*, where mutation of the *mre* genes resulted in round-shaped bacteria, indicating a role in elongation.[32] Three Mre protein types have been described: the soluble actin homolog MreB (and the likes Mbl and MreBH) that are absent in ovococci, MreC and MreD, two membrane proteins. The exact role of MreC and MreD remains elusive to date. In *S. pneumoniae*, both are localized at the PG insertion site.[13] They were shown to be essential in some *S. pneumoniae* strains lacking suppressor mutations in *pbp1a* or other genes of unknown function.[13] In this background, MreC and MreD depletion led to cell rounding and lysis, the first experimental evidence for a role of these proteins in the elongation of an ovococcus.[13] The *mreC* and *mreD* genes are absent in *S. pyogenes*, which may be consistent with the fact that this species does not elongate and accordingly lacks other components of the elongation system such as PBP2b and RodA.

Table 1. Proteins Thought to Participate in the Elongation of Ovococci and *Bacillus subtilis*

Species/strain	*PBP2b*	*PBP1a*	*RodA*	*MreC*	*MreD*	*RodZ*	*GpsB*
Streptococcus pneumoniae R6	P0A3M6 spr1517	Q8DR59 spr0329	Q8DQE8 spr0712	Q8DMY2 spr2023	Q8DMY3 spr2022	Q8DMX7 spr2028	Q8DR57 spr0332
Streptococcus thermophilus CNRZ1066	Q5M0P5 str0613	Q5M1K8 str0230	Q5LZC8 str1229	Q5M214 str0020	Q5M213 str0021	Q5LXJ1 str2011	Q5M1K5 str0233
Streptococcus mutans UA159	Q8DVA0 SMU_597	Q8DVL4 SMU_467	Q8CWX3 SMU_1279c	Q8DW M4 SMU_20	I6L914 SMU_21	Q8DRR7 SMU_2152c	Q8DVL1 SMU_471
Streptococcus agalactiae 2603V/R	Q8E0G8 SAG0765	Q8E1Q5 SAG0298	Q8E0U9 SAG0621	A	A	A	Q8E1Q1 SAG0302
Streptococcus dysgalactiae D166B	E8QC63 SDE12394_07630	E8QD40 SDE12394_08565	E8Q947 SDE12394_03755	A	A	E8Q9U7 SDE12394_10950	C5WIH6 SDEG_1708
Streptococcus pyogenes MGAS10394	A	Q5XAM7 M6_Spy1401	A	A	A	Q5X9B3 M6_Spy1865	Q5XAN0 M6_Spy1398
Lactococcus lactis IL1403	Q9CIL7 LL0339	Q9CI23 LL0543	Q9CH43 LL0896	Q9CDI9 LL2231	Q9CDJ0 LL2230	Q9CE74 LL1971	Q9CF28 LL1653
Enterococcus faecalis V583	Q830D1 EF_2857	Q836G4 EF_1148	Q820T7 EF_2502	Q82ZJ4 EF_3062	Q82ZJ5 EF_3061	Q82ZB7 EF_3149	Q836G2 EF_1151
Enterococcus faecium Aus0004	H8LF48 EFAU004_01299	H8LE97 EFAU004_00997	H8LD04 EFAU004_00554	H8LAP3 EFAU004_02614	H8LAP3 EFAU004_02614	H8LAS4 EFAU004_02645	H8LE94 EFAU004_00994
Enterococcus hirae ATCC 9790	I6T1G1 EHR_14050	I6S3M8 EHR_12290	I6T427 EHR_01910	I6S0D1 EHR_05725	I6T5X3 EHR_05730	I6T5U6 EHR_05540	I6T0I4 EHR_12275
B. subtilis 168	P54488 BSU25000 (PBP2a YqgF)	P39793 BSU22320 (PBP1)	P39604 BSU38120	Q01466 BSU28020	Q01467 BSU28010	O31771 BSU16910 (YmfM)	P0CI74 BSU22180

For each protein, the UniprotKB number is given with the ordered locus name. The *B. subtilis* protein name is given in parenthesis if different.
A, absent.

Intriguingly, *mreC* and *mreD* are also missing in *Streptococcus agalactiae* and *Streptococcus dysgalactiae* (Table 1)[1], which is consistent with the fact that these species appear shorter than other ovococci.

SEDS (for shape, elongation, division, and sporulation) proteins are integral membrane proteins with 10 membrane-spanning segments.[8] In genomes, their genes are often located in the same operons with genes encoding monofunctional class B PBPs, suggesting that they belong to the same machinery.[38] These membrane proteins allow flipping of the lipid II from the inside to the outside of the cell, providing PBPs with their substrate.[16] Ovococci have generally two SEDS, RodA and FtsW that are involved in elongation and division, respectively.[38] Some exceptions are to be noted: *S. pyogenes* lacks RodA, whereas *Enterococcus faecium* has one and *E. faecalis* and *E. hirae* have two additional SEDS proteins (Table 1). The absence of RodA in *S. pyogenes* is consistent with the absence of PBP2b and elongation in this species. The presence of at least one additional SEDS protein in enterococci is consistent with the additional class B PBP5, which has a low affinity for β-lactams. RodA depletion in *S. thermophilus* results in the same round phenotype as PBP2b depletion[26] supporting its role in elongation. Land and Winkler reported preliminary results suggesting the essentiality of RodA in *S. pneumoniae*.[13]

More recently, other proteins were proposed to participate in the elongation of ovococci.

In *B. subtilis*, the small protein GpsB interacts with PBP1 (PBP1a's orthologue) to allow its transition between the division and elongation machineries through the cell cycle.[5] Given that GpsB is present in ovococci and that PBP1a probably participates in the elongation of pneumococci, Land and Winkler proposed that GpsB also participates in the peripheral growth of ovococci.[13] However, subsequent studies of this essential protein showed that GpsB is rather implicated in the division of *S. pneumoniae*, as depletion caused cell elongation.[12] In *B. subtilis*, GpsB was proposed to act primarily as a vector to relocate PBP1 from the division to the elongation machinery, with a minor effect on the reverse transition from the elongation to the division machinery. If a similar mechanism operates in ovococci, it could function in the opposite way, explaining that depletion leads to aborted division.

RodZ is a nonessential bitopic membrane protein of *E. coli*, where its depletion results in shorter cells, and its overexpression causes elongation of the bacteria,[23] suggesting a role in elongation. RodZ was proposed to play a role in MreB cytoskeleton polymerization and stability[2] and was shown to link MreB filaments to the membrane.[28] RodZ is widely conserved in bacteria, but is generally absent in species devoid of MreB with the exception of ovococci where it is present.[1] The conserved function of RodZ might therefore be linked to that of the MreC/MreD complex, which is found in ovococci, rather than to MreB. Note that *S. agalactiae*, which lacks MreC and MreD proteins, also lacks RodZ, but *S. pyogenes* and *S. dysgalactiae* that also lack MreC and MreD nevertheless encode RodZ in their genome (Table 1).

To ensure a proper insertion of PG strands in the peripheral wall, additional bifunctional class A PBPs and hydrolases may be required.[38]

An outstanding observation should be taken into consideration here. In a study of the localization of wall-anchored proteins in *S. pyogenes*, Raz *et al.* noted that methicillin treatment at precisely 0.2 μg/ml induced coccus to rod transition.[20] This observation is counterintuitive as *S. pyogenes* does not encode for the elongation proteins PBP2b, RodA, MreC, and MreD (Table 1). Methicillin is known to inhibit specifically PBP2x in ovoid bacteria such as *S. pneumoniae*[12] and *L. lactis*.[19] At 0.2 μg/ml of methicillin, the activity of PBP2x may be inhibited, and the three bifunctional class A PBPs of *S. pyogenes* may remain functional at a level that allows peripheral PG insertion in the cell wall.

Characterized Interactions Between Elongation Proteins

In *E. coli*, two-hybrid assays showed that MreC interacts with both MreB and MreD and they were proposed to form a complex with RodA and PBP2, the monofunctional class B PBP assigned to elongation.[11] These five proteins have a similar localization in *E. coli*.[31] Surprisingly, however, MreB, MreC, MreD, and RodA are each able to localize correctly in the absence of the four other proteins. Only PBP2 localization appears to depend on the presence of MreC.[31] In *Helicobacter pylori*, MreC and PBP2 (the orthologue of PBP2b) form a complex that is required for the elongation.[7] In *B. subtilis*, two-hybrid experiments have shown that PBP1 (the orthologue of PBP1a) interacts with MreC and GpsB.[5]

RodZ was shown to interact with MreB in *E. coli* by a two-hybrid assay, and the crystal structure of a complex between MreB and the cytoplasmic domain of RodZ was solved for proteins from *Thermotoga maritima*.[28] Van den Ent *et al.* proposed that MreB is brought close to the membrane by interacting with RodZ, which favors its interaction with MreC and links the cytoskeleton to the PG synthesis machinery.[28]

No such studies have been performed with elongation proteins from ovococci. In these organisms, although some of the same interaction patterns likely occur, significant differences are also expected. For example, the MreB-interacting domain of RodZ is present in the protein from ovococci although MreB is absent.

Recently, some complexes, including up to five proteins of the divisome (a complex, including all the division proteins) of *S. pneumoniae* (namely, DivIC, DivIB, FtsL, FtsW, and PBP2x), were reconstituted *in vitro*.[18] However, to our knowledge, no characterized protein–protein interactions have demonstrated the presence of an elongasome (a complex, including all the elongation proteins) in ovococcus species. We report in this study, the reconstitution of two complexes of recombinant proteins of *S. pneumoniae* (Fig. 2).

The bitopic membrane protein MreC and the integral membrane protein MreD (five predicted transmembrane domains) were fused to distinct affinity tags (Strep-MreC and His_8-MreD). Coexpression in *E. coli* followed by two successive purification steps on Strep-Tactin® and Ni-NTA resin allowed us to recover the complex *in vitro* (Fig. 2). Size-exclusion chromatography–multi-angle laser light scattering analysis of the sample suggested a complex comprising a dimer of MreC and a single MreD unit, although a large portion of the sample was aggregated (55%) affecting the interpretation.

Similarly, a membrane protein complex of recombinant PBP2b and RodA from *S. pneumoniae* was isolated in the

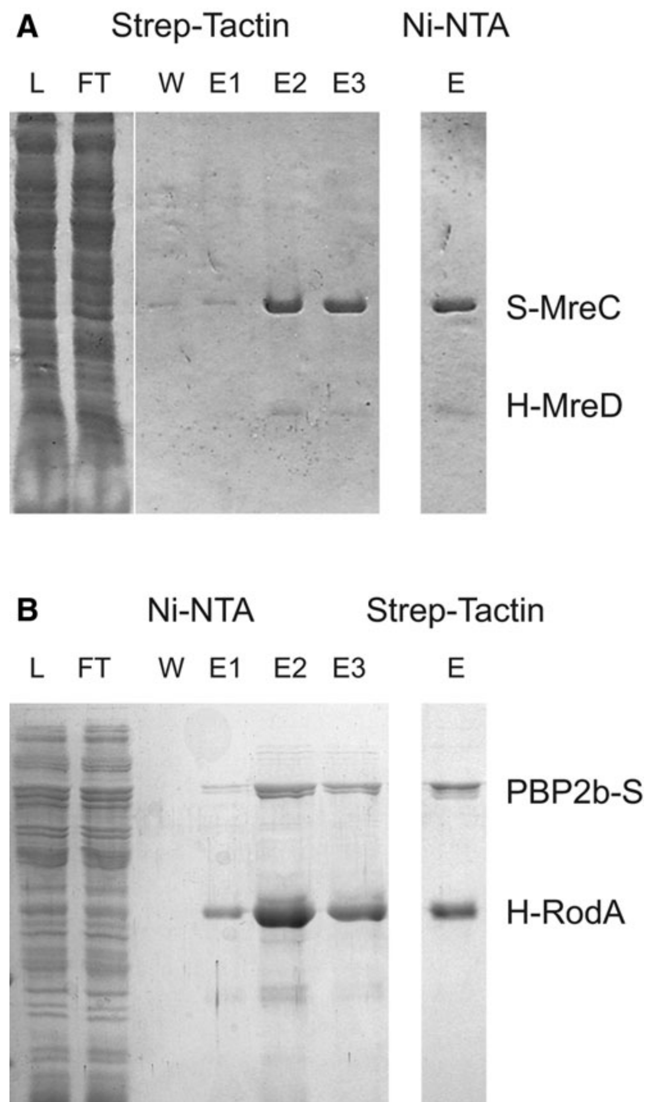

FIG. 2. Isolation of recombinant MreC/MreD and PBP2b/RodA complexes from pneumococcus. **(A)** MreC and MreD were expressed in *Escherichia coli* from an artificial operon fused with N-terminal Strep- and His_8-tags, respectively. Membranes were isolated and solubilized with *n*-dodecyl-β-D-maltopyranoside. The complex was isolated by two successive affinity chromatography steps on Strep-Tactin® and Ni-NTA. **(B)** PBP2b with a C-terminal Strep-tag and RodA with an N-terminal His_8-tag were similarly expressed and the complex isolated, in that case, first by Ni-NTA chromatography followed by a Strep-Tactin® purification. L, W, and E stand for load, wash, and elution. Samples were analyzed by Coomassie-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

laboratory (Fig. 2). In this case, PBP2b harbored a Strep-tag, whereas RodA was fused to a His_8-tag.

Our attempts to isolate a larger elongation protein complex, including the four proteins MreC, MreD, RodA, and PBP2, were not successful so far. The trivial technical explanation cannot be discounted since it is difficult to find appropriate conditions to copurify stable membrane protein complexes as these should solubilize the proteins, while preserving their interaction, two competing goals. Nevertheless, such a complex may indeed not exist. Colocalization does not necessarily imply interaction, and protein may participate in a common function without interacting. If interactions take place, they may occur successively between different partners during the cell cycle, and thus preclude the isolation of one single large assembly. Finally, additional hitherto unknown partners may be required to form stable complexes that can be isolated recombinantly.

Relationship Between the Elongation and the Division Machineries

In 1990, Lleo *et al.* gave the evidence for the existence of two distinct machineries for the elongation and the division of ovococci. They also hypothesized that these machineries would be independent and mutually exclusive.[14] Some experimental observations are in conflict with this latter statement, as several ovococci appear to simultaneously elongate and synthesize a septum, such as *E. hirae*, *S. pneumonia*, and *E. faecalis*.[9,33]

The link between the two PG synthesis machineries remains unclear in ovococci. In *B. subtilis*, GpsB was proposed to shuttle between the elongasome and the divisome, forming a link between elongation and division machineries. Proteins from both machineries were shown to colocalize throughout the cell cycle of *S. pneumoniae* at the resolution of epifluorescence optical microscopy.[17,38] However, and most importantly, recent observations with improved resolution using three-dimensional structured illumination microscopy revealed a clear difference in the localization of PBP1a and PBP2x during septum formation of *S. pneumoniae*.[12] The constriction of the PBP1a ring was found to lag behind that of PBP2x. This first observation that different PBPs are in distinct localization at some point during the cell cycle is in strong support of the two-machinery model. PBP1a remaining at the periphery of the closing septum is consistent with the primary role in peripheral growth. Nevertheless, as PBP1a and PBP2x are colocalized at the onset of the cell cycle, it is not excluded that PBP1a plays a role in both machineries, as it was proposed in *B. subtilis*.[5] Such a dual role of PBP1a would also be consistent with intriguing β-lactam resistance phenotypes that hint at an interaction of PBP2x with PBP1a.[39] Also, of note, in these high-resolution microscopy experiments is the finding that the rings of the different proteins (PBPs or FtsZ) are discontinuous and constituted of foci of different sizes arranged irregularly in circles.[12]

In *E. coli*, FtsZ and PBP2 (the orthologue of PBP2b) can act together in inserting PG in the side wall of cells when MreB is inhibited.[30] Also, in these organisms, division is preceded by a short period of PG insertion at the midcell dependent on FtsZ and the elongasome. This phase of the cell cycle is particularly visible when septation is inhibited (*e.g.*, de Pedro *et al.*[6]). Consistent with these observations of the PG, it was recently shown by immunofluorescence that elongation proteins are transiently colocalized with those of the division before the septal PG synthesis phases in *E. coli*.[29] Also, fluorescence resonance energy transfer analysis gave evidence of an interaction between the class B PBP2 and PBP3 that belong to the elongation and division, respectively.[29] Interestingly, in ovococci, the elongation or peripheral growth occurs while the FtsZ-ring is assembled

and possibly even during its constriction. In this respect, peripheral PG synthesis in ovococci is more akin to the localized preseptal PG synthesis observed in rod-shaped bacteria than to their true elongation phase.[31]

Today, we lack evidence for the existence of two physically separated machineries of PG assembly in ovococci, with the exception of the distinct localization of PBP1a and PBP2x. Nevertheless, a comprehensive model was recently proposed that includes all the morphogenesis proteins in a single large machinery comprising two complexes located at the midcell, the site of insertion of new PG in *S. pneumoniae*[15]: the division proteins acting on the leading edge of the closing septum and the elongation proteins on the outer edge of the septal disc. It is expected that various features of this model will be tested in the coming years by various novel multicolor super-resolution microscopy techniques and *in vitro* reconstitutions.

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Disclosure Statement

No conflicts of interest.

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