THE CELL WALL OF *STREPTOCOCCUS PNEUMONIAE*

Waldemar Vollmer[1], Orietta Massidda[2] and Alexander Tomasz[3]

[1] Institute for Cell and Molecular Biosciences, The Centre for Bacterial Cell Biology, Newcastle University, Newcastle upon Tyne, United Kingdom.

[2] Centre for Integrative Biology, University of Trento, Trento, Italy.

[3]The Rockefeller University, New York, NY, USA.

Summary

Streptococcus pneumoniae has a complex cell wall that plays key roles in cell shape maintenance, growth and cell division, and interactions with components of the human host. The peptidoglycan has a heterogeneous composition with more than 50 different subunits (muropeptides) – products of several peptidoglycan modifying enzymes. The amidation of glutamate residues in the stem peptide is needed for efficient peptide cross-linking, and peptides with a dipeptide branch prevail in some beta-lactam resistant strains. The glycan strands are modified by deacetylation of N-acetylglucosamine residues and O-acetylation of N-acetylmuramic acid residues, and both modifications contribute to pneumococcal resistance to lysozyme. The glycan strands carry covalently attached wall teichoic acid and capsular polysaccharide. Pneumococci are unique in that the wall teichoic acid and lipoteichoic acid contain the same, unusually complex repeating units decorated with phosphoryl choline residues, which anchor the choline-binding proteins. The structures of lipoteichoic acid and the attachment site of wall teichoic acid to peptidoglycan have recently been revised. During growth pneumococci assemble their cell wall at mid-cell in coordinated rounds of cell elongation and division, leading to the typical ovococcal cell shape. Cell wall growth depends on the cytoskeletal FtsA and FtsZ proteins and is regulated by several morphogenesis proteins that also show patterns of dynamic localization at mid-cell. Some of the key regulators are phosphorylated by StkP and dephosphorylated by PhpP, to facilitate robust selection of the division site and plane, and maintain cell shape.

Introduction

Since the last edition of this book (Year 2006) a number of important publications appeared in the literature the inclusion of which made it necessary to limit the topics of this chapter to information that has bearing on the biochemical and genetic aspects of covalently linked components of the pneumococcal cell wall. Information on proteins non-covalently attached to the cell wall (Chapter 24); cell walls and phase variation (Chapter 22) and inflammatory activity of cell walls (Chapter 21) are reviewed separately.

Historically, studies of the pneumococcal cell wall were motivated by such unique features as the presence of choline in the teichoic acids and the pleiomorphic changes that accompany removal or alteration of choline residues; and structural changes in peptidoglycan that are associated with penicillin resistance. Most recent contributions to the field include immunofluorescence (1-7) or fluorescent tagged (8, 9)(microscopic localization of cell wall synthetic enzymes at sites of wall synthesis; identification of genetic determinants and enzymes that are involved with the chemical modification of peptidoglycan precursors and peptidoglycan itself, the synthesis of teichoic acid precursors and the assembly of lipoteichoic acid (LTA) and wall teichoic acid (WTA); and the mechanisms and regulation of the cell wall machinery at the central growth zone.

Functional anatomy of pneumococcal cell wall.

The overwhelming majority of “natural” isolates of pneumococci are enwrapped on their outermost surface by one or the other of the more than 90 chemically different capsular polysaccharides that this bacterial species is capable of synthesizing (10)).

Under these diverse structures lies the cell wall which, as far as the resolution of currently used analytical techniques can tell, is much more uniform in its chemistry: it is composed of a peptidoglycan covalently linked to chains of an unusually complex teichoic acid (11-13) which contain as structural components phosphoryl choline residues (14, 15). The phosphoryl choline residues play multiple roles in the physiology and virulence of the pneumococcus (see below). The two polymers, peptidoglycan and teichoic acid, make up the bulk of the cell wall in roughly equal (mg to mg) proportions (16). In most but not all cases, chains of the capsular polysaccharide are attached by covalent bonds to the underlying peptidoglycan (17) (Fig. 1).

In electron microscopic thin sections (18) prepared by the method of Kellenberger (osmium tetraoxide and glutaraldehyde fixation followed by uranylacetate and lead citrate staining) the cell wall of *Streptococcus pneumoniae* strain R36A appears as a band of uniform width composed of two electron dense lines (30-40 nm each) enclosing a wider low density layer (60-80 nm). The distribution of teichoic acid chains appears to be uniform within this wall layer (19) and this is presumably also true for the peptidoglycan. Some anatomically differentiated areas may be identified through electron microscopy. These are (i) the equatorial areas where cell wall growth becomes centripetal (formation of septum or cross wall) and which represent the "growth zones" of the entire wall; (ii) in dividing cells a circumferential thickening ("hump") of the cell wall appears at the place of the incipient septa. (iii) Parallel, or perhaps just prior to the beginning of the formation of septum, the hump appears to be split at the center, and the two halves begin to "move" on the cell surface symmetrically to the left and to the right of the ingrowing septum coupled to the growth and eventual division of the cell into two daughter cells. Due to the conservative mode of replication of the pneumococcal cell wall (see below), these two half-humps are morphological age markers: they divide the cell wall of each pneumococcal cell

into two hemispheres which differ in age by one cell generation. The functional correlates of these morphological changes began to be identified by the use of immunofluorescence microscopy which allowed the localization of the various pneumococcal high molecular weight penicillin binding proteins (PBPs) and the FtsZ-ring to equatorial and septal areas of the bacteria in various stages of cell division (2-4, 7). A special role of two hydrolases, the carboxypeptidase PBP3 (1, 20) and the endopeptidase PcsB (21-23) in these processes was also proposed. (iv) The final stage of cell division: the separation of daughter cells may be inhibited in pneumococci by several means resulting in the formation of long chains of bacteria. Under these conditions one can observe by electron microscopy a thin bridge of cell wall material connecting neighboring cells to one another. The hydrolase, LytB (an endo-β-1,4-N-acetylglucosaminidase) has been identified as an enzyme essential for the terminal separation of daughter cells at the end of cell division (24, 25).

Structure of peptidoglycan.

Purified cell walls of the non-encapsulated strain R6St (streptomycin-resistant) of *Streptococcus pneumoniae* were hydrolyzed by the pneumococcal amidase (N-acetylmuramic acid L-alanine amidase; the product of *lytA* gene) under conditions in which this enzyme can quantitatively release the peptide units of cell wall muropeptides. A method that uses muramidase digestion of the pneumococcal cell wall or peptidoglycan followed by HPLC separation of muropeptides, through an adaptation of the method of Glauner and Schwarz, has been described (26, 27).

In the amidase method, the family of peptides were separated by high performance liquid chromatography (HPLC) and size fractionation; determination of amino acid composition and

NH2 termini. Partial sequencing of the peptides generated by HPLC and analysis by time-of-flight mass spectrometry has allowed the identification of a surprisingly large number of monomeric, dimeric and trimeric peptides (28) showing a diversity comparable to that seen among the muropeptide species identified in *E. coli*. In the structural assignments it was assumed that the amino acids within the stem peptides had the usual, alternating sequence of L and D amino acids beginning with L-alanine in position 1, followed by a D-isoglutamine and then by L-lysine. The carboxyterminus in the stem peptide is occupied by two consecutive D-alanine residues; however, such muropeptides with intact pentapeptide residue are rare in pneumococci cultivated under normal growth conditions. Extension of this analytical technique to cell walls of clinical isolates from a large variety of isolation sites and dates and expressing a variety of different capsules has led to the proposition that the cell wall muropeptide composition of *S. pneumoniae* grown in the commonly-used semisynthetic media and harvested in the late exponential phase is constant and characteristic of the species. The most abundant monomer of the pneumococcal peptidoglycan was a tripeptide, the most frequent dimer a directly crosslinked tri-tetrapeptide. Interestingly, the representation of carboxyterminal alanine was extremely rare suggesting the presence of powerful DD- and LD-carboxypeptidases. These enzymes, PBP3 and LdcB, will be discussed below.

An interesting feature of the peptide network was the presence of both directly and indirectly crosslinked components. In the latter, alanyl-serine or alanyl-alanine dipeptides formed the crosslink. In terms of crosslinking mode, the pneumococcal cell wall may be classified as either A1 or A3 depending on which dimer one chooses. A massive distortion of peptidoglycan composition in the direction of preponderance of indirectly crosslinked components was demonstrated in several penicillin resistant clinical isolates (29).

The amidase also releases the glycan chains which in *S. pneumoniae* harbor two modifications: *N*-deacetylation of some of the Glc*N*Ac residues resulting in glucosamine, and O-acetylation at some of the Mur*N*Ac residues. The latter modification is acid labile and lost during the removal of wall teichoic acid to purify peptidoglycan and therefore the glycan chains released by the amidase carry glucosamine residues the percentage of which depends on the strain and growth conditions. The isolated glycan chains can be chemically re-acetylated and analyzed by HPLC with or without partial digestion by muramidase. This analysis revealed that glycan chains of less than 25 disaccharide units are virtually absent in pneumococcal PG (27), in sharp contrast to that of staphylococci or *E. coli* in which short glycan chains prevail (30, 31). That pneumococcal PG contains relatively long glycan chains was confirmed by size exclusion chromatography (32).

The muramidase method in combination with mass spectrometry allowed detection of ~50 different muropeptides (27). It confirmed the above mentioned peptide composition and quantified the abundance of modifications in uncross-linked (monomeric) and cross-linked (dimers, trimers) structures, allowing predictions about the activities of cell wall enzymes. For example, non-amidated D-glutamate residues at position 2 were significantly less abundant in cross-linked muropeptides, suggesting that enzymes performing cross-linking reactions (the penicillin-binding proteins) require the presence of the amidated D-glutamate residue at position 2 (27, 33). The analysis also revealed the presence of cross-linked muropeptides (mainly trimers) that lacked Glc*N*Ac or the Glc*N*Ac-Mur*N*Ac disaccharide at one of the peptides, indicating *N*-acetylglucosaminidase and amidase activities, presumably those of LytB and LytA, respectively. Muropeptide analysis has recently been used to study different aspects of pneumococcal cell wall physiology (34-36).

Peptidoglycan biosynthesis and remodeling

Pneumococci contain the essential genes of the general biochemical pathway of peptidoglycan synthesis and cell morphogenesis that were studied extensively in other model species such as *E. coli* and *B. subtilis* (37, 38). Many of the peptidoglycan precursor genes and cell division proteins cluster in three chromosomal regions, instead of one single cluster present in *E. coli* and *B. subtilis*, consistent with the hypothesis that the ovococcal shaped pneumococci require less co-regulation of cell wall and division genes than rod-shaped species (39). In this section we will focus on covalent modifications in the peptidoglycan that are important for the physiology or lifestyle of pneumococci.

Modifications of the peptidoglycan precursor.

Many Gram-positive species amidate the D-isoglutamate residue at the α-carboxylic group by transfer of an amido group from L-glutamine to the cytosolic precursors UDP-MurNAc-pentapeptide or the bactoprenol-linked precursors lipid I and lipid II. This amidation reaction is catalyzed by a complex of two proteins, MurT and GatD, which were originally identified in *S. aureus* (40). MurT/GatD are essential in both, *S. aureus* and *S. pneumoniae* (40, 41), consistent with the observation that amidation of the lipid II precursor is required for efficient cross-linking in an *in vitro* assay for peptidoglycan synthesis with purified penicillin-binding proteins (33). Hence, MurT/GatD seem to be essential because they provide a modification needed for the essential peptide cross-linking reaction.

The presence of a 'branch' consisting of 2 to 7 L-amino acids “added” to the ε-amino group of L-lysine at position 3 of the stem peptide is another wide-spread modification in Gram-

positive bacteria whereby the sequence of the branch varies between species. Virtually all *S. aureus* strains have a pentaglycine branch but the peptidoglycan is less homogeneous in *S. pneumoniae* in which strains can significantly differ in the amount of three types of peptides: with no branch, with an L-Ala-L-Ala branch or with an L-Ser-L-Ala branch (42).

The branch is added to the lipid II precursor by the ligases MurM and MurN. The *in vivo* substrate of MurM is lipid II and the enzyme catalyzes the addition of a serine or alanine residue (depending on the particular *murM* allele) to the free amino group of lysine in the stem peptide – in a reaction in which the amino acid donor is presumed to be an amino acyl-tRNA (43). This reaction is followed by the Mur~~M~~N catalyzed addition of an alanine to complete the dipeptide branch (see Fig. 2). Although the peptide branches synthesized by MurM and MurN are not essential for pneumococcal growth, they are required in various strains to express high levels of β-lactam resistance. A more detailed description of the connection between the *murMN* operon and β-lactam resistance is provided in part 4 of this chapter (below).

Penicillin-binding proteins (PBPs)

S. pneumoniae has six PBPs of which three, PBP1a, PBP1b and PBP2a are class A bi-functional glycosyltransferase-transpeptidases (GTase-TPases) capable of polymerizing the precursor lipid II to form the glycan chains and performing peptide cross-linking reactions. None of these bi-functional enzymes is essential, but double mutants lacking *pbp1a* and *pbp2a* are not viable (44), and *in vitro* experiments with the purified proteins showed that both require amidated lipid II substrate for efficient transpeptidase activity (33).

S. pneumoniae also has two essential class B monofunctional transpeptidases, PBP2x and PBP2b, involved with septal and peripheral peptidoglycan synthesis, respectively (7, 45).

Consistent with their roles, cells depleted of PBP2x become elongated and cells depleted of PBP2b are shorter. PBP2x has two C-terminal PASTA (PBP and Serine/Threonine kinase associated) domains which are required to localize the protein at mid-cell (46). Genetic evidence points to an important role of branched peptide substrates for these PBPs. The remaining PBP, PBP3, is not a synthase but a carboxypeptidase for trimming of pentapeptides to tetrapeptides (see below).

Trimming of the peptides by carboxypeptidases. Newly synthesized peptidoglycan contains abundant pentapeptides with a D-alanyl-D-alanine terminus, but these are trimmed in two steps to tripeptides which prevail in the mature pneumococcal peptidoglycan. The DD-carboxypeptidase PBP3 produces tetrapeptides (47), which are the substrate for the LD-carboxypeptidase LdcB (or DacB) to produces the tripeptides. The wall peptide composition in a PBP3 defective mutant (48) and in the laboratory strain R36A growing in the presence of sub-inhibitory concentrations of clavulanic acid, a selective inhibitor of PBP3 in this bacterium (49) showed accumulation of peptide species that retained carboxyterminal D-alanine residues. Pneumococci grown in the presence of clavulanate also showed abnormal physiological properties: premature induction of stationary phase autolysis; hypersensitivity to lysozyme and reduced MIC values for deoxycholate and penicillin.

The *ldcB* gene was identified in a search for hypothetical peptidoglycan hydrolase genes, and a deletion mutant has defects in cell shape and septation (50), as has a *dacA* mutant lacking PBP3 (1). However, only *dacB* mutants were significantly attenuated in an intranasal mouse infection model, and showed increased uptake by professional macrophages and reduced adherence to lung epithelial cells (51). The *dacB* mutant is rich in tetrapeptides (50, 52), but it is

not known why the presence of tetrapeptides instead of tripeptides has these drastic effects on the physiology and pathogenicity of pneumococci. The crystal structure of LdcB shows a catalytic domain with a fold characteristic for the LAS family of Zn-dependent peptidases (51, 52).

The putative lytic transglycosylase MltG

The *mltG* (*spd_1346*) gene was recently linked to peripheral peptidoglycan synthesis based on the observation that its inactivation suppresses the requirement for PBP2b, MreCD, RodA and RodZ, i.e., genes that are all part of the pneumococcal elongation machinery (53). MltG has a YceG-family domain that ~~has been hypothesized to have~~ lytic transglycosylase activity. A model has been proposed according to which MltG removes nascent glycan strands, produced by PBP1a, from their membrane anchor to facilitate their incorporation into the old cell wall by RodA and PBP2b (53). However, direct proof for a lytic transglycosylase activity of MltG has yet to be presented, and the lytic transglycosylase products, 1,6-anhydro-*N*-acetylmuramic acid termini in glycan chains and turnover products have yet to be detected.

Peptidoglycan hydrolases involved in septum remodeling and cleavage

Several peptidoglycan hydrolases are important for pneumococcal growth and morphology. The extracellular protein PcsB localizes to the cell division site where it is required for proper septum formation and cleavage (54). The *pcsB* gene is the only essential gene under the control of the WalRK cell wall stress regulon, and the depletion of *pcsB* results in growth arrest and severe morphological defects. The active site of PcsB resides near the C-terminus and belongs to the CHAP (for Cysteine, Histidine dependent amidohydrolase/peptidase) family of peptidoglycan endopeptidases, but such an activity could only be observed for purified PcsB

when tested in an in-gel zymogram assay. In the cell, PcsB requires activation by the essential membrane-bound ABC transporter-type FtsEX complex (54, 55). The crystal structure of PcsB shows an inactive homodimer in which the V-shaped coiled-coil domain of each monomer inserts into the active site groove of the other, explaining why this dimer form is inactive (23). It was suggested that FtsEX utilizes ATP hydrolysis to induce conformational changes in the PcsB dimer to unlock this mutual inhibition. However, the precise roles of FtsEX and PcsB in septum formation and cleavage has yet to be established.

LytB is a secreted endo-β-*N*-acetylglucosaminidase with a choline-binding domain consisting of 14-18 imperfect repeats, which direct the enzyme to the septum and cell pole regions, followed by an SH3b peptidoglycan binding domain, a small carbohydrate-binding domain with unknown function and a C-terminal catalytic domain (56). LytB functions at the final stages of the division process to separate the daughter cells. Mutants lacking LytB grow in deeply constricted chains of unseparated cells (57). Purified LytB is able to disperse ~~chaining~~ cells of a *lytB* mutant. LytB cleaves at sites with fully acetylated GlcNAc residues and has preference for monomeric peptides, suggesting a substrate assisted catalytic mechanism (25).

Apart from their role in cleavage of the septum and autolysis, peptidoglycan hydrolases do not seem to be very active during pneumococcal growth in the laboratory. There is a minimum of peptidoglycan turnover at the hemispherical regions with the old peptidoglycan remaining virtually inert during growth (58, 59).

Peptidoglycan N-acetylglucosamine deacetylase PgdA

An unusually high proportion of hexosamine units in the glycan strands of the pneumococcal cell wall is not *N*-acetylated, explaining the resistance of this peptidoglycan to the hydrolytic action of lysozyme, a muramidase that cleaves in the glycan backbone. A gene, *pgdA*, was identified as encoding for the peptidoglycan N-acetylglucosamine deacetylase A with amino acid sequence similarity to fungal chitin deacetylases and rhizobial NodB chitooligosaccharide deacetylases (60). PgdA is a metal-dependent enzyme belonging to the family 4 carbohydrate esterases. Its crystal structure shows a His-His-Asp catalytic triad in its active site (61, 62). Pneumococci in which *pgdA* was inactivated produce fully *N*-acetylated glycan and become hypersensitive to exogenous lysozyme in the stationary phase of growth. The *pgdA* gene may contribute to pneumococcal virulence by providing protection against host lysozyme. A pneumococcal strain expressing capsular type II with inactivated *pgdA* showed reduced virulence in the mouse model of intraperitoneal infection (63).

Peptidoglycan O-acetyltransferase

Many bacteria modify the C6-OH group of MurNAc residues in their peptidoglycan by an O-acetyl group and this modification has been ~~implied~~ in the regulation of autolysins and resistance to lysozyme (64). The pneumococcal *adr* gene encoding the O-acetyltransferase has been initially identified in a mariner mutagenesis screen for increased sensitivity to penicillin in the background of the resistant strain Pen6 (65). *Adr* mutant cells also show higher sensitivity to lysozyme, and Adr shares sequence homology with the peptidoglycan O-acetyltransferase OatA of *S. aureus* (66). Muropeptide analysis by HPLC and mass spectrometry showed that the *adr* mutant lacked muropeptides with O-acetylated MurNAc residues, suggesting that Adr is the pneumococcal peptidoglycan O-acetyltransferase. Recent work showed that peptidoglycan O-

acetylation by Adr occurs at mid-cell and that this modification protects the cell wall from cleavage by the autolysin LytA (67).

Sortase A-dependent covalent attachment of proteins to the pneumococcal cell wall

Most virulence related pneumococcal proteins studied so far were shown to be attached to the cell surface through the choline residues of teichoic acids, and pneumococci have been described as a paradigm for the display of virulence proteins through specific but noncovalent associations with the cell surface (68). Such choline binding proteins include PspA; PsaA (see Chapters 21 and 22); the enzyme phosphoryl choline esterase (69, 70); and LytA, in which a 20-amino-acid repeat was shown to recognize choline residues in wall or membrane teichoic acids (71). The observations described in this report demonstrate that the mechanism of surface display of proteins does not depend on the choline binding paradigm alone; pneumococci also use covalent anchoring for some surface proteins. Data available from the genome of *S. pneumoniae* strain R6 (72) indicate that there are at least 23 proteins carrying the LPXTG motif and 15 of these have this recognition sequence at the C terminus, as expected for proteins that are processed by a typical sortase.

Inactivation of sortase gene *srtA* in *Streptococcus pneumoniae* strain R6 caused the release of beta-galactosidase and neuraminidase A (NanA) from the cell wall into the surrounding medium. Both of these surface proteins contain the LPXTG motif in the C-terminal domain. Complementation with plasmid-borne *srtA* reversed protein release. Deletion of *murM*, a gene involved in the branching of pneumococcal peptidoglycan, also caused partial release of beta-galactosidase, suggesting preferential attachment of the protein to branched muropeptides in the cell wall. Inactivation of srtA caused decreased adherence to human pharyngeal cells in vitro

but had no effect on the virulence of a capsular type III strain of *S. pneumoniae* in the mouse intraperitoneal model. These observations suggest that similarly to other gram-positive bacteria sortase-dependent display of proteins occurs in *S. pneumoniae* and that some of these proteins may be involved in colonization of the human host (73).

Peptidoglycan composition and penicillin resistance

The first series of highly penicillin resistant clinical isolates examined by the HPLC method were from South Africa. It was in these isolates that the mechanism of resistance, namely reduction in antibiotic "affinity" of penicillin binding proteins (PBPs), was identified for the first time (74). It was also in genetic crosses with these isolates used as DNA donors that the stepwise nature of penicillin resistance (i.e., the sequential reduction in the penicillin affinity of several high molecular weight PBPs in parallel with the gradually increasing penicillin MIC value) was recognized (74).

Analysis of the penicillin resistant South African clinical isolates revealed that they produced cell walls of a radically different composition from the one seen in the penicillin susceptible and non-encapsulated laboratory isolate (75). When the HPLC analysis was extended to the walls of several penicillin susceptible clinical isolates and several resistant strains (all but one from South Africa), the striking shift towards indirectly crosslinked wall peptide composition in the resistant isolates was fully confirmed. A link between resistance to penicillin and abnormal wall composition was also suggested by the analysis of genetic crosses: a shift towards the distorted wall composition of the resistant DNA donor was observed in genetic transformants above certain MIC value. It was suggested that the anomalous wall peptide

composition reflected the altered substrate preference of the penicillin resistant PBPs, a shift from the linear to the branched wall peptide precursors (75).

The *murMN* operon

A considerable clarification concerning determinants of cell wall structure and also its relationship to penicillin resistance was obtained by the identification of the *murMN* operon which encodes enzymes involved in the synthesis of branched structured muropeptides in the pneumococcal peptidoglycan (42). The same determinants were also described independently and named *fib* by another group (76). The *murMN* operon shows homology to the *femXAB* genes of *S. aureus* which are involved with the addition of the pentaglycine branches to the epsilon amino group of lysine residues in the staphylococcal peptidoglycan (77).

Inactivation of the *murM* operon or inactivation of the *murM* gene alone did not interfere with growth of the bacteria but caused the production of a peptidoglycan composed exclusively of linear muropeptides. Another consequence of inactivation was the complete loss of the penicillin resistant phenotype. Examination of a large number of *S. pneumoniae* clinical isolates representing different genetic lineages has identified several distinct *murM* alleles carried by penicillin resistant strains which also showed different and unique muropeptide composition in their peptidoglycan (78). Analysis of the *murM* alleles from penicillin resistant isolates showed that they differed from one another and from the *murM* carried by penicillin susceptible strains in regions of considerable sequence diversity that were distributed as heterologous “patches” along the *murM* gene sequence. Different *murM* alleles from several penicillin resistant *S. pneumoniae* strains, each with a characteristic branched peptide composition were introduced on a plasmid into a common penicillin susceptible laboratory strain. All transformants remained penicillin

susceptible but their cell wall composition changed in directions which corresponded to the muropeptide pattern of the strain from which the *murM* allele was derived. This observation suggests that the muropeptide composition of *S. pneumoniae* is determined by the particular *murM* carried by the strain (43, 78) (Figs. 3A and B and Table 1).

The relationship between *murM* alleles and the penicillin resistant phenotype is less clear. In genetic transformation of high level penicillin resistance it was shown that successful expression of the resistant phenotype required that the transformants received not only the particular mosaic *pbp* genes of the DNA donor but also the *murM* allele carried by the donor strain (79). However, in other experiments the linkage between penicillin resistance and abnormal wall composition was lost in transformation experiments in which the first round of transformation was followed by a second backcross (80). Interestingly in this particular experiment the penicillin resistant secondary transformants began to show "fitness" defects: defective growth and premature autolysis in antibiotic free medium, similar to the defective physiology observed in laboratory isolates of penicillin resistant pneumococci (81).

Genetic analysis and molecular modeling of the *murM* protein has identified amino acid residues that appear to be critical for the specificity of this protein and also pinpointed domains which interact with the aminoacyl tRNA and the bactoprenyl linked substrate of the *murM* catalyzed reaction (82).

Structure of wall teichoic acid (WTA) and lipoteichoic acid (LTA)

The pneumococcal C-polysaccharide and F-antigen. In 1930, long before LTAs and TAs had been discovered and defined, pneumococcal TA was described as pneumococcal C-polysaccharide by Tillet and coworkers (83). Thirteen years later, pneumococcal LTA was

isolated by Goebel and his colleagues in 1943 and named lipocarbohydrate or pneumococcal F-antigen owing to its fatty acid content and immunological properties (84). In these early studies, a structural relationship between C-polysaccharide and lipocarbohydrate was suggested and in contrast to the various strain-specific capsular polysaccharides, lipocarbohydrate and C-polysaccharide were considered pneumococcal common antigens. This was confirmed by serological methods which showed that all 90 known capsular types of *Streptococcus pneumoniae* possess C-polysaccharide and F-antigen (19). The two polymers differ immunologically, as Forssman antigenicity is associated with the LTA (85, 86). As shown by immunoelectron microscopy, C-polysaccharide is uniformly distributed on both the inside and outside of the cell walls, and LTA is located on the surface of the cytoplasmic membrane (12).

Choline, the surface signature of pneumococci, was identified as a component of TA and LTA (14, 15). The complex structures of WTA and LTA could not be unraveled before modern analytical techniques became available (87). In 1980, Jennings and coworkers published the first complete structure of pneumococcal TA (11). The structure of pneumococcal LTA was clarified in 1992 (85) and revised in 2013 (88), and subsequent reinvestigation of the WTA, isolated from the same strain from which the LTA had been isolated, revealed that both polymers possess identical chain structures (13, 82). The linkage structure of WTA to PG was recently established (13), and all genes required for TA synthesis were predicted by sequence comparisons (89).

Structure of teichoic acid (TA) and Lipoteichoic acid (LTA). The chains of LTA and TA contain 4 to 8 identical pseudo-pentasaccharide repeating units which consist of the rare positively charged amino sugar 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose (AATGal); D-glucose; ribitol 5-phosphate; two N-acetyl-D-galactosaminyl (GalNAc) residues and one or two

phosphocholine residues. Hydroxyl groups of ribitol can be substituted by D-alanine. The repeats are joined together by an α-1,4 glycosidic linkage between AATGal and a GalNAc residue of the adjacent repeat. The functionally important phosphocholine residues are phosphodiester-linked to O6 of the N-acetyl-D-galactosaminyl residues. The number of phosphocholine residues per repeat is strain specific: in LTA and WTA of strain R6 the majority of repeats carry two phosphocholine residues, whereas most of the repeats in strain Rx1 are substituted with one (Fig. 4). Moreover, the terminal repeating unit of both WTA and LTA can occur with or without the phosphocholine substitution at both GalNAc residues. The chain of LTA is β-1,3 glycosidically linked via the AATGal residue to O3 α-D-Glc*p*(1-3)-diacylglycerol (Fig. 4).

Microheterogeneity of LTA became apparent by hydrophobic interaction chromatography and mass spectrometry analysis (85, 88). The chain of LTA may vary in length between four and eight repeats, with the range differing from sample to sample. On SDS-polyacrylamide gel electrophoresis LTA yields a ladder-like pattern of up to six bands, each differing from the next by one repeat. Species with one phosphocholine per repeat are distinguished from species with two by higher mobility of the individual bands.

In addition to the unusual complexity of the chemical structure of TA, pneumococci are also unique because their wall teichoic acid (WTA) and lipoteichoic acid (LTA) possess identical repeat and chain structures, whereas in other gram-positive bacteria WTAs and LTAs are structurally and biosynthetically distinct entities. In LTA and WTA the AATGal residues are of conformational importance because the positively charged amino groups interact electrostatically or by hydrogen bonding with the negatively charged phosphate groups on the adjacent glucosyl residues (87, 90).

Pneumococcal WTA chains have been isolated with or without attached peptidoglycan fragment and were shown to contain 4 to 8 repeating units (13, 27). WTA is linked to the peptidoglycan by a phosphodiester bond to O6 of some of the MurNAc-residues which is demonstrated by the release of MurN-6-*P* on HCl hydrolysis of cell walls (91) and of muropeptides. It is estimated that – dependent on the particular strain – between 15% up to 30% of the muramic acid residues carry WTA chains (16, 92). In other Gram-positive bacteria connecting the WTA chain and muramic acid by a phosphodiester bond typically have a linkage unit containing glycerophosphate or *N*-acetylmannosamine, but these components could not be detected either in the hydrolysate of pneumococcal cell walls or in WTA-containing muropeptides. Since no other sugar was found either, an acid-degradable sugar like AATGal was suggested as a component of the linkage unit (87). Indeed, PG chains with attached WTA could be isolated showing that the AATGal residue of the first repeating unit has an α-configuration and is directly linked to Mur*N*Ac 6-phosphate (13).

Synthesis of teichoic acids and modifications

Although only few enzymes of the pneumococcal TA pathway have been biochemically studied the complete pathway was deduced from bioinformatics analysis (89). Many TA genes cluster in three genomic regions, called lic1, lic2 and lic3. The lic1 region contains genes encoding components for the uptake of choline (LicB), the choline kinase LicA and the cytidyltransferase LicC which produces CDP-choline. In addition, the lic1 region encodes for TarI and TarJ which synthesize CDP-ribitol (93). The adjacent lic2 region contains the *tacF* gene, encoding the putative TA flippase, and the *licD1*/*licD2* genes encoding phosphotransferases for decoration of the TA subunits with phosphoryl choline residues. The lic3 region locates elsewhere on the chromosome and was identified by the presence of a gene,

called *licD3*, with sequence similarity to *licD1* and *licD2*, which is surrounded by four other putative TA genes. LicD3 is most likely the phosphotransferase for the transfer of ribitol phosphate during synthesis of the repeating unit, which involves eight additional enzymes that synthesize the repeating unit linked to the carrier lipid bactoprenol phosphate. Presumably, Spr1222 polymerizes the TA precursors to the nascent chains, which are still linked to the carrier lipid, before these are flipped across the cytoplasmic membrane by TacF. Interestingly, TacF is specific for choline-loaded TA precursor chains, ensuring that the mature TAs contains phosphoryl choline residues (94).

After reaching the outer leaflet of the cytoplasmic membrane the TA chains are either transferred to peptidoglycan to become WTA, or to the glycolipid to form the LTA. The transfer to peptidoglycan is catalyzed by members of the LCP (LytR-CpsA-Psr) family of proteins, which have been identified as phosphotransferases involved in cell wall assembly in Gram-positive bacteria (95). The three LCP proteins of *S. pneumoniae* appear to have semi-redundant roles in the attachment of capsular polysaccharides and WTA to C6-OH of MurNAc residues in peptidoglycan (96). However, strain D39 and strains with serotype 8 and 31 link the capsule polymer via a 1,6-glycosidic bond to GlcNAc residues of the peptidoglycan. Such linkage is inconsistent with catalysis by LCP enzymes, indicating that the nature of the capsule attachment enzyme is still elusive (97). In the case of LTA, it is the membrane protein TacL that transfers the TA precursor chains onto the glycolipid anchor. Interestingly, mutants lacking *tacL* grow normally under laboratory condition but show attenuated virulence in mouse models of acute and/or systemic infections by *S. pneumoniae* (13).

Pneumococci contain a *dlt* operon the products of which are responsible for the decoration of LTA and WTA repeating units with D-alanine residues, which are attached via an

ester linkage to OH groups in ribitol (98). This modification introduces positive charges to TAs and thus increases resistance to cationic antimicrobial peptides. While *S. pneumoniae* strain D39 modifies its TAs by alanylation, this modification is not present in the laboratory strain R6 and in strain TIGR4 due to mutations in the *dlt* operon rendering it non-functional.

Phosphoryl choline esterase – PCE – enzymatic removal of phosphoryl choline residues from the pneumococcal cell wall. The *pce* encoding for a teichoic acid phosphoryl choline esterase (Pce) – an enzyme capable of removing phosphorylcholine residues from the cell wall teichoic acid and lipoteichoic acid – was identified independently by two research groups (69, 70). Pce carries an N-terminal signal sequence, contains a C-terminal choline-binding domain with 10 homologous repeating units similar to those found in other pneumococcal surface proteins. The catalytic (phosphorylcholine esterase) activity is localized on the N-terminal part of the protein. The mature protein was over-expressed in *Escherichia coli* and purified in a one-step procedure by choline-affinity chromatography. The product of the enzymatic digestion of ^{3}H-choline-labelled cell walls was shown to be phosphorylcholine. Inactivation of the *pce* gene in *S. pneumoniae* strains by insertion-duplication mutagenesis caused a unique change in colony morphology and a striking increase in virulence of a capsular type III strain in the intraperitoneal mouse model. Pce may be a regulatory element involved with the interaction of *S. pneumoniae* with its human host. Sequence comparison indicates that PCE, the protein originally identified through it's unique enzymatic activity (99) is identical to choline binding protein E (CbpE) – described by Masure and colleagues as a protein implicated in the attachment of pneumococci to nasopharyngeal cells (100). The crystal structure of Pce shows a catalytic site with two zinc ions and an elongated binding domain recognizing the phosphocholine residues of teichoic acid (101).

Multiple functions of cell wall choline residues

Nutritional requirement for choline. Choline is an essential growth factor for all natural isolates of pneumococci which have to import this nutrient from the growth medium (102). Choline may be replaced by other amino alcohols such as ethanolamine which can incorporate into LTA and TA at the same positions as phosphocholine but it can not replace phosphocholine functionally. Ethanolamine-grown cells show a number of striking abnormalities which include inhibition of cell separation (growth in long chains); inhibition of DNA uptake in genetic transformation; lack of autolysis during treatment with penicillin and other wall inhibitors and detergents (103); production of an "immature – enzymatically inactive – form of the autolytic enzyme LytA (104); production of a cell wall that cannot absorb LytA (105) and is completely resistant to the hydrolytic action of LytA (106). However, the requirement of LytA for built-in phosphocholine residues is no longer seen when solubilized cell wall polymers are degraded to muropeptides (12) or when the TA is removed from peptidoglycan (48). These observations may be interpreted to indicate that TA prevents the access of autolysin to its cell wall substrate and that this effect is overcome by binding of the enzyme to the phosphocholine residues.

There are several proteins in pneumococci – including LytA, LytB and LytC – that specifically recognize and bind to phosphocholine residues. These proteins contain distinct domains which are responsible for their specific biological activities, whereas the choline-binding domains are homologous and contain 6 to 10 choline-recognizing repeats of ~20 amino acids each. The structure of crystallized LytA and LytC and their interaction with choline residues in the cell wall has been described (107, 108).

Choline-independent strains. A choline-independent strain, R6Cho$^-$, was recovered from a heterologous cross with DNA from *Streptococcus oralis* (48). *S. oralis* also incorporates phosphocholine into its cell wall TA, but, unlike pneumococci has no nutritional requirement for choline. Other choline-independent strains, for example JY2190 and R6Chi are mutants generated by serial passage of strains in chemically defined medium containing decreasing concentrations of ethanolamine with each passage (94, 109). None of these strains had acquired the capability to synthesize choline or ethanolamine because phosphorylated amino alcohols could not be detected in TA and LTA. In spite of the absence of phosphocholine there was no alteration either in the structure of LTA and TA or in cell wall composition, including the stem peptide profile. Only the phosphate content of cell walls was reduced, consistent with the absence of phosphocholine. In vivo, the lack of active autolysins became apparent by impaired cell separation at the end of cell division and by resistance against stationary phase and penicillin-induced lysis. Due to the absence of choline from LTA, PspA was lost into the surrounding medium whereas, in spite of choline-free TA, the amidase was retained on the cells. Both choline-independent strains retained the capacity to incorporate choline into teichoic acids: when grown in the presence of choline, PspA was retained, cells separated normally, became penicillin-sensitive, and phosphocholine was discovered on LTA and TA.

In the case of strain R6Chi and related strains, the nutritional requirement for choline was explained by the loss of specificity of the teichoic acid flippase TacF for choline loaded TA precursor chains (94). R6Chi contains a single point mutation in the *tacF* gene, rendering the cells choline-independent. When growing in the presence of choline, R6Chi still incorporated phosphocholine into its teichoic acids, suggesting that the mutated TacF can transport choline-loaded and -unloaded TA precursor chains. The presence of the *tacF* mutation also allowed for

the deletion of otherwise essential choline utilization genes *licABC*, producing a strain that did not incorporate choline into its cell wall even when growing in the presence of exogenous choline. An encapsulated version of this mutant was used to demonstrate the crucial role of cell wall choline residues in immune evasion (110).

Cell growth regulation during the cell cycle

Growth zone and cell wall segregation. Similarly to other streptococci, pneumococci incorporate new cell wall units into the pre-existing wall material at a single growth zone located at the cell equator (mid-cell). Both peptidoglycan and teichoic acids units and also capsular polysaccharide (111) enter the pneumococcal surface at this growth zone which could be visualized by exploiting the unique selectivity of a pneumococcal enzyme, the peptidoglycan hydrolase LytA for choline-containing segments of the cell wall (58). Pneumococci require choline for growth and the design of this experiment was based on the observation that the choline component of the wall teichoic acid can be replaced by structural analogues such as ethanolamine. Pneumococci grown in ethanolamine containing medium show several striking abnormalities: unlike the choline grown bacteria, pneumococci utilizing ethanolamine grow in long chains and are completely resistant to the cell wall degrading activity of LytA (103). Upon addition of trace amounts of radiolabeled choline to a culture grown on ethanolamine the bacteria immediately shifted to the utilization of choline so that the nascent wall units that began to incorporate into the cell surface contained choline residues in the teichoic acid component of the nascent cell wall and produced regions that were susceptible to hydrolysis by exogenous LytA enzyme added to the medium. It was possible to show by electron microscopy that under these conditions the LytA enzyme performed an enzymatic “microsurgery” on the bacteria: it

has selectively removed a thin equatorially located band of cell wall, thus identifying the anatomical site of wall incorporation and growth zone (58).

Another abnormality of the ethanolamine-grown pneumococci, the complete inhibition of cell separation, has allowed the design of experiments to test the mode of inheritance of pneumococcal cell walls. Pneumococci labeled in their wall by titrated choline were shifted to an ethanolamine-containing medium in which the bacteria continued to grow in the form of chains of cells, i.e. “linear clones” in which the distribution of radioactively labeled bacteria could be followed (by autoradiography) as a function of cell generations in the ethanolamine containing medium. Since the teichoic acid choline does not exhibit turnover during growth, the localization of radioactively labeled cells within the chains of bacteria could provide clues as to the mode of wall segregation. The finding was that the radioactive label remained in large clusters in association with cells that were located either at the tips or at the center of chains. The results demonstrate the conservation of large hemispherical segments of the cell wall which are passed on intact to daughter cells during cell division (112).

Novel methods such as staining of nascent peptidoglycan with fluorescent vancomycin (113), incorporation of fluorescent D-amino acids (114) and super-resolution microscopy (32) confirmed that pneumococci incorporate new cell wall units into the pre-existing wall material at a single growth zone located at mid-cell.

Cell wall growth and cell division complexes. The mode of growth and cell wall segregation described above implies that *S. pneumoniae*, as the model rods *Escherichia coli* and *B. subtilis*, grows ~~by alternating cycles~~ of peripheral (side-wall) and septal peptidoglycan synthesis. Consistent with this, the *S. pneumoniae* chromosome was found to encode proteins that are part

of the elongation machinery (elongasome), such as MreC, MreD, MltG, RodZ, RodA, PBP2b, PBP1a and CozE, and others that are part of cell division machinery (divisome), such as FtsZ, FtsA, ZapA, ZapB, EzrA, FtsE, FtsX, FtsK, FtsL, FtsB(DivIC), FtsQ(DivIC), SepF, GpsB, DivIVA, FtsW, PBP1a, PBP2x, PcsB and LytB, as well as proteins that have a regulatory role in these processes (4, 39, 53, 115-118).

Nevertheless, the characteristic ovococcal shape of pneumococci suggests that there must be major differences in the cell wall growth mechanism compared to those of the rods. One central difference is that pneumoccoci, despite conservation of all the other components of the elongation complex lack the rod-shape determinant MreB, *i.e.* the actin-like protein that in rod-shaped model bacteria provides the dynamic cytoskeletal scaffold to maintain rod-shape (119). The second difference is that, despite previous reports (2), both components of the elongasome and divisome show a clear septal localization, with no substantial variation in timing of recruitment, although they may show differences in localization profile. The latter is best reflected by the specific mid-cell localization of peptidoglycan synthases PBP2b and PBP2x, which are involved with peripheral and septal cell wall growth, respectively (7). Importantly, these differences from rods have led to the proposal that in *S. pneumoniae* and in other oval-shaped cocci the elongation and septal machineries coexist in a single, intimately interconnected and tightly regulated complex, or super-complex (Fig. 5) rather than in two separated ones (39, 115). Subsequent studies supported this model and provided insights into the coordination and regulation of the complex during the cell cycle (120-123).

Information regarding gene essentiality among *S. pneumoniae* proteins involved in cell growth and division have been available thanks to both global and dedicated studies. However, it was not possible to assess directly the effect of inactivation of these genes until genetic systems

allowing the generation of merodiploids and conditional lethal mutants became available. Moreover, a recent work employing a CRISPRi method (41) provides an additional powerful tool to confirm results obtained with other methods and identify and characterize new essential genes.

Genes encoding pneumococcal elongasome proteins were found to be essential in *S. pneumoniae* D39 strain (53, 123, 124). However, some of these genes were not essential in strain R6, a laboratory derivative of D39, and similar strains (53, 122-124). Presumably, these laboratory strains contain pre-existing suppressor mutations that could compensate for the loss of otherwise essential genes. Interestingly, inactivation of *mreC*, *mreD*, *mltG* and *rodZ* in *S. pneumoniae* D39 are directly suppressed by mutations that inactivate *pbp1a* function or upon *pbp1a* deletion (53, 124). Strain R6 contains an allelic variant of PBP1a (T124A D388E), the functional importance of which has yet to be determined. In agreement, allele swapping experiments showed that the *pbp1a* allele from R6 was sufficient to compensate for *mreCD* loss in the R6 genetic background but less so in the D39 background, suggesting that other mutations present in R6 must be involved in the strong suppression of *mreCD* essentiality (124). This result is also supported by the fact that *pbp2a*, which shows a synthetic lethal relationship with *pbp1a*, can be readily inactivated in strain R6 (44).

A previously uncharacterized gene, *spd_0768* (*spr0777* in R6) encoding the membrane protein named CozE (for Coordinator of zonal elongation) was identified in a Tn-seq screen for genes dispensable in D39 with inactivated *pbp1a* (118). Inactivation of *cozE* or *mreCD* in the presence of PBP1a resulted in an aberrant relocalization of PBP1a and peptidoglycan synthesis from mid-cell to the whole cell periphery and cells became increasingly spherical before they lysed. Similarly to *mreCD*, *cozE* is dispensable in the R6 genetic background. CozE interacts

directly with MreCD and PBP1a. Together the results were interpreted as CozE being part of a coordination mechanism, critical for proper zonal cell wall synthesis, by connecting PBP1a with the other components of the elongation machinery *via* direct interactions (118).

With the exception of *pcsB* and *gpsB*, which showed strain-dependent essentiality, inactivation of cell division genes were more consistent between strains. Of these, *ftsZ*, *ftsA*, *ezrA*, *ftsEX*, *ftsL*, *ftsB* (*divIC*) and *pbp2x* were demonstrated to be essential in different commonly used genetic backgrounds (7, 45, 46, 54, 121, 125).

As in other bacteria, in *S. pneumoniae* cell division starts with the localization of the tubulin-like FtsZ and the actin-like FtsA cell division proteins (126) that assemble at mid-cell to form the Z-ring required for the recruitment of the other components to complete cytokinesis.

Photoactivated localization microscopy (PALM) showed that as in other bacteria the Z-ring in *S. pneumoniae* displayed a patchy structure rather than forming a homogenous ring, providing important insight into its *in vivo* organization (127). Following the fluorescent tagged-FtsZ localization and the Z-ring diameter, it was observed that FtsZ molecules assemble at mid-cell at the beginning of the cell cycle to form a single patchy ring, that thickens and then disassembles during constriction, while new Z-rings assemble at the future division sites (or new equators) of the newborn cells. Notably, unconstricted double Z-rings, suggesting short-lived intermediates, were observed in a small percentage of cells at mid-cell but not at the future division site (127).

Once the Z-ring is formed the later pneumococcal cell division proteins localize to mid-cell, and a few to mid-cell and cell poles, in exponentially growing pneumococcal cells. A hierarchical order of recruitment to the septum has not been determined and it may not even be a linear sequence of events, albeit co-localization studies based on fluorescence microscopy suggest that divisome assembly occurs in at least two steps. In the absence of MreB it is still

unknown how elongation components are targeted to mid-cell and how lateral and septal growth are coordinated. FtsZ is believed to be the best candidate to act as a scaffold to coordinate both side-wall and septal synthesis (127), although this role has not been experimentally verified, and the effect on *S. pneumoniae* cells of depletion of essential proteins involved in the initial steps of cell division were not available until recently.

A recent study characterized conditional lethal mutants for the cell division protein FtsA providing insights about how integration of the elongation and septation machineries at a single mid-cell location in *S. pneumoniae* may result in distinct cell growth pattern characteristic of oval-shaped cocci. Unexpectedly, the complete depletion of FtsA in the pneumococcus resulted in cell ballooning and ultimately lysis, in sharp contrast to the cell filamentation phenotype observed in rods (121). FtsZ rings and peptidoglycan synthesis delocalized upon FtsA inactivation, suggesting that the cells could neither elongate nor divide. In contrast, inactivation of genes encoding other cell division components, such as GpsB (5~~, and~~ *~~al.~~*~~, 2013)~~ and SepF (128), which in *B. subtilis* are synthetically lethal with *ftsA* (129, 130), resulted in enlarged and elongated cells, with multiple FtsZ rings but not cell ~~enlargement~~ or lysis. Consistent with these results, and in contrast to the model rods where FtsA localizes after FtsZ, in *S. pneumoniae* FtsZ and FtsA are targeted to mid-cell at the earliest stages of the process and both form a ring that remains co-localized throughout the cell cycle, while GpsB and SepF are recruited later, after the Z-ring is assembled (121).

Taken together these observations led to the hypothesis that, in oval-shape cocci, the actin-like FtsA, and not FtsZ, could play a major role in coordinating peripheral and septal peptidoglycan synthesis, likely carrying out a similar role as the actin-like MreB in pre-septal synthesis in *E. coli*. However, since FtsA is needed to tether FtsZ to the membrane (131) and

FtsZ may be required for FtsA to be targeted to the septum, additional work is necessary to distinguish any direct role for FtsA in coordinating both modes of PG synthesis from that of FtsZ.

While the elucidation of the mechanisms that in *S. pneumoniae* control the intimate coordination between the two PG biosynthetic machineries still awaits further study, new insights came from an unanticipated source, the screening for mutations that bypass the need of PBP2b. This approach identified the membrane-bound ~~hypothetical~~ endo-lytic transglycosylase, MltG (see above), which is involved in cell elongation (53). More recently, the inactivation of two other genes, *spd_0675* and *spd_1849* (*spr0683* and *spr1851* in R6, respectively) was shown to remove the requirement for PBP2b, MreCD, RodA, RodZ and GpsB in *S. pneumoniae* D39 (123). The corresponding proteins, named KhpA and KhpB, form an RNA-binding complex which regulates cell division post-transcriptionally. Inactivation of *khpA* or *khpB* resulted in cells with a significant reduction in cell volume, ~~but~~ they overall maintained a normal aspect-ratio, while inducing a strong increase in the expression of the WalRK cell wall stress regulon and modulating the level of FtsA through a post-transcriptional mechanism. Indeed, overproduction of FtsA was found necessary and sufficient to compensate for the loss of PBP2b, MreC, MreD and RodA, but not ~~of RodZ~~ GpsB, indicating that KhpAB must also regulate other genes in addition to *ftsA* (123).

Two independent studies also identified Spr1851/Spd_1849, either as a novel substrate for phosphorylation by the Ser/Thr protein kinase, StkP, in the laboratory strain *S. pneumoniae* Rx1 (132) or as a suppressor of *pbp2b* in the R704 derivative of laboratory strain R6 (133) and named it Jag (for Jag-domain protein) or EloR (for Elongasome-regulating protein), respectively. Jag was found to be phosphorylated at least at T89 (132). Experiments with null or phosphoablative

(T89A) alleles of *eloR* in the R704 strain revealed that both *pbp2b* and *rodA* could be deleted, while its phosphomimetic form (T89D or T89E) could only be tolerated in strains that acquired suppressor mutations in *mreC* and *rodZ* (133). In contrast, no phenotype was observed in either KhpB phosphoablative or phosphomimetic derivatives in *S. pneumoniae* D39, indicating that T89~P phosphorylation of KhpB is not necessary for its function in the ~~parent strain~~ (123). Despite discrepancies between strains, these studies highlight the crucial yet complex role of StkP in the regulation of pneumococcal cell cycle.

Regulators of cell wall growth and division and role of phosphorylation. Similarly to other Gram-positive bacteria, *S. pneumoniae* possesses, in addition to ~~the~~ histidine kinase two component~~s~~ system (TCSs), a conserved signaling system consisting of a Ser/Thr protein kinase, StkP, and a cognate PP2C-type, phosphatase, PhpP (134, 135). StkP belongs to the subfamily of eukaryotic-type Ser/Thr kinases, ESTKs, and consists of a cytoplasmic kinase domain, a transmembrane region and a C-terminal part outside the cell made up of four PASTA domain also found in PBP2x (136).

StkP acts as a dimer and forms a signaling pair with PhpP (137, 138). Several StkP substrates playing a role in cell wall metabolism and cell division were identified to be phosphorylated *in vivo* in a global study of the pneumococcal phosphoproteome (139). However, only some of them have been confirmed to be specifically phosphorylated by StkP *in vitro* and/or *in vivo*. These include the phosphoglucosamine mutase GlmM, the PG precursor biosynthesis enzyme MurC, the cell division proteins DivIVA(135, 140-144) and the recently discovered substrates MapZ/LocZ (~~120~~, ~~145~~) and Jag/EloR/KhpB (123, 132, 133). Although StkP is not essential in *S. pneumoniae*, the multiple phenotypes of an *stkP* mutant suggested that it was

involved in the regulation of cell growth and cell division (140, 141), virulence, competence (134) and stress resistance (146). It was later shown that both StkP and its cognate PhpP localize to the division site with active peptidoglycan synthesis and that the PASTA domains of StkP were required for its septal localization, indicating that they bind to newly synthesized and still uncross-linked peptidoglycan chains *in vivo*. Inactivation of *stkP*, or overproduction of PhpP, resulted in elongated cells with multiple and often unconstricted division rings, perturbed in cell wall synthesis. In contrast, *S. pneumoniae* cells overproducing StkP, or lacking functional PhpP, were significantly smaller and rounder (132, 143). These data indicate that StkP and PhpP play an important role in coordinating cell wall synthesis during growth and division to achieve and maintain the characteristic ovococcal shape. StkP was proposed to act as a molecular switch that, through phosphorylation of key division substrates, signals the shift from peripheral to septal cell wall synthesis (143). A parallel study on the role of StkP in pneumococcal cell division partially confirmed these results and proposed diverse functions for the different StkP domains in the R6 derived strain R800 (142). In this strain, deletion of the *stkP* gene or expression of a truncated protein lacking the kinase domain (StkP-PASTA-TMH) resulted in round and chaining cells rather than in elongated cells, while the elongated morphology was only observed in mutants expressing a truncated protein lacking the PASTA domains (StkP-KD-TMH) or the catalytically inactivated StkP(K42M) (142), in agreement with what was already reported for the *S. pneumoniae* mutant StkP(K42R) and other *stkP* mutants lacking the PASTA domains obtained in different genetic backgrounds (140, 141, 143). The round and chaining phenotype, connected with the *stkP* deletion or truncation (142), was not observed in other studies and was interpreted to be likely due to differences in genetic background, growth conditions or to suppressor mutations in that specific *stkP* null strains (39). To date, the molecular mechanisms of StkP's

regulatory function on its substrates is still unclear, although recent studies have reported some advances in this direction.

In 2014, Fleurie *et al*. reported that the DivIVA paralog, GpsB, a putative component of the pneumococcal cell elongation and cell division complex, was required for proper localization and activation of StkP in the *S. pneumoniae* R6 derivative strain R800 (120). R800 cells deleted for *gpsB* were viable but displayed an elongated and twisted-towels phenotype, more severe than that previously reported for *S. pneumoniae* D39 cells depleted of *gpsB*, where this gene is essential (5). Both FtsZ and PG synthesis were shown to have an helical pattern of localization in Δ*gpsB* R800 cells, in which phosphorylation of all StkP substrates, including itself, was abolished, consistent with the StkP delocalization observed. The R800 Δ*divIVA* phenotype, instead, was consistent with the Δ*divIVA* phenotype previously reported for *S. pneumoniae* Rx1 strain (3); however its characteristic rounder and chainy morphology was reinterpreted as a major defect in cell elongation (120) rather than a defect in septum closure and pole maturation as originally proposed (3). In addition, *divIVA* inactivation was found to suppress the elongated phenotype of Δ*gpsB* cells. Together these observations formed the basis for a model in which DivIVA (required for cell elongation) and GpsB (required for cell division) constituted the mechanism for the molecular switch to coordinate peripheral and septal PG synthesis, connecting them to the Z-ring through the cell division protein EzrA: GpsB and DivIVA did not interact directly with FtsZ but both interacted directly with EzrA, that in turn interacted with FtsZ (120). In this model, GpsB, which has been shown to be phosphorylated in *S. agalactiae* (147) and *B. subtilis* (148) but not in *S. pneumoniae*, would be needed for StkP localization at mid-cell and for consequent phosphorylation of itself and its substrates, in particular DivIVA, to activate septum closure. The elongated phenotype of the *S. pneumoniae* R800 cells expressing the

phosphoablative DivIVA allele T201A, the only amino acid residue of the protein shown to be phosphorylated (142), further supported this interplay between GpsB, DivIVA and StkP.

In 2016, Straume *et al.* reported that *S. pneumoniae* R6 depleted for PBP2b, which grows as long chains of characteristic lentil-shaped cells, has an altered stem peptide composition and is hypersensitive to the peptidoglycan hydrolase CbpD, which is produced during competence (45). They developed a genetic screen to identify genes that, when inactivated, would display a PBP2b-like CbpD-dependent lytic phenotype, encoding proteins functionally related to PBP2b (~~149~~). Four out of the 20 proteins tested, RodA, DivIVA, Spr0777 (later identified as Spd_0768/CozE) and MreD (but not of MreC), showed a PBP2b-like competence-induced autolytic response and consequently belong to the cell elongation complex (45). Intriguingly, this study revealed that the *mreD* mutant differed from the expected morphology observed in other studies for *mreD* mutants in the same or similar genetic backgrounds (39, 118, 124), highlighting once more the difficulties to understand individual effects of gene inactivation in *S. pneumoniae*.

The CbpD-dependent lysis data were then correlated with protein-protein interactions between the identified proteins using the Bacterial Two-Hybrid Analysis (BACTH) system (~~150~~). PBP2b interacted with RodA, confirming that these proteins have a close functional relationship also in *S. pneumoniae*. Other interactions detected were between DivIVA and PBP2b, DivIVA and Spr0777(CozE), and also the known DivIVA self-interaction (3, ~~149~~).

Taken together, these data were interpreted as DivIVA being part of, and required for, proper localization of the pneumococcal elongasome, likely recruited by its interacting partner CozE, supported by the observations that truncations determining the loss of DivIVA localization were also associated with the loss of the elongasome function (~~149~~). However, direct experimental proof for the lack of localization of DivIVA in the absence of CozE or known

components of the elongasome in the absence of DivIVA, is presently missing. Moreover, Δ*divIVA* cells showed a significantly smaller increase in the amount of branched stem peptide incorporated in the PG with respect to that of cells depleted for PBP2b, RodA or CozE (~~149~~). As DivIVA is dispensable and shows a similar phenotype in all *S. pneumoniae* genetic backgrounds, while other members of the elongation complex are not, a deeper understanding of the localization profile and time of arrival at midcell of the different cell cycle proteins would be needed to reveal their precise function.

The regulatory function in cell wall biosynthesis of its paralog, GpsB, appears to become more clear. Similar to DivIVA, GpsB, is also found in Gram-positive bacteria and contains a conserved N-terminal domain, which recognizes the negative membrane curvature at the nascent division septum (130, ~~151-153~~).

The *gpsB* gene is essential in *S. pneumoniae* D39 strain but not in its laboratory derivatives R6 or R800 strains (5, 120). The recent work of Rued *et al.* (2017) showed that GpsB was not strictly required for StkP localization or peptidoglycan synthesis at division septa. GpsB depletion resulted in elongated cells with multiple Z-rings but no helical localization of FtsZ in twisted-towels cells was observed. However, D39 cells depleted for GpsB showed decreased StkP-mediated phosphorylation that could be suppressed by functional inactivation (mutation or deletion) of its cognate PhpP phosphatase, which also relieved the cells from the need of GpsB. A similar suppressor mutation inactivating PhpP was detected in Δ*gpsB* mutants obtained in the other, commonly used, D39 laboratory derivative, Rx1(122). Importantly, GpsB forms a complex with EzrA, MreC, StkP, PBP2a and PBP2x and directly interacts with some of these proteins. Moreover, functional relationships were identified, based on synthetic lethality with Δ*pbp1a* or Δ*pbp2a*, with PBP2a and also with PBP2x, in which transpeptidase activity was

prevented from localizing at the center of division septa in the absence of GpsB (122). A previous work reported that, although PBP2x is not an obvious StkP substrate, the two proteins form a complex in the *S. pneumoniae* membrane and the PASTA domains of StkP interacted directly with the extracellular region of PBP2x, likely allowing correct localization of both protein at the division site (6).

Taken together, these results highlight a key role for GpsB in regulating the peripheral and septal synthesis, through the regulation of PBP activities during the cell cycle, in agreement with what is observed for other Gram-positive bacteria (~~154~~, ~~155~~). A revised model for GpsB function would then be that, on one hand, GpsB activates PBP2a and StkP-PBP2x to close the septal ring but, on the other hand, it negatively regulates peripheral elongation by inhibiting the activity of PBP2b and MreC (122).

While these aspects of the function of GpsB are relatively well understood, the role of phosphorylation for GpsB function is not. The recent studies on GpsB (120, 122) both agreed that inactivation of GpsB reduces StkP-mediated phosphorylation and, consequently, that GpsB is important for optimal phosphorylation. However, because GpsB does not appear to be a substrate of StkP, the functional relationship between both proteins remains unclear. Hence, whether StkP phosphorylates DivIVA as part of a molecular switch that together with GpsB and StkP control peripheral *vs*. septal PG synthesis remains controversial. In contrast with what was reported for the *S. pneumoniae* R800 strain (120), Δ*divIVA* mutations were not found to be epistatic to Δ*gpsB* mutations in strains D39 and its derivatives R6 and Rx1 (122), and Δ*divIVA* cells did not show any detectable morphological phenotype when producing the phosphoablative DivIVA T201A allele (122, ~~149~~), also different from what was observed in the *S. pneumoniae* R800 strain (120, 142) ~~(Fleurie *et al.*, 2012, Fleurie *et al.*, 2014)~~.

Division site selection (MapZ/LocZ) and origin of replication. Until recently, no mechanisms for targeting FtsZ and the division complex to the nascent septum were identified and no counterparts of the widespread or specific systems described in other bacteria, were found to be present in *S. pneumoniae*. Rod-shaped model species use the Min system and nucleoid occlusion to prevent asymmetric cell division and/or the septum from constricting over unsegregated chromosomes but, in contrast to the striking conservation of the proteins that constitute the cell elongation and division complexes, the proteins involved are poorly conserved reviewed in (~~156-158~~).

It came as a surprise when two independent studies reported the characterization of a previously unknown substrate of StkP, annotated as Spr0334 (SPD_0342 in D39) and showed that a specific system for correctly identifying the midcell did indeed exist also in *S. pneumoniae* (~~145, 159~~). The protein was named MapZ (for Mid-cell-*a*nchored *p*rotein Z) (~~159~~) or LocZ (for Localizing at the midcell of Z) (~~145~~), and shown to be a membrane protein consisting of a cytoplasmic domain and two extracellular domains (EC) separated by a highly entropic serine-rich region. MapZ/LocZ has a rather narrow phylogenetic distribution, being present only in streptococci, enterococci and lactococci.

Consistent with its proposed function, MapZ/LocZ was found to localize as a ring at midcell at the very early stages of cell division and before FtsZ (~~159~~) or FtsZ/FtsA (145). ~~However, differently from any other cell division proteins, soon after its localization~~ MapZ/LocZ splits into two rings that move away with the so called “wall bands” or “equatorial rings” until they reach the equators of the newly forming daughter cells, marking the future division sites (~~145, 159~~).

The gene encoding MapZ/LocZ was found to be dispensable in four different genetic backgrounds, R800 (~~159~~), D39, R6 and Rx1 (~~145~~), and its deletion resulted in misshapen cells often showing misplaced septa (~~145, 159~~). Moreover, in the absence of MapZ/LocZ, FtsZ (and FtsA) rings formed but were not localized and/or correctly oriented with respect to the longitudinal axis of the cells. A direct interaction between MapZ cytoplasmic domain and FtsZ was detected and MapZ alleles lacking the N-terminal domain were unable to promote correct FtsZ placement (~~159~~). Finally, both studies identified that MapZ/LocZ was phosphorylated by StkP at Thr67 and Thr78 and that both phosphomimetic (T67-78E) and phosphoablative (T67-78A) allelic variants localized to midcell, although the phenotypes of the strains differed completely, ranging from severe defects in cell shape and viability with aberrant FtsZ structures and reduced number of Z-ring per cell (~~159~~) to no evident morphological defects and a wild-type morphology (~~145~~).

Thus, albeit the overall conclusions of the MapZ/LocZ studies agreed on the protein function, the two works differed in important details: (i) the growth and morphological defects are significantly more severe in the R800 background than in the others; (ii) in R800 there is a third MapZ ring forming at mid-cell after the initial ring splits into two and moved apart from the cell center, but this was not seen for LocZ in other strains; ~~(iii) in R800, FtsZ was reported to move together with the MapZ rings to the future division site, whereas in the other strains FtsZ disassembles from mid-cell to assemble *de novo* at the future division site~~. While these issues need to be clarified, further analysis of MapZ, using both a genetic and a structural approach, demonstrated the importance of the second EC domain, which shows a marked flexibility at acid pH, while demonstrating that the first EC domain is likely a molecular scaffold or pedestal, required to mark the division site (~~160~~).

A more recent study indicated that MapZ/LocZ is not involved in division site selection, but rather in the selection of the correct division plane and that the replication status and the position of the chromosome have, instead, a crucial impact on spatial regulation of cell division. In particular, the position of the chromosome origin of replication precedes MapZ/LocZ at midcell and promotes the use of the correct division site (~~161~~). These conclusions were based on the reasoning that Δ*mapZ*/Δ*locZ* mutants are not elongated, but rather shorter and with only minor changes in cell morphology with respect to wild-type cells. Advanced fluorescent microscopy and image analysis showed that FtsZ localization is not affected in cells lacking MapZ, suggesting that it would not be ~~not~~ critical for accurate timing of the Z-ring assembly at midcell (~~161~~). The localization profile of MapZ/LocZ during the cell cycle was consistent with previous reports, however two and not three MapZ-rings were observed, in line with what was previously observed for *S. pneumoniae* D39 and related derivatives (~~145~~) and not for *S. pneumoniae* R800 (~~159~~).

The reported mislocalization of FtsZ in Δ*mapZ*/Δ*locZ* cells was attributed to the FtsZ fusion protein used in the previous studies to detect FtsZ localization in the null mutants (~~145~~, ~~159~~). ~~However, caution should be taken when using fluorescent tags~~. This consideration applies only to the MapZ study, where a GFP-FtsZ fusion used was the only source of FtsZ in the cell (~~159~~) but not to the LocZ study, where the CFP-FtsZ fusion was ectopically produced, in addition to native FtsZ (~~145~~). Nevertheless, the Δ*mapZ*/Δ*locZ* phenotype was reinterpreted in a new light and suggested that MapZ/LocZ could function in cell wall remodeling rather than in site selection, similarly to peptidoglycan hydrolases and LMW-PBPs (~~161~~). However, this intriguing hypothesis awaits experimental validation.

In conclusion, the study of the cell cycle of *S. pneumoniae* has benefited greatly from the work on other organisms, in particular the model rods with which the pneumococcus has many similarities as well as many marked differences. The gap in knowledge between *S. pneumoniae* and the model organisms is rapidly closing and it will be interesting to see how further works develop. Regulation of the cell cycle through phosphorylation is a relatively new and active area of research (~~162~~) that will likely provide new insights on how *S. pneumoniae* succeeded to manage peripheral and septal growth that emanates from a single insertion site located at the cell center or mid-cell.

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FIGURE LEGENDS

FIG. 1. Diagrammatic sketch of the cell wall complex of pneumococci. Some of the Mur*N*Ac and Glc*N*Ac residues in the glycan chains of peptidoglycan are modified by O-acetylation or N-deacetylation, respectively. Direct and indirect peptide cross-links are shown. Capsular polysaccharides have been assumed to be connected to Mur*N*Ac residues in peptidoglycan but recent work showed that they might be connected to Glc*N*Ac residues. Surface proteins are covalently liked to peptides in peptidoglycan, choline-binding proteins attach non-covalently to phosphoryl choline residues in wall teichoic acid.

FIG. 2. Role of MurM and MurN in cell wall branching. The substrate of the MurM- and MurN-catalyzed branchgin reaction is lipid II, which is composed of N-acetylated disaccharide units of glucosamine (hexagon with G) and muramic acid (hexagon with M) with the pentapeptide attached to the M residues. Lipid II is anchored on the plasma membrane through the carrier lipid bactoprenyl phosphate (the zig-zag line). Attachment of the completed precursor to the pre-existing cell wall occurs on the outer surface of the plasma membrane by the activity of glycosyltransferases and transpeptidases. Reproduced with permission from Ref (82).

Figs 3A & B: HPLC elution profiles of stem peptides of the peptidoglycan from the penicillin-susceptible strain R36A and several penicillin-resistant strains that carry different abnormal *murM* alleles. Structures of cell wall stem peptides identified in the pneumococcal peptidoglycan of penicillin-susceptible and –resistant strains of pneumococci. Reproduced with permission from Ref (42).

FIG. 4: Structure of the pneumococcal lipoteichoic acid and wall teichoic acid. Both types of teichoic acid have identical chains (top part) which carry phosphoryl choline and D-alanine residues. In LTA the teichoic acid chains are β-glycosidically linked from AATGal to the lipid anchor (bottom left), in WTA the linkage occurs via an α-linkage from AATGal to MurNAc-phosphate in peptidoglycan. The figure was kindly provided by Nicolas Gisch (Research Centre Borstel, Germany).

FIG. 5. Cartoon of a cell wall growth and division complex at mid-cell showing elongation and cell division proteins, and the peptidoglycan hydrolases PcsB and LytB which cleave the septum for pole formation and cell separation.

Table 1: Cell wall peptide composition of several strains of *S. pneumoniae*. Reproduced with permission from Ref (78).

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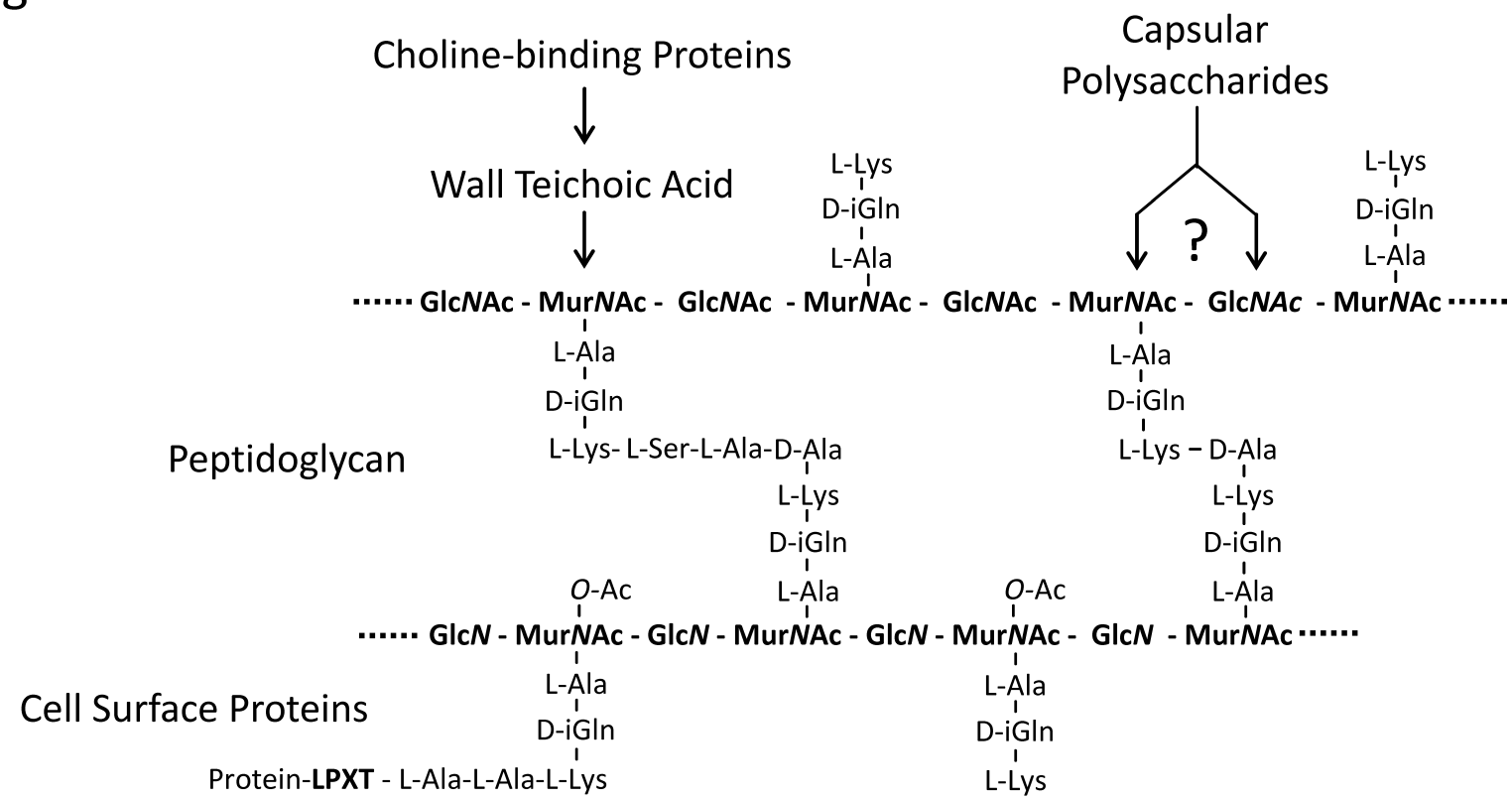
TABLE 1. Cell wall peptide composition of several strains of *S. pneumoniae.*

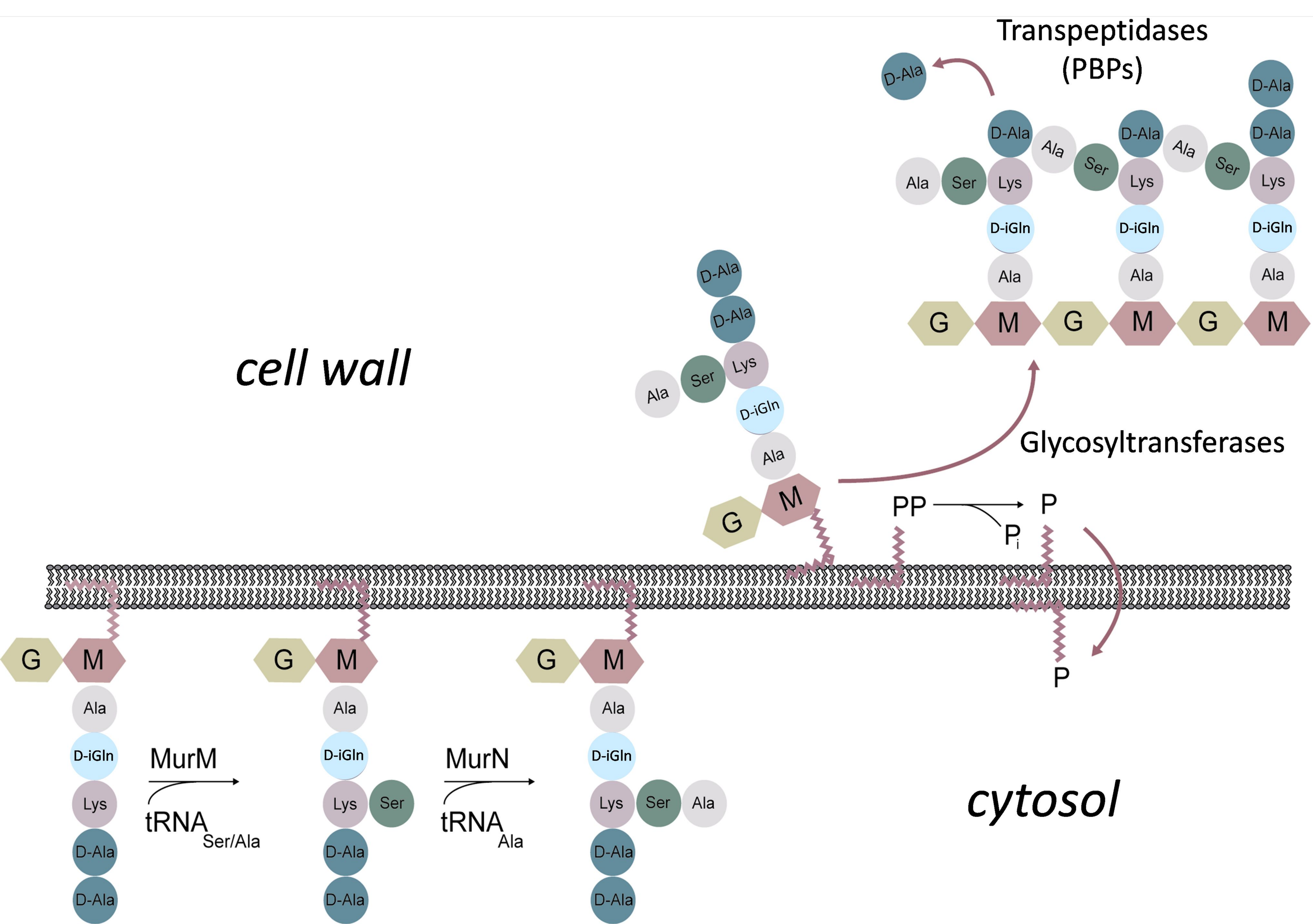
Peptide	% Peptide in				
	R36A (*murMA*)	Pen6 (*murMB2*)	Hun45 (*murMB1*)	DE1 (*murMB3*)	KY17 (*murMB5*)
1	13.3	2.6	4.5	10.8	3.4
2	3.4	0.7	0.5	1.4	0.6
3	2.8	14.3	7.2	1.0	28.5
I	1.8	14.4	24.1	23.4	11.2
II	3.4	2.7	5.1	3.1	0.8
4	21.0	3.2	4.4	6.0	7.2
III	1.3	3.5	6.7	6.9	2.1
5	12.7	2.2	0.9	0.5	2.9
6	7.1	2.5	2.8	9.3	1.9
7	7.4	11.1	1.4	0.4	19.7
IV	2.6	6.5	4.7	0.6	4.1
V	2.8	12.3	8.1	3.7	5.2
8	7.8	2.5	1.4	0.6	5.8
VI	6.6	10.1	19.1	27.5	2.0
9	6.0	11.6	9.0	4.9	4.5
Total	100	100	100	100	100
Monomers	26	38	48	47	47
Multimers	74	62	52	53	53
Linear	50	12	14	23	17
Branched	50	88	86	77	83
Branched/linear	1.0	7.4	6.4	3.3	4.9

1422

1423

Figure 1


Choline-binding Proteins
Wall Teichoic Acid
Capsular Polysaccharides
?
L-Lys
D-iGln
L-Ala
L-Lys
D-iGln
L-Ala
····· GlcNAc - MurNAc - GlcNAc - MurNAc - GlcNAc - MurNAc - GlcNAc - MurNAc ·····
L-Ala
D-iGln
L-Lys- L-Ser-L-Ala-D-Ala
L-Ala
D-iGln
L-Lys – D-Ala
Peptidoglycan
L-Lys
D-iGln
L-Ala
L-Lys
D-iGln
L-Ala
O-Ac
O-Ac
····· GlcN - MurNAc - GlcN - MurNAc - GlcN - MurNAc - GlcN - MurNAc ·····
L-Ala
D-iGln
L-Ala
D-iGln
L-Lys
Cell Surface Proteins
Protein-LPXT - L-Ala-L-Ala-L-Lys


Transpeptidases
(PBPs)
D-Ala
D-Ala
D-Ala
Ala
Ser
D-Ala
Ala
Ser
D-Ala
D-Ala
Ala
Ser
Lys
Lys
Lys
D-iGln
D-iGln
D-iGln
Ala
Ala
Ala
G
M
G
M
G
M
cell wall
D-Ala
D-Ala
Lys
Ser
Ala
D-iGln
Ala
G
M
Glycosyltransferases
PP
P
P_i
P
G
M
Ala
D-iGln
Lys
D-Ala
D-Ala
MurM
tRNA Ser/Ala
G
M
Ala
D-iGln
Lys
Ser
D-Ala
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Ser
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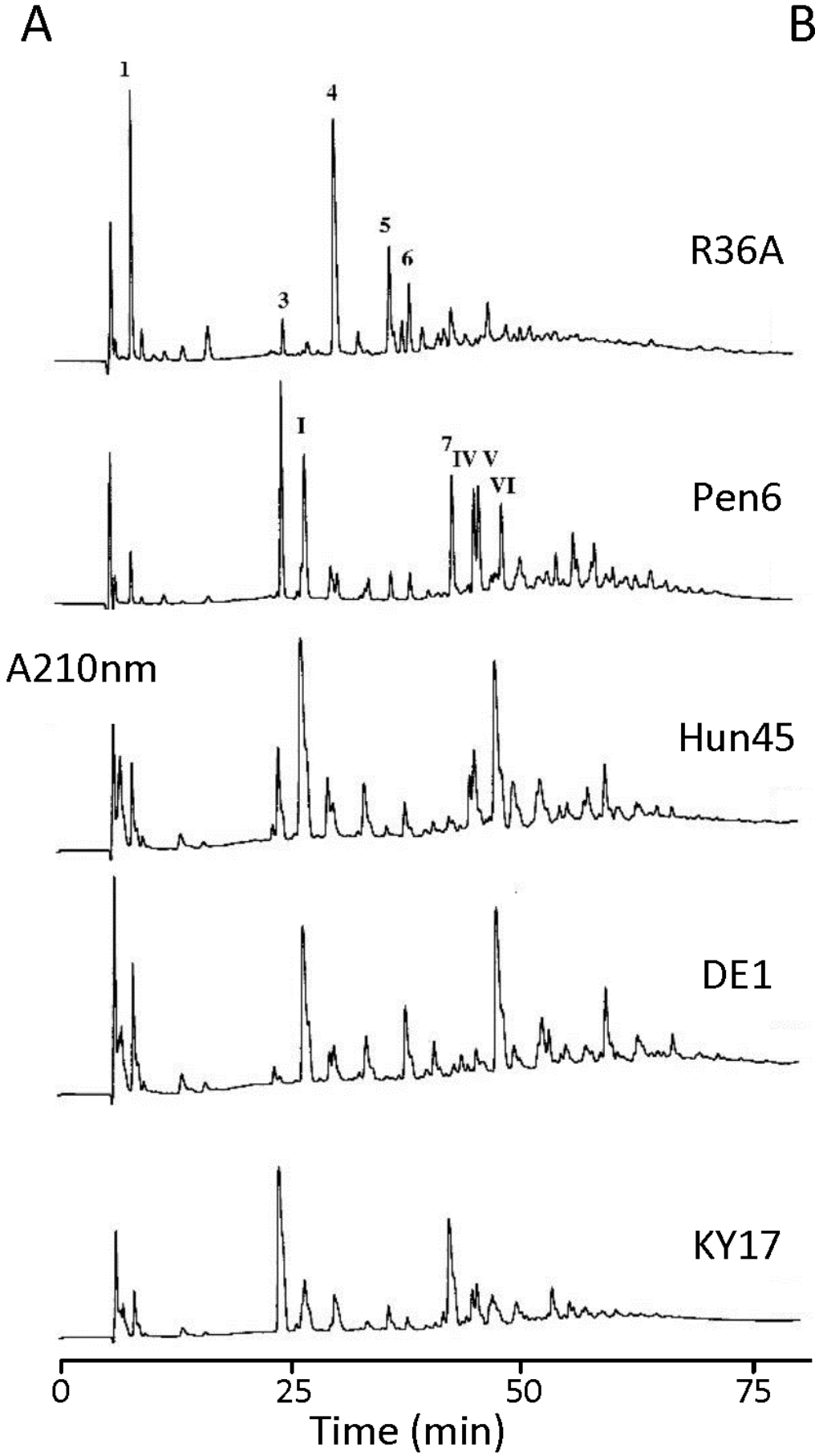
D-Ala
cytosol

Figure 3


A
B
1
4
5
6
3
R36A
I
7
IV
V
VI
Pen6
A210nm
Hun45
DE1
KY17
0
25
50
75
Time (min)

B


1
Ala
iGln
Lys
2
Ala
iGln
Lys
Ala
Ala
3
Ala
iGln
Ala -Ser-Lys


I
Ala
iGln
Ala —Ala —Lys
II
Ala
iGln
Ala -Ser -Lys
Ala
Ala
III
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Ala -Ala -Lys
Ala
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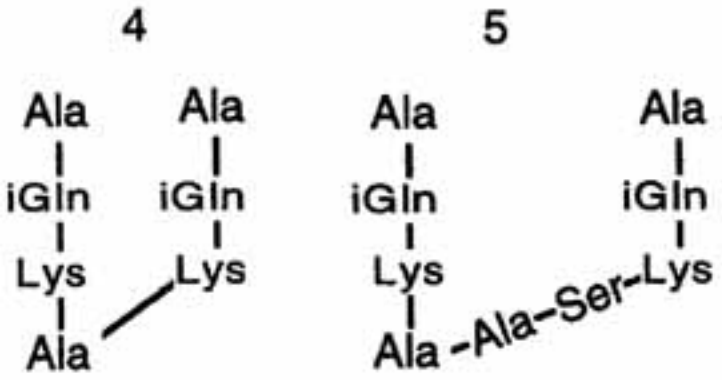
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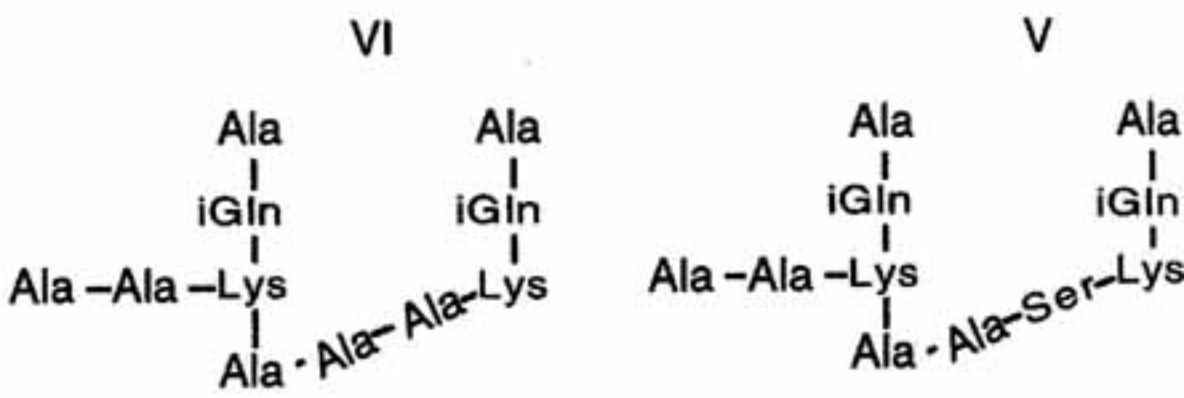
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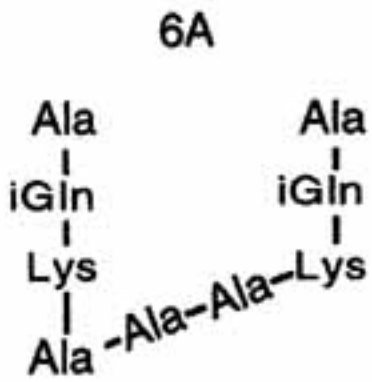
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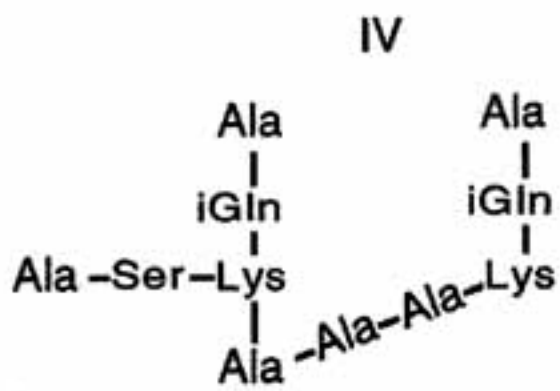
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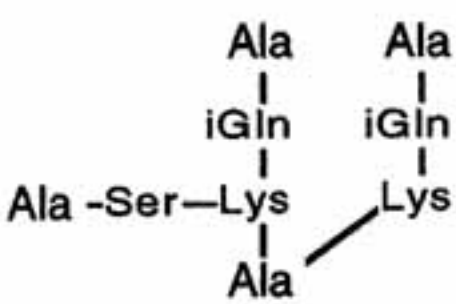
Ala -Ser–Lys
Ala -Ala-Ala-Lys
Ala
iGln


6B
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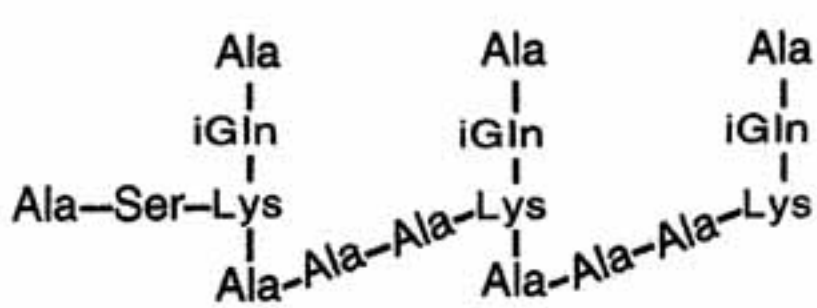
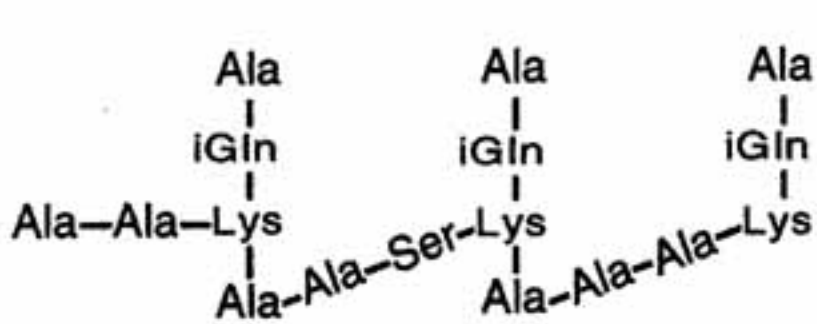
iGln
Ala -Ser—Lys
Ala
Ala
iGln
Lys


VII
Ala
iGln
Ala—Ser–Lys
Ala-Ala-Ala-Lys
Ala
iGln
Ala-Ala-Ala-Lys
Ala
iGln

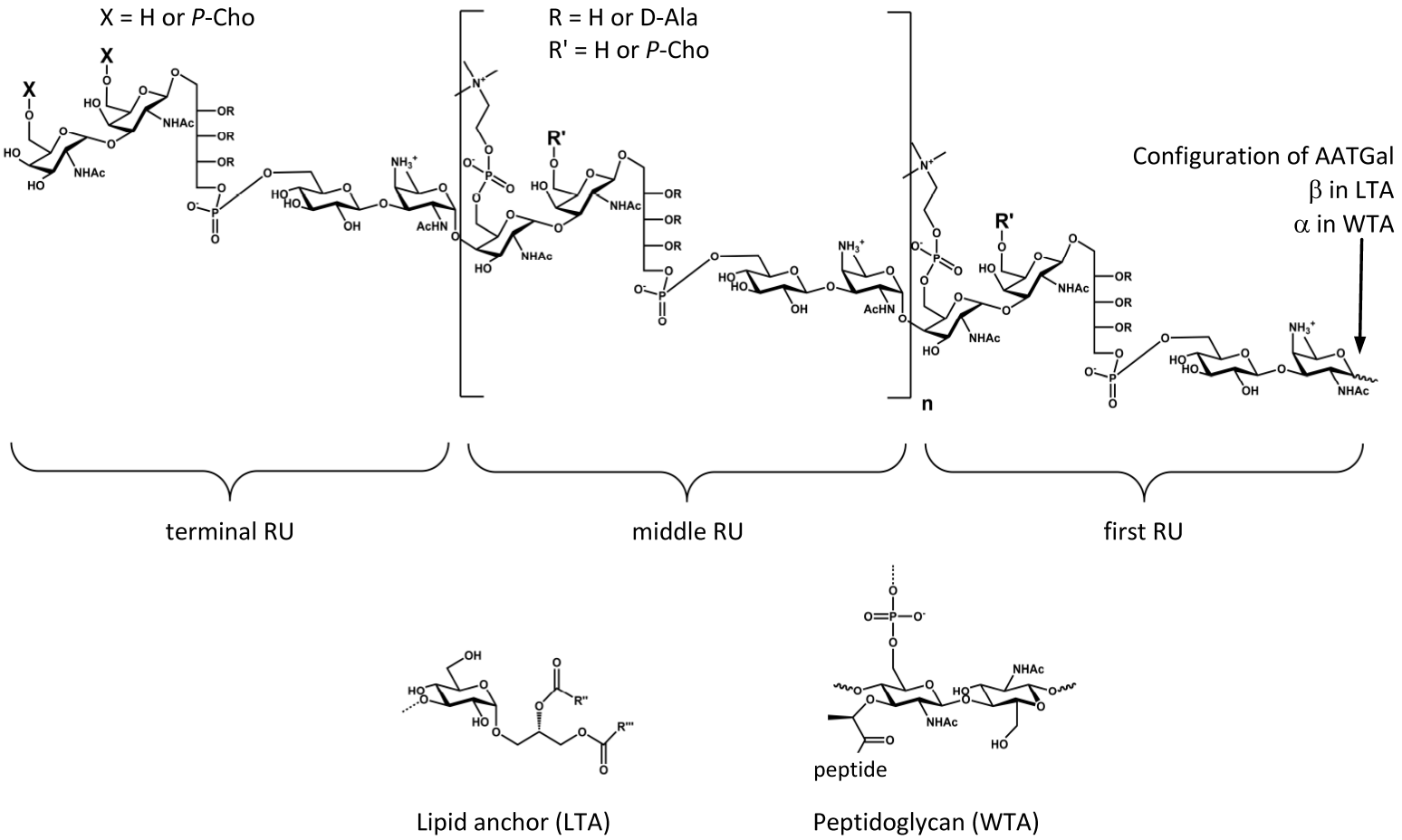

7
Ala
iGln
Ala -Ser—Lys
Ala- Ala-Ser–Lys
Ala
iGln


VIII
Ala
iGln
Ala—Ala—Lys
Ala-Ala-Ser-Lys
Ala
iGln
Ala-Ala-Ala-Lys
Ala
iGln


8

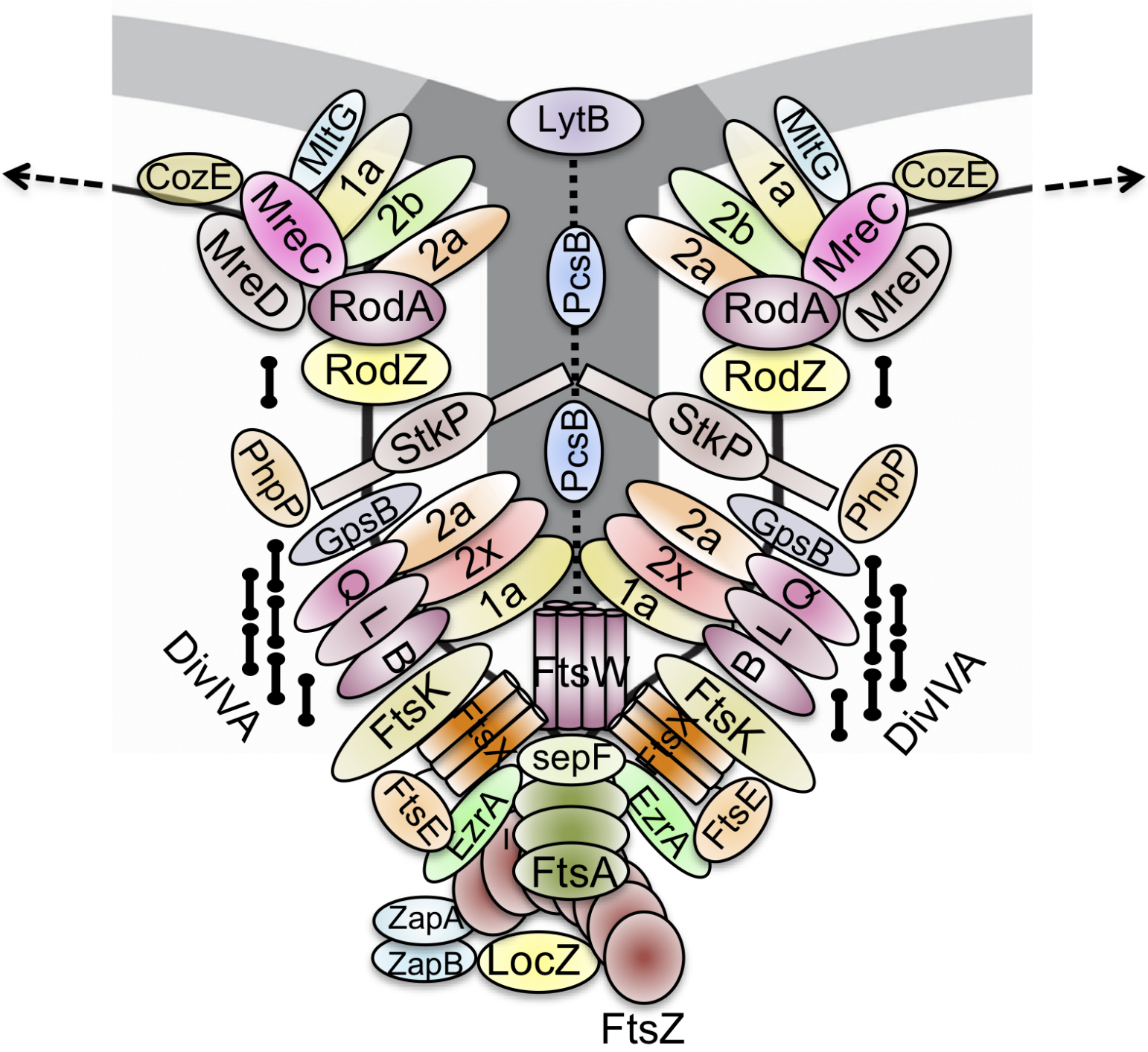

IX

Figure 4



Lipid anchor (LTA)

Peptidoglycan (WTA)


LytB
MltG
CozE
1a
2b
MreC
2a
MreD
RodA
RodZ
PcsB
StkP
PhpP
GpsB
2x
Q
1a
B
FtsK
FtsW
DivIVA
sepF
FtsX
FtsE
EzrA
FtsA
ZapA
ZapB
LocZ
FtsZ