Antonie van Leeuwenhoek **76:** 159–184, 1999.

The biosynthesis and functionality of the cell-wall of lactic acid bacteria

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Key words: autolysin, cell wall, D-alanine, D-serine, lactic acid bacteria, peptidoglycan, polysaccharide, S-layer, teichoic acid, vancomycin

Abstract

The cell wall of lactic acid bacteria has the typical Gram-positive structure made of a thick, multilayered peptidoglycan sacculus decorated with proteins, teichoic acids and polysaccharides, and surrounded in some species by an outer shell of proteins packed in a paracrystalline layer (S-layer). Specific biochemical or genetic data on the biosynthesis pathways of the cell wall constituents are scarce in lactic acid bacteria, but together with genomics information they indicate close similarities with those described in *Escherichia coli* and *Bacillus subtilis*, with one notable exception regarding the peptidoglycan precursor. In several species or strains of enterococci and lactobacilli, the terminal D-alanine residue of the muramyl pentapeptide is replaced by D-lactate or D-serine, which entails resistance to the glycopeptide antibiotic vancomycin. Diverse physiological functions may be assigned to the cell wall, which contribute to the technological and health-related attributes of lactic acid bacteria. For instance, phage receptor activity relates to the presence of specific substituents on teichoic acids and polysaccharides; resistance to stress (UV radiation, acidic pH) depends on genes involved in peptidoglycan and teichoic acid biosynthesis; autolysis is controlled by the degree of esterification of teichoic acids with D-alanine; mucosal immunostimulation may result from interactions between epithelial cells and peptidoglycan or teichoic acids.

Introduction

Already in 1675, Antonie van Leeuwenhoek had noticed the distinctive coccal, rod-like and spiral shapes of 'clear globules', as he described bacteria, 'without being able to discern any film that hold them together, or contained them' (quoted in Salton 1994). Three centuries later, Chapman and Hillier (1953) visualized the bacterial cell wall for the first time in a thin section of *Bacillus cereus* under the electron microscope. Today, fifty more years have elapsed, and a tremendous amount of information has been gained, allowing futurist applications such as using crystalline S-layer proteins for the construction of molecular nanodevices to come closer to reality (Pum & Sleytr 1999). Being specifically focused on lactic acid bacteria (LAB), this review will nevertheless address more general questions on the structure and function of the four components of the Gram-positive cell wall: peptidoglycan (PG, also called murein), teichoic acids, S-layer, and polysaccharides (Figure 1).

The sacculus

The bacterial bag-shaped PG enveloppe (sacculus) is the structural equivalent of the exoskeleton of insects. Like chitin, PG is basically a polymer of N-acetylglucosamine, but it is much more resistant to cracking and shearing than the insect cuticle, due to the covalent nature of the crosslinking of individual chains, as opposed to weak bonds holding together the chitin glycan units. The best image for describing a PG layer is that of a fisherman's net containing the bacterial cellular contents (Figure 2). The mesh of the net is made of two segments of parallel, rather inextensible glycan threads, held together by two small elastic peptide crosslinks allowing the net to expand or shrink. Thus, the whole surface is made of a single gigantic covalently closed macromolecule with remarkable elasticity and tensile strength. Single-layered PG is proper to Gram-negative bacteria, which can stand turgor pressures of the order of 2-5 atm (more than that of a car tyre!). Gram-positives have a much thicker

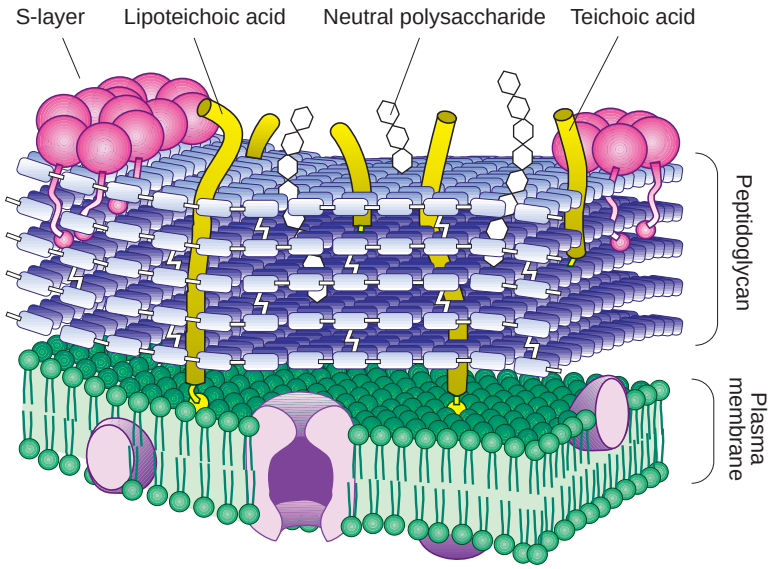

Figure 1. Artist's view of the cell wall of a Gram-positive bacterium. The bilipidic plasma membrane with embedded proteins is covered by a mutilayered peptidoglycan shell decorated with neutral polysaccharides, lipoteichoic acids and teichoic acids, surrounded by an outer envelope of S-layer proteins; for the sake of clarity, cell wall-associated proteins are not depicted.

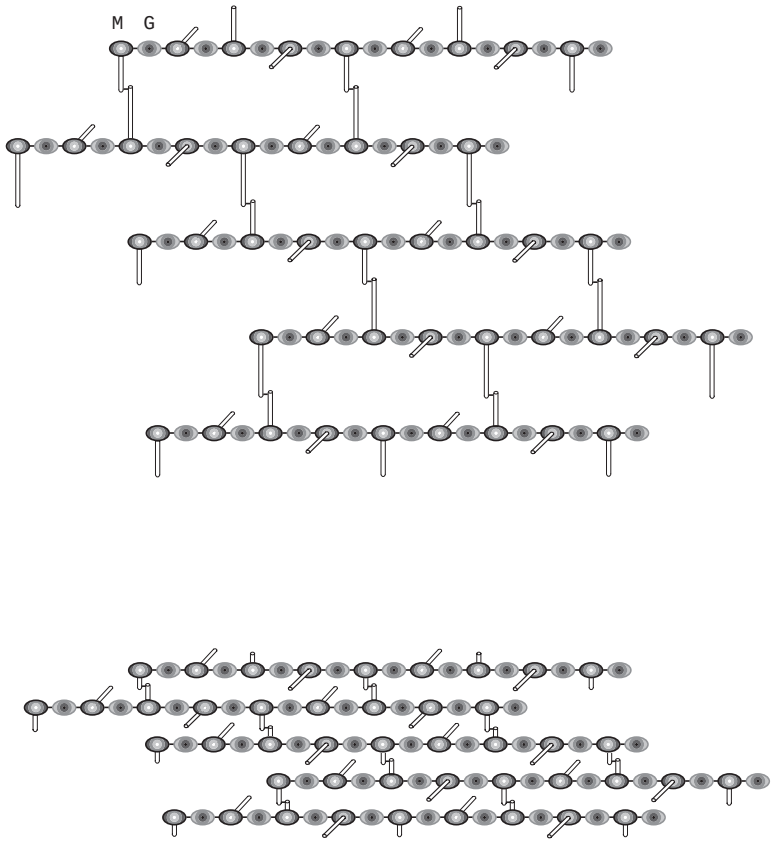

Figure 2. Schematic representation of the peptidoglycan network structure of LAB. Expanded (above) and shrunken (below) configurations are drawn to scale. M = MurNAc, G = GlcNAc. Bars represent the donor (short) and acceptor (long) crosslinking peptides, respectively; for the sake of clarity, higher-order crosslinks are omitted. The view shown corresponds to either a radial or a tangential cut of the PG layer.

cell-wall (typically 50 nm), which allows them to stand turgor pressures of the order of 20 atm. In this case, several concentric PG layers are covalently held together by additional radial crosslinks which create transverse meshes re-inforcing the whole structure. Diverse 2- and 3-dimensional PG architectures are made possible because each glycan thread has potential half-crosslinks available every other monomer, and these are pointing perpendicular to the glycan axis at right angles of each other (Figure 2). How PG biosynthesis is controlled in time and space so as to ensure faithful shaping and division of bacteria with so many diverse morphologies remains a largely open question which lies beyond the scope of this paper (for recent reviews on cell growth and division, see Ayala et al. 1994; Koch 1995; Rothfield & Zhao 1996; Rothfield & Justice 1997; Giesbrecht et al. 1998; Höltje 1998; Nanninga 1998).

The fisherman's net organization of the peptidoglycan sheets not only provides the sacculus with a container function, but makes it also behave as a sieve (Dijkstra & Keck 1996). Recent studies on isolated sacculi from *E. coli* and *B. subtilis* (Demchick & Koch 1996) indicate that in both cases the mean diameter of the meshes is approximately 4 nm, which corresponds to a cut-off value of about 25 kDa for globular proteins. Taking into account the increase in mesh size resulting from the turgor pressure imposed on the net *in vivo*, these authors conclude that bacterial cells should exclude molecules larger than 55 kDa. This sieving effect imposes restrictions to the secretion of large proteins, as that of levansucrase in *B. subtilis* which has been shown to be unable to freely diffuse through the cell wall and can only rely on the 'inside-to-outside' cell wall expansion process (see below) to be dragged towards the outer face of the thick PG enveloppe and ultimately be released passively into the medium (Kemper et al. 1993). The sieving properties of the cell wall has recently been exploited for the development of an assay for cell wall integrity using fluorescent *in situ* hybridization (Bidnenko et al. 1998).

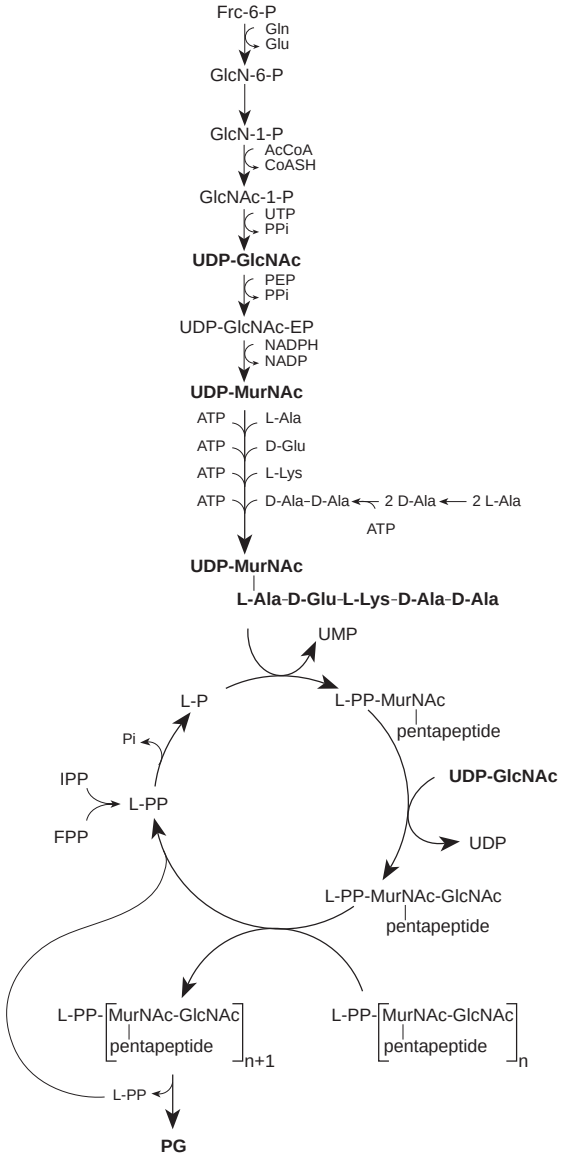

Figure 3. Pathway of peptidoglycan synthesis. L = undecaprenol (see text for explanations).

N-acetyl-glucosamine (GlcNAc) and N-acetyl-muramic acid (MurNAc), the basic components of the PG glycan thread

In LAB, like in all eubacteria, the PG glycan thread is a polymer of the disaccharide N-acetyl-glucosamine-$\beta(1\rightarrow4)$-N-acetyl-muramic acid. UDP-derivatives of the constituent hexoses are used as activated precursors in PG synthesis (Figure 3). Following polymerization and incorporation into the cell wall PG (see below), GlcNAC and/or MurNAC may undergo three different modifications. First, acetyl groups can be removed from cell wall PG through the action of N-deacetylase, as has been described for some bacilli (Araki et al. 1980; Zipperle et al. 1984) and *Lactobacillus fermentum* (Logardt & Neujahr 1975). This can have an incidence on the physiology of the cell wall *in vivo* (for instance, increased sens-

itivity to autolysis) as well as on the behaviour of the cells *in vitro* when subjected to common experimental lysis procedures, as glycosidic bonds adjacent to non-acetylated MurNAC residues are refractory to most muramidases (which cleave the $\beta 1 \rightarrow 4$ linkages between disaccharide units), including the phage T4 and egg-white lysozymes (Amano et al. 1977; Kawagishi et al. 1980; Kleppe et al. 1981). Second, the cell wall MurNAC residues can be 6-O-Acetylated. This has been observed in 11 species of Gram-negative and Gram-positive bacteria, including the pathogen *Staphylococcus aureus* as well as several species of LAB such as *Enterococcus faecalis*, *Lactobacillus casei*, *Lactobacillus acidophilus*, and *L. fermentum* (for a review, see Clarke & Dupont 1992). The extent of naturally occuring O-acetylated residues is quite variable in different species, and amounts to 60% in *L. acidophilus* (Coyette & Ghuysen 1970) and *L. casei* (Billot-Klein et al. 1997). The contribution of O-acetylation to the physiology of bacteria is unknown, but again it is noteworthy that most muramidases (including egg-white lysozyme) are inactive against glycosidic bonds adjacent to O-acetylated MurNAc residues, with the notable exception of mutanolysin (Hamada et al. 1978; for a review on bacterial lysozymes, see Höltje 1996). Finally, C6 of MurNAc can be substituted by cell wall teichoic and teichuronic acids, as will be discussed later.

The pentapeptide, the canonical crosslinking agent

The next stage in the process of PG biosynthesis is the attachment on the D-lactyl carboxyl group of MurNAc of a pentapeptide classically made of alternating L- and D-aminoacids (Figure 4; for a review on the biosynthesis of the PG unit, see van Heijenoort 1994). The first three aminoacids are attached successively on MurNAc, while the last two residues are first united by a D-alanine/D-alanine ligase to form a dipeptide which is then added to the tripeptide to yield the complete MurNAC-pentapeptide (Figure 3). In LAB, the consensus sequence (from the D-lactyl residue) is L-alanine/D-glutamate/*meso*-diaminopimelic acid or L-lysine/D-alanine/D-alanine. In *L. fermentum* as well as in some species of the genus *Bifidus*, L-ornithine is found at the central position, and in a few others (see below), D-serine or D-lactate replace D-ala at the C-terminus. Most residues are derived from normal metabolism: D-lactate is the end-product of fermentation, L-alanine and L-lysine are common aminoacids,

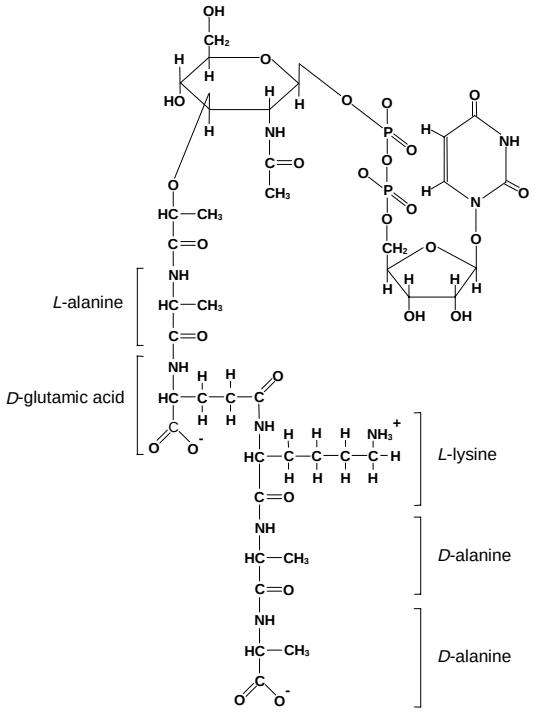

Figure 4. Structure of the disaccharide unit with its attached pentapeptide. Note the peculiar peptide linkage between the γ-carboxyl group of D-glu and the α-amino group of L-lys.

meso-diaminopimelic acid is the precursor of L-lysine and L-ornithine is an intermediate in L-arginine biosynthesis. However, the synthesis of D-aminoacids requires specific transamination or racemisation steps (Hols et al. 1997; Fotheringham et al. 1998).

The pentadepsipeptide, a peculiarity of some LAB

In early 1992, Arthur et al. provided evidence that in a vancomycin-resistant strain of *Enterococcus faecium* (a species which normally produces L-lactate), the C-terminal D-alanine residue of the MurNAc-pentapeptide was substituted by D-lactate (hence the name 'pentadepsipeptide'). This was experimentally confirmed a few months later by three different groups (Allen et al. 1992; Handwerger et al. 1992; Messer et al. 1992). In the strains studied, the resistance phenotype was shown to result from the presence of a large transposon (Tn*1546*). In addition to the transposase and resolvase genes needed for its mobility, Tn*1546* was shown to encode 7 *van* genes among which *vanH*, a D-lactate dehydrogenase, and *vanA*, a D-alanine/D-lactate ligase (Arthur et al., 1993). The strains were

shown to be inducibly resistant to high doses of vancomycin and teichoplanin (a related glycopeptide). This vancomycin resistance phenotype was defined as VanA. Two other related phenotypes of vancomycin resistance were soon reported: VanB (inducibly resistant to high doses of vancomycin only) and VanC (constitutively resistant to low doses of vancomycin). VanB strains were found to also produce D-lactate-ending depsipeptides, whereas in the VanC group D-serine was found to replace D-lactate (for a recent review, see Hakenbeck 1994; Arthur et al. 1996). At first, substitution of the terminal D-alanine seemed to be restricted to vancomycin-resistant strains of enterococci, but a number of other LAB species known as intrinsically resistant to vancomycin were later shown to also use D-lactate-ending UDP-MurNAc-pentadepsipeptides as PG precursors: *L. casei, Lactobacillus plantarum, Leuconostoc mesenteroides,* and *Pediococcus pentosaceus* (Billot-Klein et al. 1994; Handwerger et al. 1994; Ferain et al. 1996).

In order to understand why LAB endowed with depsipeptide-bearing PG precursors are resistant to vancomycin, one must refer to the mechanism of action of this glycopeptide antibiotic. Vancomycin comes into contact with the PG precursors on the cell wall side of the cytoplasmic membrane and binds to the D-ala/D-ala terminus of the pentapeptide, preventing polymerization of PG precursors. In the case of VanA and VanB resistance, the affinity of vancomycin for the depsipeptide is reduced about 1000-fold due to the loss of a single hydrogen bond (out of 5) between an oxygen from vancomycin and the NH group of the amide D-ala-D-ala linkage (Bugg et al. 1991). As to VanC, the hydrogen-binding network is preserved, but the bulkier side chain of serine sterically hinders the interaction (Healy et al. 1998).

The production and assembly of muramyl-pentadepsipeptide precursors ending in D-lac first requires that D-lactate is available. As explained above, VanA-type enterococci (L-lactate producers) may acquire this ability horizontally through the uptake of the Tn *1546* transposon. Likewise, the VanB phenotype of a clinical vancomycin-resistant *E. faecium* isolate was recently shown to result from the presence of another transposon, Tn*5382* (Carias et al. 1998). D/L-lactate producers such as *L. plantarum, L. mesenteroides* or *P. pentosaceous* pose no problems, but the source of D-lactate in *L. casei* (a L-lactate producer) is not known; the source of D-serine in VanC resistance is not known either. Second, the ligase must be able to accept D-lac or D-ser instead of D-ala. We have seen that Tn*1546* encodes the VanA ligase which meets this requirement. In the case of LAB intrinsically resistant to vancomycin, the resident ligase most probably acquired this property through evolution of the ancestral enzyme. The advantage of using D-lactate (a waste end-product of metabolism) rather than alanine (a precious aminoacid) is obvious, but the production and use of D-serine makes less sense. Recent reports clearly show that the *E. coli* D-ala-D-ala ligase can easily be changed into a D-ala-D-lac or D-ala-D-ser enzyme through a limited number of directed aminoacid substitutions (Park et al. 1996; Healy et al. 1998). In our laboratory, we have recently shown that a double *ldhL/ldhD* knockout mutant of *L. plantarum* produces about 40% of its muramyl precursors with D-ala C-termini as compared to 100% D-lac in the wild-type (Ferain et al. 1996). In this case, further investigations are needed to establish whether the resident ligase is either ambivalent, mutated or replaced by an alternative enzyme.

Lipid II, the membrane carrier

Lipid II is composed of the lipid-soluble undecaprenol (a C-55 isoprenoid compound also known as bactoprenol) attached through a pyrophosphate linkage to the C-1 of the GlcNAC-$\beta(1\rightarrow4)$-MurNAC-penta(depsi)peptide disaccharide unit (Figure 5). As will become clear throughout this review, the undecaprenyl moiety of lipid II is used as lipid carrier in several macromolecular syntheses related to the biosynthesis of cell wall components. Undecaprenyl pyrophosphate synthetase (UPPS), the enzyme responsible for the last step in undecaprenyl pyrophosphate synthesis, has been characterized in several species, and most extensively studied in *L. plantarum* (Allen 1985); yet, despite the interest in the biochemistry of this enzyme, its gene has only been identified very recently by Apfel et al. (1999). These authors have cloned and characterized the wanted *uppS* gene from *E. coli* using a genomics strategy, and found 27 putative *uppS* genes in genomic databases from a variety of species, including *B. subtilis* and *E. faecalis*; they provide evidence that the gene is essential in *E. coli*.

The main role of lipid II in PG biosynthesis is to convey the pentapeptide disaccharide building blocks pre-fabricated in the cytoplasm up to their site of polymerization and crosslinking on the external face of the cytoplasmic membrane (Figure 5). The physical and

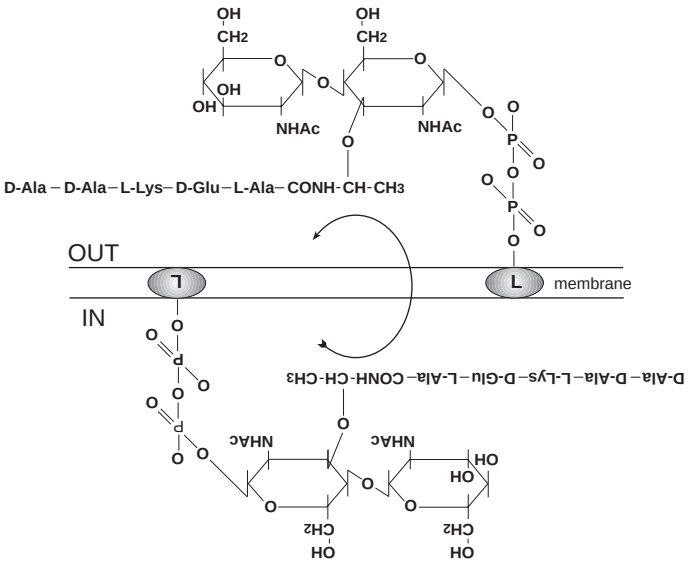

Figure 5. Structure of Lipid II and representation of its crossing through the membrane (L = undecaprenol).

chemical properties of the penta(depsi)peptide made of alternating L- and D-isomers are best suited for allowing an easy passage through the membrane, as energy minimization calculations predict a compact folding with a hydrophobic surface (Labichinski et al. 1985; Koch 1991). The mechanisms allowing lipid II to cross the membrane remain largely unknown. The physical properties and the size of undecaprenol are compatible with a passive process allowing the precursor to go across and curl back through the phospholipid membrane (Koch 1995), but the need for an active mechanism requiring a 'flippase' is often invoked in view of the high rate of translocation required to match the rate of PG synthesis (Nanninga 1998).

In some species, before crossing the cell membrane, lipid II may undergo additional processing. As will be discussed later, cross-linking of the glycan threads takes place in LAB between the third and fourth residues of facing pentapeptides. Depending on the species, the crosslink may either be direct, or require a 'cross-bridge' of one (frequently D-asp in LAB) or several aminoacids hooked on lipid II through (consecutive) peptide linkage(s) to the ω-amino group of the central diaminoacid. Since in some instances this process requires aminoacyl-tRNAs and ATP (Matsuhashi et al. 1967; Green & Vold 1993), it can only take place on lipid II still facing the cytoplasm. In addition, the α-position of D-glu, the ϵ-position of *meso*-diaminopimelic acid or the α-position of the cross-bridge D-asp may be amidated (Linnet & Strominger 1974).

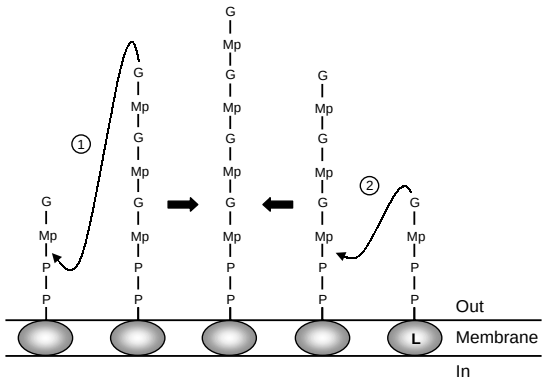

Figure 6. The two modes of transglycosylation during elongation of the peptidoglycan chain: (1) = elongation from the reducing end; (2) = elongation from the non-reducing end. g = GlcNAc; Mp = MurNAc-pentapeptide; L = undecaprenol.

Assembly of nascent PG chains

This step of PG synthesis is the target of several classes of antibiotics, in particular penicillin and its analogs in the β-lactam family (see below), or vancomycin and related glycopeptides (as already discussed). Polymerization of lipid II-bound disaccharides takes place through transglycosylation on the cell wall side of the cytoplasmic membrane. Two opposite directions of chain elongation are known. Either the terminal GlcNAC 4-OH of the nascent chain splits the energetic pyrophosphate bond linking the incoming disaccharide to its undecaprenol carrier (β4→1

elongation from the reducing end of the nascent chain; reaction 1, Figure 6). Or else, the GlcNAC 4-OH of the incoming disaccharide splits the energetic pyrophosphate linkage attaching the nascent chain to its own undecaprenol carrier ($\beta 1\rightarrow$ elongation from the non-reducing end of the nascent chain; reaction 2, Figure 6). No information is available on the direction of elongation in LAB.

The main actors of PG assembly are membrane-bound enzymes known as 'penicillin-binding proteins' (PBPs) (for a review, see Ghuysen 1991). PBPs can be divided in two groups: the multimodular, high molecular weight PBPs (>60 kDa, further clustered into the A and B subclasses), and the monofunctional, low molecular weight PBPs. They all possess a penicillin-binding module (PB) with an acyl serine transferase activity homologous to that of β-lactamases responsible for inactivation of β-lactam antibiotics such as penicillin. This module provides PBPs with the ability to break D-alanyl-D-alanine bonds in two ways: the D,D-transpeptidase activity of high molecular weight PBPs is responsible for cross-linking, whereas the D,D-carboxypeptidase activity of low molecular weight PBPs is involved in PG maturation (see below). In addition to acyl serine transferase activity, high molecular weight PBPs are provided with another, non-penicillin binding module (nPB) responsible for the polymerization of disaccharides through transglycosylation in Class A PBPs, and for the shaping or the division of the cells in class B PBPs (Matsuhashi 1994; Ayala et al. 1994; for a recent review on high molecular weight PBPs, see Goffin & Ghuysen 1998). Exceptionally, the major transglycosylase activity in *Streptococcus pneumoniae* does not belong to the PBP family (Park et al. 1985). Whether this would hold true for lactic streptococci is not known.

In LAB, PBPs have been mostly studied in enterococci (El Kharroubi et al. 1991; Piras et al. 1993; Signoretto et al. 1994; Zorzi et al. 1996; Pucci et al. 1997; Duez et al. 1998). Recent work in two laboratories have provided information on PBPs in dairy LAB. In *Streptococcus thermophilus*, a mutant with a *Tn916*-insertion in the *pbp2b* gene encoding a class B high molecular weight PBP was obtained (Stingele & Mollet 1996). The mutant grows slowly in curled and twisted chains, and the cells appear asymmetrical and nearly wedge-shaped, in agreement with a specific role of PBP2-type penicillin-binding proteins in shaping the cell morphology. Interestingly, the mutant is also defective in the production of extracellular polysaccharides, probably because of an expansion of the pool of vacant lipid II (as PG assembly is reduced in the mutant), resulting in a shortage of undecaprenyl-phosphate carriers also needed for EPS production (Stingele & Mollet 1996). In *L. lactis*, IS*S1*-insertion mutants were obtained in three distinct genes encoding ORFs closely related to PBP2A and PBP2B from *S. pneumoniae*, and PBP1A from *B. subtilis*, respectively (F. Rallu & E. Maguin, personal communication). These *pbp* mutations were selected as extragenic suppressors of IS*S1*-insertion mutations responsible for resistance to acid stress: the first two restored acid stress sensitivity in a mutant with an IS*S1* insertion into an ORF closely related to the *relA* gene encoding ppGpp synthetase in *Streptococcus equisimilis* (Mechold et al. 1993), while the third one suppressed acid stress resistance in another mutant with an IS*S1* insertion into an ORF related to the Acr regulator of *E. coli* controlling the expression of an ion efflux pump (Ma et al. 1996). The physiological mechanisms underlying these genetic interactions remain to be elucidated.

As indicated above, the D,D-transpeptidase activity of high molecular weight PBPs is responsible for the cross-linking of the nascent polysaccharide threads. In LAB, the ω-amino group of the central diaminoacid of one penta(depsi)peptide (or the N-terminus of its cross-bridge peptide) is esterified to the α-carboxyl group of the fourth residue (D-alanine) freed by cleavage of the D-ala-D-ala or D-ala-D-lac (depsi)peptide bond belonging to an opposite penta(depsi)peptide from a parallel nascent oligosaccharide chain (Figure 7) . Cross-linking is only possible with a complete penta(depsi)peptide as donor, as the reaction takes place outside the cytoplasm and can only rely on the energy stored in the D-ala-D-ala or D-ala-D-lac ester bond.

The level of cross-linking in the cell wall PG is usually about 20% in single-layered Gram-negative bacteria, but may go close to 100% in multi-layered Gram-positives (Labischinski & Maidhof 1994). This depends on many factors. The 2- or 3-dimensional structure of PG is of importance, due to the helical orientation of the successive peptides along the polysaccharide thread (Figure 3). The length, folding and flexibility of the cross-links also play a role in making contacts between the partner peptides more or less easy. Cross-linking is also dependent on the D,D-carboxypeptidase activity of the low-molecular weight PBPs which clip off the C-terminal D-alanine in the pentapeptide or the D-lactate in the pentadepsipeptide, making them inapt as transpeptidation donors. Further

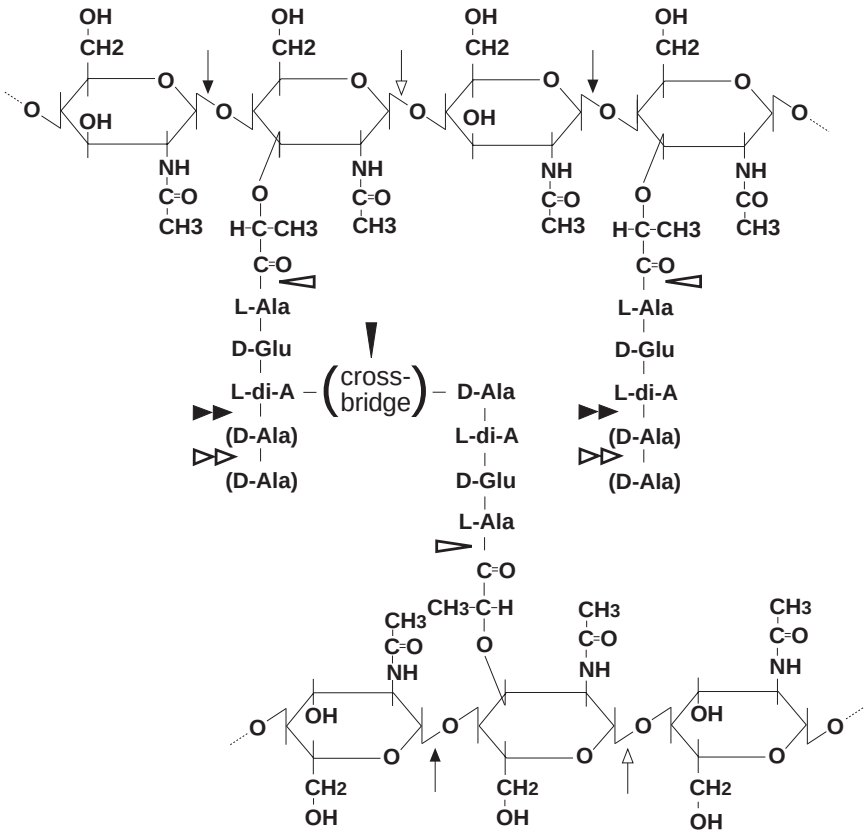

Figure 7. Modes of action of peptidoglycan hydrolases. Open arrows: *N*-acetylmuramidase; filled arrows: *N*-acetylglucosaminidase; open single arrowheads: *N*-acetylmuramoyl-L-alanine amidase; filled single arrowheads: endopeptidase; double open arrowheads: D,D-carboxypeptidase; double filled arrowheads: L,D-carboxypeptidase.

trimming or cutting of the peptides, either vacant or engaged into crosslinks, is performed by a variety of peptidases involved in the maturation and expansion of PG, and possibly also in determining whether a cell will elongate or divide (Begg et al. 1990). As a consequence, several dozens of different fragments are obtained when PG is cleaved with muramidase, as examplified by the complexity of the pattern obtained by Billot-Klein et al. (1997) with *L. casei*.

In cell wall PG, the ω-amino group of the central diaminoacid of vacant pentapeptides (or the N-terminus of its attached cross-bridge) may undergo covalent bonding with a class of cell wall-bound proteins sharing the LPXTG consensus (Navarre & Schneewind 1994, 1999). This anchoring system has been exploited for the cell wall display of various heterologous proteins (for a review, see Stahl & Uhlen 1997), and in particular for the cell wall presentation of antigens or epitopes in LAB (Pozzi et al. 1992; Piard et al. 1997; Steidler et al. 1998; Navarre & Schneewind 1999; see also Leenhouts, this symposium).

The structural diversity of PG cross-links is an important tool in systematic bacteriology (Ghuysen 1968; Sneath et al. 1986). In eubacteria, there are two main groups of cross-linkages (A and B) depending on the ranking of the acceptor residue on the penta(depsi)peptide (third or second, respectively) (Schleifer & Kandler 1972). All LAB have type A crosslinks. We have indicated earlier that besides differences in aminoacid sequence, cross-links also differ by secondary modifications consisting in various degrees of amidation of their free carboxyl groups. The resulting changes in the overall charge of the peptides and of their corresponding cross-links are likely to have an incidence on their folding, flexibility, or sensitivity to cleavage, with consequences on global charge, elasticity and stability of the whole PG envelope.

PG expansion during cell growth

The bacterial cell can be viewed as a container under pressure, due to the high osmolarity of the cytoplasm as compared to most external media to which the cells are exposed. The tensile strength of PG is the only defense against bursting, and the structural integrity of the net has therefore to be maintained in all circumstances. How this is compatible with the need for PG expansion during cell growth, which requires cleaving the meshes to covalently insert new material, remains one of the most challenging areas of bacterial physiology. Bacterial shapes are so diverse and PG architectures so different (more than 100 different PG structures are known in eubacteria), that no universal mechanism can be envisioned. Two general principles seem to prevail in Gram-positives: 'make before break' and 'inside to outside'. The first rule implies that incorporation of new PG material takes place through its cross-linking into a pre-existing, covalently closed PG sheat, and precedes cleavage of the cross-links at the site of insertion, thus making sure that shears be anticipatively repaired. The second rule states that new, un-stressed material be first assembled at the internal side of the wall in close contact with the cell membrane, and then be pushed out radially by more recent PG material.

In some way, PG expansion can be compared to molting during the larval stages of insects, where a shrunken, un-stressed envelope assembles underneath the stressed cuticle. In PG, the glycan threads are rather inextensible, and elasticity is mainly due to the cross-links which can stretch considerably (up to four-fold), from an (un-stressed) globular folding to a (stressed) extended configuration, allowing the meshes to enlarge (Koch 1991; Koch & Woeste 1992). The 'surface stress theory' of PG expansion (for a review, see Koch, 1985, 1995) states that PG layers closer to their site of synthesis on the cytoplasmic membrane have meshes with compact and unstressed cross-links, and bear appendant, 'made-before-break' glycans. While the layers are being pushed towards more peripheral zones and in reaction to the turgor pressure, their meshes first enlarge through the stretching of the cross-links. Then, the cross-links start to be stressed and undergo facilitated enzymatic cleavage by endopeptidase (see below), and the 'made-before-break' cross-linked polysaccharides automatically fill the cracks. The most peripheral layers are those under the strongest stress, and when all the 'made-before-break' chains have been used, PG shears without further repair and autolyses, giving a fibrous appearance to the cell wall periphery, as clearly seen on electron-microscopic thin sections prepared by freeze-drying (Graham & Beveridge 1990).

LAB belong to three morphological groups: bacilli, cocci, and bifidi. As each specific shape is maintained through successive generations, PG expansion must take place in a well-controlled way preventing for instance the diameter from widening or the length from increasing. Bifidobacteria have no model system to refer to with respect to the mode of cell growth. As to the bacilli and cocci, reports on *B. subtilis* or *Enterococcus hirae*, respectively, could enlight our understanding of how the PG fabric expands. The growth of *B. subtilis* has been approximated to that of an expanding cylinder with two inert hemispherical caps, and rules governing the extension of a cylindrical soap bubble have been shown to apply, implying that numerous foci of PG expansion are scattered along the whole surface of the cylinder so as to make it behave as a fluid surface (for a review, see Koch 1985). How the diameter of the cylinder is kept constant during growth is still a matter of discussion. In one of the models proposed for *B. subtilis*, the polysaccharide threads in each concentrical layer are laid in the hoop direction, so that incorporation of new chains through cross-linking will only lengthen the cylinder (Labischinski et al. 1985). In *B. subtilis*, the average PG chain is approximately 100 disaccharides long, and it has been estimated that it would take about 16 chains to circle the cylinder (Ward 1973). The rules in lactobacilli are not known, but it seems doubtful that lactobacilli with such variable morphologies as for instance *L. delbrueckii* ssp. *bulgaricus* (Botazzi 1988) would follow a simple mode of PG expansion. As to *E. hirae*, it has the shape of two elliptic poles sealed through a central annulus and cell wall expansion in this case is zonal rather than dispersive. Following the splitting of the annulus, the old poles remain almost inert while new poles grow in between the split annuli concomitantly to the formation of an inward-growing annular septum. Here too, the mode of lateral growth can be mimicked by that of two soap bubbles hold on each side and fused through a central septum (Koch 1995). Again, whether a given lactic coccus will follow the rules prevailing in *E. hirae* is hardly predictable.

PG hydrolases

Introduction of new PG material into the murein net requires that cross-links be hydrolyzed, and the inside-to-outside mode of cell wall growth of Gram-positive bacteria involves the constant removal of the most peripheral murein layers. A variety of cellular hydrolases are known to be able to cleave covalent bonds in PG (Figure 7). Murein hydrolases can be grouped into two different categories. The first group is made of enzymes which do not weaken the 2- or 3-dimensional meshing of the net, such as D,D-carboxypeptidases and L,D-carboxypeptidases. As indicated previously, D,D-carboxypeptidase activity is contributed by low-molecular weight PBPs. The existence of L,D-carboxypeptidase activity is attested in some species by the existence of crosslinks with tripeptide acceptors, notably in *E. faecium* where the ratio of murotripeptides over murotetrapeptides is very high (Billot-Klein et al. 1996), but the responsible enzyme has so far remained elusive. The second group comprises murein hydrolases (known as autolysins) which split glycan bonds and cross-links, and therefore unknit the PG fabric. Autolysins have been described in a variety of Gram-positive and Gram-negative bacteria (for general reviews, see Höltje & Tuomanen 1991; Shockman & Höltje 1994; Shockman et al. 1996; Höltje 1996; for a review on *B. subtilis* autolysins, see Doyle & Koch 1987; for reviews on LAB autolysins, see Chapot-Chartier 1996; Gasson 1996). Several bands of autolytic activity are usually resolved on zymograms following SDS-PAGE, indicating the complexity of the cell autolytic machinery (for LAB, see examples in Valence & Lortal 1995; Ostlie et al. 1995; Lepeuple et al. 1998; Blackman et al. 1998). Strikingly, the *B. subtilis* complete genome sequence reveals the presence of 16 genes encoding murein hydrolases, among which 11 *N*-acetylmuramoyl-L-alanine amidases (Kunst et al. 1997).

Autolysins are primarily needed for the separation of daughter cells following septation, as shown by the filamentous growth of some autolysin-defective mutants (as an example, see Buist et al. 1995). Interestingly, *B. subtilis* wild-type cells grow as filaments when cultured at 48° under high aeration, and these can be resolved into single cells by the addition of exogenous muramidase such as egg-white lysozyme (Fan 1970). Conflicting reports have been published on *B. subtilis* lacking both glucosaminidase (LytD) and the major amidase (LytC): according to Margot et al. (1994) , the mutant showed no detectable morphological changes and grew and divided normally, whereas a recent paper by Blackman et al. (1998) reports notable filamentation changes compared to the parent strain. Differences in experimental conditions probably explain this difference in phenotype.

Another role must be specifically devoted to autolysins in Gram-positive bacteria, whose cell wall is not only increasing in surface during growth, but also in thickness. Whereas surface enlargement is taken care of by cell division, the only way to compensate for wall thickening is to continuously shed the peripheral PG layers, as in skin desquamation. Thus, a delicate balance between PG synthesis and degradation is needed to maintain cell wall integrity, and it is therefore not too surprising that inhibitors of PG synthesis, in particular β-lactam antibiotics, trigger autolysis in growing cells (Pisabarro et al. 1990). Many conditions have been reported to induce autolysis, especially in Gram-positive bacteria. For instance, a number of strains of *L. lactis* autolyse upon prolonged incubation in culture medium, but to a different extent according to the carbon source or to the temperature (Sandholm & Sarimo 1981; Vegarud et al. 1983; Buist et al. 1997). A variety of LAB strains autolyse when incubated in buffer (Mou et al. 1976; Lortal et al. 1989, 1991), but to a much lower extent at acidic pH (Riepe et al. 1997). Autolysis of *B. subtilis* has been shown to depend on the state of energization of the cellular membrane (Joliffe et al. 1981) and can be triggered by cold-shock (Yamanaka et al. 1997).

Our knowledge of the biochemical and genetical mechanisms responsible for the induction or prevention of autolysis remains disappointingly scarce. Interestingly, stationary phase-induced autolysis in *B. subtilis* has been shown to be drastically enhanced in mutants defective for one or several proteins belonging to the cold-shock family (Graumann et al. 1997). In the same species, the LytC amidase has been reported to be transcriptionally down-regulated by *lytR*, and its activity was shown to switch from random to processive under the control of its modifier LytB (Herbold & Glaser 1975a, b; Lazarevic et al. 1992; Margot & Karamata 1992). In *E. hirae*, genetic evidence suggests that the export of muramidase-2 into the medium is under the control of the *arpU* gene (Lleo et al. 1995). In the same species, the sensitivity of exponential and stationary phase cells to hen egg white lysozyme as well as autolysis in buffer or during stationary phase (Massidda et al. 1996), seem to

be controlled by the *psr* gene (a negative regulator of *pbp5* expression, see Ligozzi et al. 1993).

A few reports concern autolysin genes in LAB. Three muramidase genes have been cloned in *E. faecalis* (Beliveau et al. 1991), *E. hirae* (Chu et al. 1992), and *Lactococcus lactis* (*acmA* gene, Buist et al. 1995), respectively. The encoded proteins comprise several repeats in their C-terminal sequence which are thought to direct cell wall binding of the enzyme (Joris et al. 1992). The function of the *L. lactis* muramidase has been studied by examining the phenotype of knockout mutants. The AcmA muramidase is the only autolysin present in the strain MG1363 of *L. lactis* subsp. *cremoris*, as the zymogram is completely negative in the knockout mutant (Buist et al. 1995). It must be recalled that the second major muramidase found in the AM2 strain of *L. lactis* subsp. *cremoris* has recently been shown to be of prophage origin (Lepeuple et al. 1998). Knockout of *acmA* prevents autolysis upon prolonged incubation of the MG1363 strain (Buist et al. 1995), or upon suspension of the CO strain in buffer (Riepe et al. 1997). The role of AcmA is mainly related to cell separation, as AcmA-deficient cells grow in long chains (Buist et al. 1995; Riepe et al. 1997). Interestingly, the separation defect in the MG1363 AcmA mutant can be reverted by the addition of culture supernatant from the wild-type strain (Buist et al. 1995). A recent report also shows that the AcmA muramidase is sensitive to the proteolytic action of the membrane-bound proteinase PrtP (Buist et al. 1998). Moreover, induced overproduction of AcmA has been achieved in MG1363, leading to increased autolysis (Buist et al. 1997). Altogether, these observations open interesting perpectives for the design of food-grade lysis systems aiming at a better control of cheese ripening (Crow et al. 1995).

It must be recalled that LAB, together with numerous other groups of bacteria, are found in close contact with, and sometimes adhere to, the mucosal surfaces of the gastro-intestinal, urogenital and broncho-nasal tracts. It is estimated, for instance, that the normal healthy microflora of the female urogenital tract is dominated by lactobacilli (Redondo-Lopez et al. 1990). Some LAB species or strains found in these ecological niches are thought to be endowed with 'probiotic' properties contributing to the maintenance of health, including the ability to stimulate the mucosal immune system so as to enhance the non-specific (immunostimulation) or specific (immunoadjuvanticity) responses (Hamann et al. 1998; for a review on probiotics, see Tannock, 1999). Interestingly, PG fragments released at these sites through autolysis or as a result of exogenous lysozyme attack could contribute to this protective action, as muramylpeptides derived from *L. plantarum* ATCC8014 for instance are known to display immunoadjuvant activity (Kotani et al. 1975; for a review on the immunological properties of muramylpeptides, see Adam et al. 1981). In contrast, there are indications in the literature that cell wall fragments from members of the normal enteric flora (including *E. faecium*) may have the potential to induce a wide range of inflammatory responses, from transient acute to chronic erosive joint disease (Stimpson et al. 1986; Schwab 1993). Attention should be paid, when selecting LAB for probiotic use, to these potentially beneficial or harmful effects of cell wall (auto)lysis on human health.

Teichoic acids

Besides PG, the cell wall of most Gram-positive bacteria also comprises teichoic acids (*sensu lato*) which may account for more than half of the weight of the wall. LAB deserve a special mention when considering these components of paramount importance for the functionality of the cell wall, as their discovery sprung forty years ago (Armstrong et al. 1958) from investigations conducted by Baddiley and his colleagues in search of a function for glycerol- and ribitol-cytidine diphosphates serendipitously found as 'contaminants' in bacterial extracts from *L. arabinosus* (now *L. plantarum*) prepared for the study of coenzyme A biosynthesis (for a recollection, see Baddiley 1989). Teichoic acids are quite diverse in structure and abundance, depending on the species or strain, stage or rate of growth, pH of the medium, carbon source, availability of phosphate, etc. (for general reviews on teichoic acids, see Archibald & Baddiley 1986; Fischer 1988, 1990, 1994; Pooley & Karamata 1994; for a review on anionic polymers from bacilli, see Archibald et al. 1993). The structures of some teichoic acids found in LAB are given in Figure 8. As will be discussed below, they are synthesized on the outer face of the cytoplasmic membrane, and their size and physicochemical properties are compatible with an extended configuration across the cell wall. By virtue of their lipid anchor, lipoteichoic acids (LTA) and lipoglycans (LG) may remain attached to the cytoplasmic membrane, but a fraction of them are found free in the cell wall or even released into the medium; teichoic acids (TA) and teichuronic acids (TUA), on the other

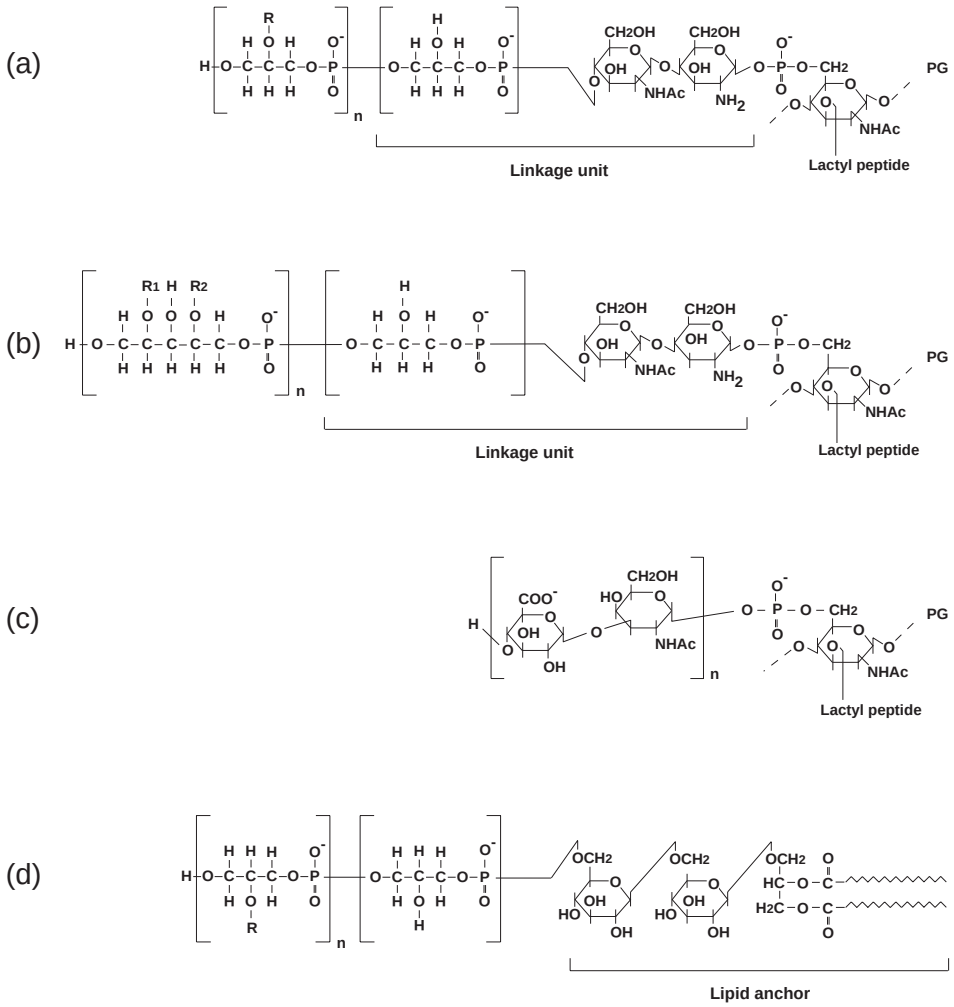

Figure 8. Examples of structures of teichoic acids *sensu lato*. (a): poly(glycerol phosphate) teichoic acid (*Bacillus subtilis*); (b): poly(ribitol phosphate) teichoic acid (*Lactobacillus plantarum*); (c): teichuronic acid (*Bacillus subtilis*); (d): lipoteichoic acid (*Bacillus subtilis*); – PG: peptidoglycan chain; R, R_1, R_2 = substituent (glucose, D-alanine, GlcNAc...) (see text for explanations).

hand, are covalently bound to PG and are sometimes generically called 'wall teichoic acids' (WTA).

Teichoic acids contribute in many respects to the functionality of the cell wall. Those which are made of repeating units of glycerol- or ribitol-phosphate constitute a reservoir of phosphate which can be mobilized when phosphate becomes scarce (Grant 1979). As anionic polymers, they play a role as scavenger of cations, in particular Mg^{++} (Hughes et al. 1970; Lambert et al. 1975b), and may create a pH gradient across the wall by sequestering the protons expelled through the cytoplasmic membrane as a result of metabolism (Urrutia et al. 1992; Kemper et al. 1993). Teichoic acids are also known to influence the activity of autolysins (Höltje & Tomasz 1975; Yamada et al. 1975; Bierbaum & Sahl 1987) and are involved in phage adsorption (Archibald 1976; Briehl et al. 1989). LTA provide the main component of the hydrophobicity of the cell enveloppe (Miörner et al. 1983), and may contribute in this way to its adhesiveness (for a review on hydrophobic interactions in relation to bacterial adhesion, see Rosenberg & Kjelleberg 1986). In this respect, it was shown recently that the adhesion of the *Lactobacillus johnsonii* La1 strain to Caco-2 human intestinal cells could be inhibited in a concentration-dependent way by LTA purified from that strain, as well as by *L. johnsonii* La1 culture supernatant containing LTA (Granato et al. 1999). Last but not least,

teichoic acids are potent immunogens and can be regarded as the gram-positive equivalent of the gram-negative lipopolysaccharides (Knox & Wicken 1973; Wicken & Knox 1975, 1980). LTA is able to weakly stimulate cytokine synthesis, and this effect is lost upon removal of the D-alanine substituents (see below) (Bhakdi et al. 1991; Tsutsui et al. 1991; for a general review on bacterial modulins, see Henderson et al. 1996).

Biosynthesis of wall teichoic acids

As shown in Figure 8, both poly(glycerol phosphate) and poly(ribitol phosphate) teichoic acids have the same overall structure made of a chain of phosphodiester-bound glycerol or ribitol residues attached to a terminal 'linkage unit' allowing their covalent fixation on PG. The structure of the linkage unit of the *L. plantarum* poly(ribitol phosphate) TA is shown in Figure 8b as an example in LAB (Kojima et al. 1985a). It is made of the disaccharide *N*-acetylmannosaminyl$\beta(1\rightarrow4)$glucosamine followed by glycerol phosphate. This type of structure seems to be well conserved despite the wide variety of TA; differences may occur in the nature of the sugars and the number of glycerol phosphate residues (Archibald et al. 1993). The two teichoic acids may be found together in the same species (but attached to distinct PG glycan chains); however, in *L. casei* (Neuhaus 1985) and in *L. lactis* subsp. *cremoris* (Valyasevi et al. 1990), no wall teichoic acids are present and the anionic character of the cell envelope is exclusively due to LTA. The biosynthesis of both types of TA follows a similar scheme (Figure 9). TA and PG biosyntheses share the same lipid carrier (undecaprenyl phosphate), and the formation of the TA linkage unit starts with the same precursor as that used in priming PG synthesis (UDP-GlcNAc); both PG and main chain TA precursors are nucleotide-activated monomers synthesized in the cytoplasm, although CDP-glycerol or CDP-ribitol are used in TA biosynthesis instead of UDP-bound sugars in PG. Biosynthesis of the polymer takes place most probably on the outer side of the cytoplasmic membrane. It starts with the assembly of the linkage unit on the lipid carrier, followed by the cyclic addition of a few dozens of glycerol or ribitol phoshate units. Monomers are added either at the end remote from the anchor lipid (as in *B. subtilis*, Archibald et al. 1993), or at the end adjoining the linkage unit, depending on the species. In the course of polymerization, the C-2 hydroxyl of glycerol can be variably substituted with α-D-glucose or D-alanine ester (TA and LTA substitution will be discussed below); in the case of poly(ribitol phosphate) TA, the C-2 and C-4 hydroxyls can also be substituted in a similar way. The completed TA is finally hooked through its linkage unit on the C-6 of a MurNAc residue of a growing PG glycan chain.

Most of the genes involved in TA biosynthesis have been identified in *B. subtilis*, except those responsible for the synthesis of the linkage unit (Mauël et al. 1991; Pooley & Karamata 1994; Lazarevic et al. 1995). Insertional mutagenesis studies indicate that TA is essential in this species (Mauël et al. 1989), and conditional-lethal (thermosensitive) mutations could only be obtained, in particular in the *tagD* gene encoding glycerol-3-phosphate cytidylyltransferase (Pooley et al. 1991). Thermosensitive Tag^- mutants display grossly deformed cell shapes when incubated at the restrictive temperature, demonstrating complex metabolic interactions between TA and PG syntheses (Briehl et al., 1989). Two genes from the *B. subtilis tag* divergon (*tagGH*) have been reported to encode a two-component ABC transporter likely to be responsible for the translocation through the membrane of poly(glycerol phosphate) as well as a minor poly(glucose-galactosamine phosphate) TA, or their precursors (Lazarevic & Karamata 1995). Genes related to TA biosynthesis have not yet been described in LAB.

Biosynthesis of teichuronic acids

The phosphate-free teichuronic acids have only been described so far in a few species (mainly bacilli), but the phylogenic relatedness of the genus *Bacillus* to LAB makes it possible that they would also exist in that group. The structure of TUA differs from that of TA in two respects: the main chain is made of sugar monomers directly linked by glysosidic bonds, and no linkage unit is present (Figure 8c). The nature of the sugar monomers varies in the different species studied. In *B. subtilis* (strain W23) it is a disaccharide composed of N-acetyl-galactosamine linked to glucuronic acid, but more complex tri- or tetra-saccharide may be found in other bacilli (Ward 1981). The negative charges in TUA are provided by the carboxyl groups of glucuronic acid. In *B. megaterium*, TUA are the only anionic polymers present, but in *B. subtilis* TUA replace TA under phosphate starvation conditions (Lang

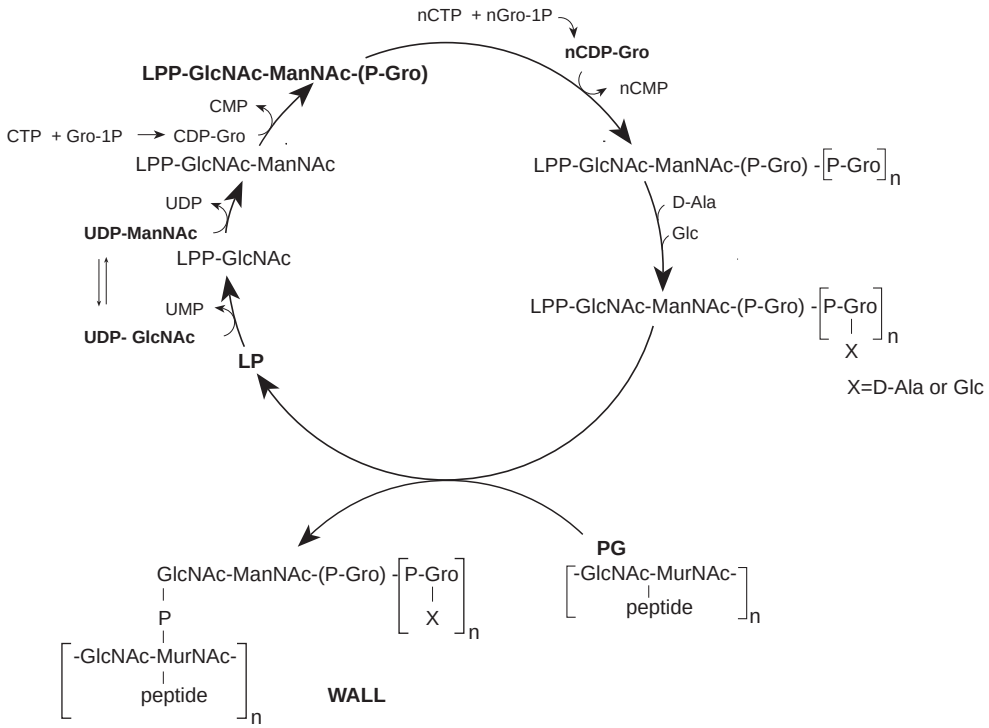

Figure 9. Pathway of teichoic acid synthesis. Gro = glycerol; L = undecaprenol; ManNAc = *N*-acetyl mannosamine (see text for explanations).

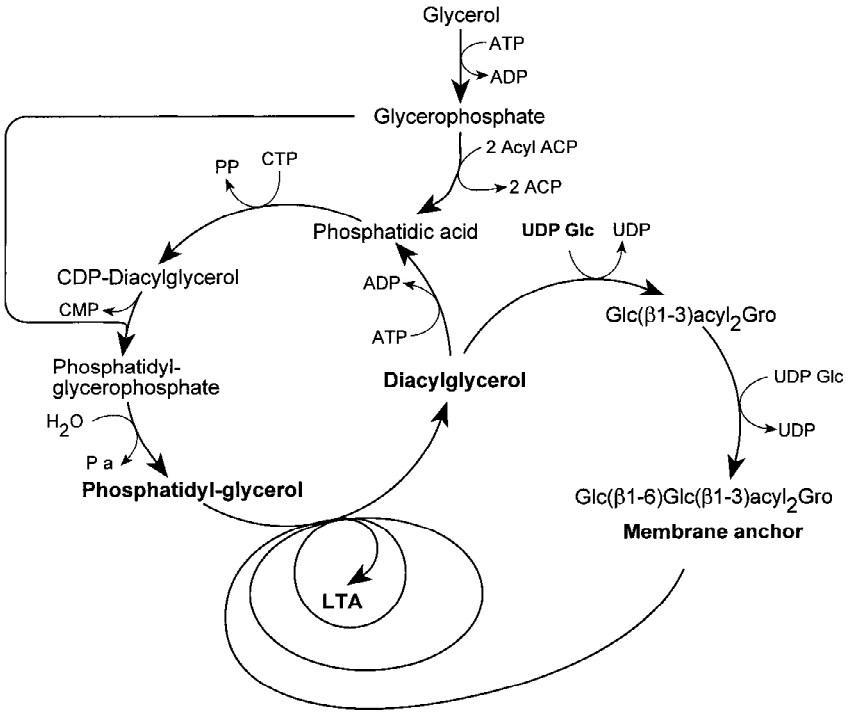

Figure 10. Pathway of lipoteichoic acid synthesis (see text for explanations).

et al. 1982). Recent genetic studies clearly show in this species that transcriptional repression of the *tag* divergon (responsible for TA synthesis) together with transcriptional induction of the *tuaA* promoter (needed for TUA synthesis, see below) is governed by the two-component PhoP-PhoR regulation system in response to phosphate concentration (Liu et al. 1998; Qi & Hulett 1998). Interestingly, genetic evidence indicates that TUA contributes to the ability of an alkalophilic *Bacillus* sp. to grow at alkaline pH (Aono & Ohtani 1990; for a review, see Krulwich et al. 1997).

Biosynthesis of TUA follows the same logics as that of TA. Undecaprenyl phosphate serves as lipid anchor for the growing chain, and nucleotide-activated sugars are used as precursors (UDP-GalNAc and UDP-GlcA in *B. subtilis* W23). The disaccharide monomers are cyclically inserted between the lipid carrier and the nascent TUA chain (elongation from the reducing end), and the completed polymer is finally cleaved from its undecaprenylphosphate anchor (together with one phosphate) to be attached through a phosphodiester bond to the C-6 of MurNAC on a growing PG chain. Recently, the 8-cistron *tua* operon encoding the genes needed for TUA biosynthesis has been characterized in *B. subtilis* strain 168 (Soldo et al. 1999). Comparison of GC contents of *tua* and *tag* loci suggests that TUA is an ancestral bacillar anionic polymer, while operons involved in the synthesis of TA were acquired by horizontal transfer from low-GC content species. This strengthens the possibility that *tua* genes could also be found in some LAB genomes.

Biosynthesis of lipoteichoic acids

LTA are widely distributed in LAB and have been found in enterococci, lactobacilli, lactococci, leuconostocs, and streptococci (Fischer 1994). LTA with a poly(glycerol phosphate) main chain (Figure 8d) represents the most common type of membrane-anchored anionic polymer. Interestingly, it was first discovered in the lactic acid bacterium *L. fermentum* (Wicken & Knox 1970). In *L. lactis*, it has been estimated that the glycolipid anchor (see below) of poly(glycerol phosphate) LTA constitutes every fifth lipid molecule of the outer leaflet of the cytoplasmic membrane (Fischer 1981). Other types of LTA are known, as for example in *Lactococcus garvieae* where the repeating units are made of (α1-6)-digalactosyl-glycerophosphate with no D-alanyl substituent (Koch & Fischer 1978). Several species of bifidobacteria have lipoglycans of various structures instead of LTA, and these are substituted with L-alanine instead of the usual D-isomer (Iwasaki et al. 1990; Sutcliffe & Shaw 1991). The main chain of poly(glycerol phosphate) LTA is very similar to that of poly(glycerol phosphate) TA except that the chiralities of glycerol are opposite, reflecting differences in their respective modes of biosynthesis (see below). The reducing end of the main chain is linked by a phosphodiester bond to the O-6 of the non-reducing hexosyl terminus of a glyceroglycolipid anchor whose precise structure may vary according to the genus or species, but obeys the same general pattern, i.e. diacylglycerol bound to a di- or tri-saccharide (itself bearing sometimes an O-6-acyl or -phosphatidyl substituent). Membrane anchoring is ensured by the hydrocarbon chains of the fatty acids. As in TA, the C-2 hydroxyls of the main chain LTA glycerolphosphate residues carry glucosyl (mostly D-glucose, D-galactose or GlcNAc) or D-alanyl substituents (to be discussed below).

The pathway of biosynthesis of poly(glycerol phosphate) LTA in bacilli is outlined in Figure 10. It starts with the recruitment of a free membrane glycolipid to which the *sn*-glycerol-1-phosphate moiety of phosphatidylglycerol is esterified, together with the release of diacylglycerol. Elongation proceeds by further addition of glycerophosphate residues to the glycerol-terminal end of the growing chain, using again phosphatidylglycerol precursors (and not CDP-glycerol as in TA, hence the difference in chirality). Substitution with glycosyl or alanyl residues takes place concurrently on the same chain during elongation, but may also continue on completed chains. The majority of the released diacylglycerol is recycled through phosphorylation to phosphatidic acid, itself a precursor of phosphatidylglycerol, while the remainder is used for membrane glycolipid synthesis. LTA chains in a given strain are not only variable in size, but also in the extent of substitution and in the identity of the lipid anchor fatty acids, as illustrated by the polydisperse pattern obtained from *E. faecalis* LTA (Fischer 1994). Although LTA are born anchored to the cytoplasmic membrane and may remain attached when completed, a fraction of the chains may be released through deacylation, while others still holding their glyceroglycolipid anchor may become loose, possibly through dragging by the inside-to-outside PG expansion mechanism. Finally, the heterogeneous 'secreted' fraction of LTA will assemble into various kinds of micelles (Wicken et al. 1986) and contribute to the glycocalyx or be released into the medium (for

a general review on bacterial cell surface amphiphiles, see Wicken & Knox 1980).

TA and LTA substitution

The main chains of TA and LTA are decorated with glycosyl or D-alanyl substituents which contribute greatly to the functionality of these anionic polymers, as evidenced by the phenotypes of strains with genetic changes altering the substitution process. It was shown more than 30 years ago that glucosylation of TA was required for the adsorption of certain phages on *B. subtilis* (Young 1967) and phage-resistant mutants deficient in glucosylation were mapped in three distinct loci named *gtaA*, *B*, and *C* (Young et al. 1969). The corresponding genes were later shown to encode the three genes needed in turn for glucosylation: phosphoglucomutase (*gtaC*) ensuring the production of glucose-1-P from the glycolytic intermediate glucose-6-P (Pooley et al. 1987), UDPglucose pyrophosphorylase (*gtaB*, also known as *galU*) responsible for the production of UDPglucose from glucose-1-P (Pooley et al. 1987), and UDP poly(glycerol phosphate) α-glucosyltransferase (*gtaA*, also known as *tagE* or *rodC*) allowing the transfer of the glucose moiety from UDPglucose onto TA (Honeyman & Stewart 1989). UDPglucose is brought to the membrane-bound GtaA protein following coupling to undecaprenyl phosphate, thereby relating the TA glucosylation process to other membrane-bound macromolecular syntheses requiring the same lipid carrier (PG, EPS). Recently, a mutant (*gtcA*) deficient in poly(ribitol phosphate) TA galactosylation has been obtained in *Listeria monocytogenes* (Promadej et al. 1999). Curiously, a *gtcA*-related gene is present in the genomic sequence of *B. subtilis* (Glaser et al. 1993), a species not described as having galactose-decorated TA. Little information is available on glycosylation in LAB. An early report showed that the loss of glucose substitution in poly(ribitol phosphate) TA confers phage resistance to a mutant of *L. plantarum* (Douglas & Wolin 1971); likewise, the plasmid-encoded resistance of the *L. lactis* subsp. *cremoris* strain SK110 to phage sk11G (de Vos et al. 1984) results from the acquisition of galactosylated LTA (Sijtsma et al. 1988, 1990). In neither case, did the changes in the glycosylated status of anionic polymers bring any phenotypic traits other than phage resistance. In contrast, knockout of the *galE* gene in *L. lactis*, which encodes the UDP-glucose 4-epimerase allowing UDP-glucose to be converted into UDP-galactose during growth on glucose, was shown recently to impact on the separation of cells following division, leading to the formation of long chains of cells connected with tiny peptidoglycan filaments. This peculiar mode of growth in long chains is reminiscent of the phenotypes of the AcmA (Buist et al. 1995) and DltD (Duwat et al. 1997; see below) mutants, and it can be speculated that the lack of UDP-galactose, by preventing substitution of LTA with galactosyl residues, would lead to an increased inhibition of autolytic activity by LTA (B. Grossiord, personal communication).

D-alanylation takes place during the assembly of the LTA main chain, but may also occurs postsynthetically through transesterification from one site to another within LTA (Childs III et al. 1985). Whether D-alanylation of TA can also be accomplished by the same enzymatic machinery as that used in LTA substitution is still unclear (Perego et al. 1995). Pulse-chase experiments strongly suggest that in *Staphilococcus aureus* D-alanine substituents can be transesterified from LTA to TA (Koch et al. 1985), but this does not exclude that primary D-alanyl substitution of TA could also take place through a direct (common or distinct) process.

Altering the process of D-alanylation usually has a dramatic incidence on the functionality of the cell wall (for a review, see Heaton and Neuhaus 1993). This can easily be understood by reference to the variety of functions devoted to anionic polymers in connexion with their acidic nature, keeping in mind that D-alanine is esterified to TA and LTA, and therefore brings its positive charge as a counterion to those of phosphate or carboxyl. It is known that a reduction in D-alanine substitution increases the binding capacity of LTA for magnesium ions (Heptinstall et al. 1970; Lambert et al. 1975a). Early *in vitro* studies also showed that the inhibitory effect of LTA on *S. aureus* autolysin activity was negatively correlated to the level of D-alanylation (Fischer et al. 1981). This conclusion was recently supported *in vivo* by the reduced autolytic activity of alanine racemase mutants in the same species (Kullik et al. 1998), although in this case D-alanine deprivation may also affect PG synthesis and/or cross-linking. More convincingly, *L. casei* mutants defective in LTA D-alanyl esters show aberrant C-shaped morphologies and defects in cell separation indicative of reduced autolytic activity (Ntamere et al. 1987). Opposite evidence supporting a repressor role of D-alanine substitution on autolysis is provided by the increased autolysis of alanine racemase mutants in *B. subtilis* (Ferrari et al. 1985;

Heaton et al. 1988), and *L. plantarum* or *L. lactis* (Hols et al. in preparation), but again these mutations may have pleiotropic effects. A stronger argument is provided by the enhanced buffer- and β-lactam-induced lysis of *B. subtilis* mutants solely defective in D-alanylation (Wecke et al. 1996, 1997); no changes in growth or morphology were observed in this case. It seems difficult at first sight to reconcile these apparent conflicting results, although species-related diversity in autolysins and anionic polymers is expected to bring differences in the control of autolysis.

Recently, in a search for UV-sensitive *ISS1* insertion mutants in *L. lactis*, Duwat et al. (1997) have found a hit in the *dltD* gene involved in LTA D-alanylation (see below). Besides being UV-sensitive, the mutant was reported to grow slowly in long chains, to be less efficient in conjugation, and not to require glycine for becoming electrocompetent, all indicative of alterations in the functionality of the cell wall. Using a similar *ISS1* insertion approach, F. Rallu & E. Maguin (personal communication) have selected two distinct mutations in the *dltD* gene, which respectively suppress the acid stress resistance of the RelA and AcrR mutants already mentioned (see above). The molecular mechanisms accounting for these genetic interactions are unknown.

The biochemistry and molecular biology of D-alanylation have been studied in *B. subtilis* and in *L. casei* (for a review, see Neuhaus et al., 1996). In *B. subtilis*, 5 genes responsible for D-alanine decoration of both LTA and TA have been found clustered (*dltA*→*E*,in that order) in an operon driven by three consecutive promoters (σ^A-σ^D-σ^A); the same type of organization has been observed in *L. casei*, although in this case a single, vegetative promoter was detected. The encoded proteins are similar in both species, and the following common mechanism can be envisioned. D-alanine is first activated by the DltA enzyme (also called Dcl in *L. casei*) in the presence of ATP and Mg^{++} to yield a D-alanyl adenylate. The same enzyme transfers its D-alanyl moiety to the DltC (Dcp) carrier protein (an analog of acyl-carrier proteins involved in fatty acid synthesis). The next steps are still a matter of debate. It is thought (Perego et al. 1995; Neuhaus et al. 1996) that the membrane-bound DltB protein (for which no enzymatic activity has been demonstrated so far) transfers D-alanine to the lipid carrier undecaprenyl phosphate, and that DltD is finally responsible for D-alanyl transesterification of LTA (and possibly TA, see above) on the outer face of the cytoplasmic membrane. No functions have been allocated to the *dltE* gene yet; this gene, unlike the four others, is dispensable for the process.

S-Layer

The bacterial envelope may comprise one (or two) superimposed surface layer(s) (S-layer) made of protein subunits, sometimes glycosylated, packed in a paracrystalline hexagonal or tetragonal monolayer. S-layers have been found in more than 400 species belonging to about 100 eubacterial and archaebacterial genera, and more than 30 different S-layer protein genes have already been cloned. As the subject has been reviewed extensively over the last three years (Boot & Pouwels 1996; Bahl et al. 1997; Beveridge et al. 1997; Messner et al. 1997; Rachel et al. 1997; Sleytr 1997; Sleytr & Sara 1997; Sleytr et al. 1997; Pum & Sleytr 1999), this chapter will only address a few points of specific interest to lactic acid bacteria.

In LAB, S-layers have been found so far only in the genus *Lactobacillus* (Masuda & Kawata 1983; Messner & Sleytr 1992; Lortal 1993). S-layer proteins have been studied in some details in *L. helveticus* (Lortal et al. 1992; Mozes & Lortal 1995), *L. brevis* (Vidgren et al. 1992; Yasui et al. 1995), *L. acidophilus* (Boot et al. 1993), and *L. crispatus* (Toba et al. 1995). Genes have been cloned and characterized in *L. brevis* (Vidgren et al. 1992), *L. acidophilus* (Boot et al. 1993), and *L. helveticus* (Callegari et al. 1998). In *L. acidophilus*, two *slp* genes are present in opposite orientation in the same locus: one (*slpA*) is higly expressed in the majority of the cells, while the other (*slpB*) is silent except in a small fraction of the population where it has been exchanged with *slpA* by inversional homologous recombination. This situation is reminiscent of the phenomenon of antigenic variation of S-protein described in several pathogens (Boot et al. 1995, 1996a).

The S-layer lattice is strongly (yet, non-covalently) anchored to the underlying PG through a repetitive modular element (SLH motif) that occurs at the amino- or carboxy-terminal end of the S-layers monomers and also of many other cell-wall associated proteins (Lupas et al. 1994; Lupas 1996; Sara et al. 1998). This anchoring system offers interesting perspectives of applications for the surface-display of heterologous proteins, including antigens in live recombinant vaccine vectors (Mesnage et al. 1999; for a review on LAB as live mucosal vaccine vectors, see Mercenier 1999).

Knockout of the *L. acidophilus slpA* gene has been attempted in two laboratories with no success, suggesting that the gene is essential (Boot et al. 1996a). This is not too surprising, considering the enormous burden imposed on the cell economy by the production of up to 500 S-layer subunits per second (Sleytr et al. 1993), amounting to 15–20% of the total cellular protein content (Yasui et al. 1995). This very high rate of production is due to a conjunction of strong transcription/translation/secretion signals and remarkably stable mRNA (reported half-lives: 15 min; see Boot et al. 1996b; Kahala et al. 1997). Taking advantage of these features, Savijoki et al. (1997) have developed an expression vector allowing the production of the β-lactamase reporter in various lactobacilli at a rate of up to 5×10^5molecules/cell $\times$ hr, yielding 80 mg/l of secreted protein. These are probably amongst the highest rates of heterologous expression reported so far in lactobacilli.

Despite intensive research efforts over the last 30 years, the contribution of the S-layer(s) to the functionality of the cell wall remains elusive. Several putative functions have been invoked: virulence factor in pathogenic species, sieve allowing retention of useful macromolecules or exclusion of harmful hydrolases, masking of phage receptor, adhesion factor (for a review, see Sleytr et al. 1993). In *L. crispatus*, the S-layer has been suggested to act as an adhesin, as it was reported to promote binding to components of the extracellular matrix of target cells *in vitro*, particularly collagen (Toba et al., 1995), in contrast with earlier work indicating no participation of the S-layer to adhesion to Caco-2 enterocytes *in vivo* (Greene & Klaenhammer 1994).

Cell wall polysaccharides

Cell wall polysaccharides (WPS) are ubiquitous components of the cell envelope, and form the basis of many serological typing systems, as examplified in LAB by the Lancefield classification of streptococci (Lancefield, 1933). They are sometimes called neutral polysaccharides to distinguish them from the anionic polysaccharide teichoic acids, although some may also be acidic as they may be branched with anionic substituents, in particular glycerol phosphate (Coyette & Ghuysen 1970; Kojima et al. 1985b, 1986). Conceptually, a distinction is made between the capsular polysaccharides (CPS), which form a thick outermost shell intimately associated with, and often covalently bound to, the cell wall, and WPS *sensu stricto*, which decorate the cell enveloppe and may again be either covalently bound to the PG (as an example, see Coyette & Ghuysen 1970) or loosely associated with it (as an example, see Gopal & Crow 1993). Extracellular polysaccharides (EPS) are another category of polysaccharides which in contrast to capsules do not remain attached to the cells but are released into the medium (for a review on EPS, see Whitfield 1988). Operationally, differentiation between these various classes is often difficult, as for instance EPS transiting through the thick Gram-positive cell wall may be recovered as loosely associated material, and as sample handling may remove most of the capsular material and leave only residual PG-bound polysaccharides mistakenly taken as WPS.

Capsules have been extensively studied as they contribute to the virulence of many pathogens, including pathogenic streptococci such as *S. pneumoniae* where more than 90 different serotypes have been described, each producing its own, specific CPS (Henrichsen 1995; Garcia & Lopez 1997; for a general review on CPS, see Roberts 1996). This diversity is also observed in EPS, in particular in 'ropy' dairy LAB which produce a range of different texturing polysaccharides of high technological interest (for a review on EPS in LAB, see Cerning 1990). These two classes of polysaccharides lie beyond the focus of this review and will not be covered here.

Like polysaccharides in general, WPS are indeed complicated structures which differ not only in the nature of sugar monomers, but also in their mode of linkage, branching, and substitution. A unified nomenclature for polysaccharide synthesis and gene designation has recently been proposed (Reeves et al. 1996). Further complications arise, as different WPS may co-exist in the same strain, as their structure may be strain-dependent, and as their composition may also vary with the growth conditions (Wicken et al. 1983). Quite often, the only information available is the identity of the monomers, sometimes together with their molar ratios. Rhamnose is very often found as a constituent of WPS in LAB, in particular in lactobacilli (Hall & Knox 1965; Coyette & Ghuysen 1970; Douglas & Wolin 1971; Wicken et al. 1983), and in streptococci (Schleifer & Kilpper-Bälz, 1987). The precise structures of WPS acting as co-aggregation receptors for the formation of the dental plaque have been elucidated in several oral streptococci (for a review on oral bacteria adhesion mechanisms, see Kolenbrander & London 1993; Whittaker et al. 1996),

as well as in a few non-pathogenic LAB species, in particular *L. casei* (Nagaoka et al. 1990), *Bifidobacterium longum* (Nagaoka et al. 1995), and *Bifidobacterium catenulatum* (Nagaoka et al. 1996). In the latter cases, as the polysaccharide fractions analyzed had been released from the cell wall following *N*-acetylmuramidase treatment, they are to be considered as authentic WPS covalently bound to the PG, most probably through a phosphodiester linkage between the reducing end of the WPS and the C-6 of MurNAc (Murazumi et al. 1986).

Information about WPS biosynthesis pathways is scarce. Two reports available in the litterature concern the biosynthesis of the acidic and neutral WPS in *B. subtilis* (Kojima et al. 1986; Murazumi et al. 1986). In both cases, undecaprenyl phosphate serves as lipid carrier, and nucleotide sugars are used as precursors: glucose, galactose, mannose, GlcNAc and ManNAc precursors are used as UDP derivatives, whereas the rhamnose precursor is a dTDP-derivative. This overall scheme is similar to that established for PG and WTA biosyntheses (see above), as well as to those of CPS (Roberts 1996) and EPS (Stingele et al. 1996; van Kraenenburg et al. 1997) biosyntheses. It is also reminiscent of the mode of biosynthesis of the O-antigen of the Gram-negative lipopolysaccharides (for a review on LPS, see Raetz 1996).

Some of the genes responsible for the biosynthesis of the *Streptococcus mutans* serotype c-specific WPS have recently been cloned. This polysaccharide is made of a backbone of 1,2- and 1,3-linked rhamnosyl units decorated with glucose side chains. Using a random genomic sequencing approach, Tsukioka et al. (1997a, b) have identified four genes (*rmlA, B, C* and *D*) respectively similar to the gram-negative *rbfA, B, D* and *C* genes responsible for the conversion of glucose-1-phosphate into the dTDP-rhamnose precursor used for the synthesis of the LPS O-antigen (Raetz 1996). Southern blotting analysis revealed the presence of genes hybridizing strongly to the three *rml* probes in various *Streptococcus* species (including *S. salivarius*); weaker hybridization signals were also found with *L. casei*. Two recent papers from the same group report the cloning of 7 additional genes required for the conversion of glucose-1-phosphate into the UDP-glucose precursor of side chain glucose units (*gluA*; Yamashita et al. 1998a), and for the cell wall localization and side chain formation of the antigen (*rgpA→F*; Yamashita et al. 1998b). Interestingly, the RgpC and D proteins are homologous to components of the ATP-binding cassette transporter system and may be involved in polysaccharide export, while RgpE has features typical of glycosyltransferases. Nothing is known about the enzyme(s) or the gene(s) required for WPS anchoring to the cell wall, and we ignore how this process is coordinated with PG elongation and WTA decoration (the latter taking place concomitantly on the same MurNAc C-6 site).

Our understanding of the contribution of WPS to the functionality of the cell wall is far from being clear, in particular in non-pathogenic LAB. Interestingly, knockout of the *gluA* gene in *S. mutans*, which prevents glucose decoration of the c-antigen WPS without affecting the synthesis of its rhamnose backbone, brings about an increased sensitivity to acidic pH (Yamashita et al. 1998a). The physiological explanation for this peculiar phenotype is unknown. Probably the main beneficial function of WPS in general is to mediate interactions with components of the environment. WPS may play a role in non-specific interactions with inert surfaces by contributing to the wall surface physical chemistry (Rosenberg & Kjelleberg 1986; Pelletier et al. 1997). They may also behave as specific lectin receptors mediating adhesion to other microorganisms (as for example in the case of co-aggregation of oral microorganisms in the dental plaque; Mcintire et al. 1988; Cassels & London 1989) or to eukaryotic cells (as for instance in the case of colonizing lactobacilli in the gut; Greene & Klaenhammer 1994; Adlerberth et al. 1996). Adsorption of phages has also been shown to rely on specific interactions with WPS in *L. plantarum* (Douglas & Wolin 1971), *L. casei* (Ishibashi et al., 1982), and *L. lactis* (Valyasevi et al. 1990; Monteville et al. 1994).

Concluding remarks

Over the last two decades, LAB research has witnessed fantastic progress in many different fields ranging from molecular taxonomy to metabolic engineering. Over the years, the five preceding symposia in this series have offered numerous reviews on a wide variety of subjects, of both fundamental and applied interest. Strikingly, the topic of LAB cell wall structure and function had never been addressed before in this context, for the simple reason that little was known – and still is – in this area of 'LAB genetics, metabolism and applications', as the title of the symposia series goes. Yet, we have seen that the cell wall plays an essential role in many aspects of LAB physiology, not only as an exoskeleton 'holding together' the cellular contents, but also as a surface interacting with

the environment in many ways. Truly, the physiology and molecular biology of the cell wall may not seem easily approachable, as it is made of many different polymeric components with complicated biosyntesis pathways. Fortunately, we are now entering the genomics era, as the complete genome sequences of several LAB species will shortly be available (see Ehrlich, this symposium). This will allow identification *in silico* of many genes involved in LAB cell wall biosynthesis, and offer tremendous opportunities for tackling problems of cell wall structure and function in LAB through the creation of isogenic pairs of strains differing by only a single genetic lesion.

Acknowledgements

We are grateful to Sylvie Lortal and Christophe Boonaert for the gift of personal documentation on some of the topics covered in this review. We acknowledge Emmanuelle Maguin and Benoît Grossiord for allowing us to report their unpublished results. We are indebted to Bernard Hallet for the drawing of Figure 1. We thank Marie-Paule Delcour for her kind assistance in the preparation of the manuscript. Research on the molecular biology of lactic acid bacteria has been funded in our laboratory by EC contracts (AGRE-CT90-0041, BIOT-CT94-3055, BIO4-CT96-0439, BIO4-CT96-0542, BIO4-CT96-0498 and BIO4-CT98-0118) and FNRS grants (3.4578.70 and 2.4594.96). MD and EP hold a doctoral bursary from FRIA.

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