Cell Wall Polysaccharides in Gram-Positive and Gram-Negative Bacteria

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Certain bacteria constitute a threat to humans due to their ability to escape host defenses as they easily develop drug resistance. Bacteria are classified into gram-positive and gram-negative according to the composition of the cell membrane structure. Gram-negative bacteria have an additional outer membrane (OM) that is not present in their gram-positive counterpart; the latter instead hold a thicker peptidoglycan (PG) layer. Bacterial resistance to these drugs continues to evolve, which calls for novel antibacterial approaches to be developed.

carbohydrate-based drugs | cell wall polysaccharides | bacterial membrane

1. Bacteria

1.1. Gram-Positive and Gram-Negative Bacteria: General Remarks

Bacteria are microscopic single-celled organisms that are listed among the oldest known life forms on Earth. They can be found in the soil, ocean water, ice, and underneath the earth's crust; that is because these microorganisms are often essential to other organisms' life [1][2][3]. The worldwide bacterial population is estimated to reach 2×10^{30} cells, with a major role in the ecosystem [4]. Moreover, nitrogen-fixing bacteria are essential for the natural growth of several plant species [5][6]. The bacterial fermentation process is also fundamental for the digestive systems of sheep and cattle. In the food industry area, bacteria are involved in the manufacturing of many products, including dairy products, baked goods, and alcoholic beverages [7]. In industrial microbiology, microorganisms are grown on a large scale to produce compounds such as antibiotics, enzymes, and various chemicals, while in biotechnology, genetically-modified organisms are used to synthesize, for example, human proteins for medical use. Bacteria also live in the human and animal bodies, particularly on the skin, airways, oral cavity, and the digestive, reproductive, and urinary systems, normally without causing any harm [8]. They populate the resident flora, or "microbiota", are essential to facilitate food digestion or prevent the proliferation of other more dangerous bacteria, and acting as saprophytes [9][10][11]. The intestinal microbiota plays a central role in the development of the immune system and the modulation of its function. This has led to investigations of the mechanisms that link autoimmune diseases or allergies to changes in the microbiome [12][13][14][15]. Currently, the connections between the human body and the microbiome are not limited to the gastrointestinal or immune system but concern almost all other systems. Alzheimer's disease, multiple sclerosis, and autism have been studied for a putative role of microbiota in their development [16][17][18][19]. Based on the bacterium-host relationship, these microorganisms can be divided into four categories: symbionts, that live and multiply in contact with the host without causing damage and establishing a

beneficial reciprocal relationship; diners that live and multiply in contact with the host without causing damage; pathogens that take advantage of the host causing diseases, from mild to serious ones; and opportunists, which normally are harmless but can cause disease, even serious ones if a weakening of the defense system of the organism occurs. Among them, pathogens represent a small percentage of the entire known bacterial population.

At the beginning of the 20th century, infectious diseases originating from bacterial and viral pathogens were the main cause of death. A decrease in incidence occurred through the years thanks to a greater understanding of the infectious process, the improvement of public health services, vaccination campaigns, and last but not least, the discovery of antibiotics, which made the managing of infectious diseases possible.

1.2. Morphology of Gram-Positive and Gram-Negative Bacteria

Bacteria are unicellular organisms that are formed by a prokaryotic cell, with dimensions ranging from 0.2 to 2 µm. The cell is surrounded by the plasma membrane, which regulates the transport of nutrients and waste substances. The plasma membrane is covered by a wall with different characteristics, leading to the well-known division into gram-positive and gram-negative bacteria. In the gram-negative cell wall, a lipid bilayer membrane, the outer membrane (OM) is present, while the above-mentioned plasma membrane is the inner membrane (IM). In the inter-membrane space, there is a thin layer of peptidoglycan (or murein), referred to as the periplasm, a complex polymer that is made up of amino sugars and amino acids that completely envelop the cell. The outer membrane acts as a barrier against the diffusion of many compounds, making the cell more resistant to toxic substances. Gram-positive bacteria do not have an outer membrane and their wall is mainly composed of a thick layer of peptidoglycan [20].

The different composition of the bacterial wall determines the different coloration that is taken up by the bacteria with the dye that was created by the Danish microbiologist Hans Christian Gram in 1884 and thus allows one to discern the two groups of bacteria. Gram stain is still used in normal clinical practice, as the first step in the screening of a biological sample to identify the pathogenic microorganisms present [21].

In addition to the above-described cell wall, some bacteria possess additional layers of coating (S layer, capsule, or glycocalyx) and surface appendages (pili, fimbriae, flagella). The capsule or mucous layer consists of polysaccharide or protein substances that are secreted on the cell surface by bacteria for several functions, including adhesion (e.g., on solid surfaces generating biofilms) and protection.

1.3. The Cytoplasmic Membrane

The cytoplasmic membrane is a thin barrier that surrounds the cell and separates the cytoplasm from the extracellular environment. The membrane is made of a phospholipid bilayer: phospholipids contain both hydrophobic (fatty acid) and hydrophilic (glycerol-phosphate) components. In the phospholipid membrane, the fatty acids point inward toward each other to form the hydrophobic environment, while the hydrophilic portion remains exposed to the external environment or the cytoplasm [22]. The membrane has a thickness of 6–8 nm and can be seen using an electron microscope: it appears as two dark-colored lines (glycerophosphate) that are separated by

a lighter area (fatty acids). The structure is fluid and the proteins that are immersed in the phospholipid bilayer can undergo both rotational and lateral movement. The cytoplasmic membranes of some bacteria are strengthened by molecules called hopanoids, which are rigid planar molecules and structural analogs of sterols [23]. Many membrane proteins are firmly embedded in the membrane and are named integral membrane proteins [24]. Other proteins have one portion that is anchored in the membrane and extra-membrane regions that point into or out of the cell. The so-called peripheral membrane proteins remain firmly associated with the membrane surfaces [25]. The peripheral proteins interact with integral membrane proteins in important cellular processes such as energy metabolism and transport [25].

The main function of the cytoplasmic membrane is to prevent the passive leakage of solutes into or out of the cell; it acts as a gateway for the transport of nutrients into and wastes out of the cell (permeability barrier). The membrane is also an anchor for many proteins, glucides, lipids, and complexes of the same. Some are enzymes that catalyze bioenergetic reactions whereas other transport solutes into and out of the cell [22].

Salts, sugars, amino acids, nucleotides, and many other substances need to be transported by proteins that, besides ferrying substances across the membrane, accumulate solutes against the concentration gradient. One substance that can cross the membrane in both directions is water, but its movement is accelerated by dedicated transport proteins, called “aquaporins” [26].

1.4. Pathogenic Bacteria

As previously mentioned, disease-causing bacteria are referred to as pathogens. However, in some cases, it is not possible to draw a clear distinction between pathogenic and non-pathogenic bacteria since the encounter of the human organism with the pathogen does not always generate disease. The severity and outcome depend on the complex relationship that is established between the microorganisms and the human organism. Under normal conditions, the thousands of bacteria that make up the microbiota are harmless to human health; indeed, they assist and help maintain it. The immune defenses of human body, therefore, help people to avoid the invasion of microbiota microorganisms, but when the body is compromised for various reasons, invasion can occur, and serious illness appears. Hence, the same beneficial bacteria can become pathogenic.

2. Bacterial Cell Wall Polysaccharides (CWPs) and Peptidoglycans (PGs): Structure and Functions

From a structural point of view, the bacterial cell wall can be classified into two different types, depending on gram-positive or gram-negative bacteria. The main difference between the two cell walls is the absence of a second bilayer membrane in the gram-positive bacteria, which is compensated by a thicker peptidoglycan (PG) layer. The bilayer membrane in gram-negative bacteria is highly asymmetric; the inner leaflet is composed of phospholipids while the outer leaflet mainly contains lipopolysaccharides (LPS) whose polysaccharide chains occupy the extracellular space. [27][28] The PG layer in gram-positive bacteria is a fundamental component of the cell wall placed outside the cytoplasmic membrane of almost every bacterium [29][30][31], with very rare exceptions (e.g.,

Mycoplasmas, *Planctomyces*, *Orientia* (*Rickettsia*) *tsutsugamushi*) [32][33]. Its primary role is to maintain a sustained turgor of the cell wall, preserving the integrity and counteracting the osmotic pressure of the cell [34][35], but it also represents the anchor point for proteins and cell wall polysaccharides (e.g., teichoic acids) [36][37]. Together with the assembly of bacterial peptidoglycan hydrolases, the PG is also deeply involved in the process of cell division and cell growth [38]. Given its almost ubiquitous nature, higher eukaryotes evolved several PG recognition molecules (e.g., CD13, Nod1-2, lysozymes, and amidases) representing the first defense line against bacterial infections [34][39][40]. Moreover, PG represents a robust target for almost all effective antibiotics inhibiting bacterial cell wall synthesis [35]. The presence of such a layer is of fundamental importance for the integrity of the cell. Indeed, any degradation (e.g., by lysozymes) during cell growth or inhibition of its biosynthetic path leads irremediably to cell lysis. In addition to morphological functions, the cell wall undergoes recycling where it is constantly broken down, turned over, and remodeled [41][42][43].

PG is of major importance in gram-positive bacteria given its higher abundance which accounts for almost half of the cell wall mass, while in gram-negative bacteria, the PG layer is rather thin and not directly exposed to the extracellular space due to the presence of the outer membrane. Owing to its close to the omnipresent occurrence, PG has been one of the major antibiotic targets in recent years (e.g., penicillin, cephalosporins, carbapenems, vancomycin, teicoplanin) [44].

2.1. Chemical Structure and Variability of PG and Correlated Functions

The basic scaffold of the PG layer, consists of polymeric glycan strands of two alternating β-(1→4)-linked sugars: *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc). The different strands are arranged parallel to each other and covalently connected via peptide linkers that are substituted at the d-lactyl group of each MurNAc residue. The most common peptide linker is composed of l-Ala-γ-d-Glu-*meso*-A2pm (or l-Lys)-d-Ala-d-Ala. The last d-alanine residue is lost in the mature macromolecule and the previous d-Ala condenses with the corresponding d-Ala residue of another strand's linker, thus creating the distinctive net of the PG layer.

Variations from the general structure of PG have largely been divided into three categories, *viz*., of: (i) the glycan strand structure and substitution pattern, (ii) the lipid linker structure and condensation point, and (iii) the glycosylation point by extracellular glycans. The most commonly reported modification in the first case is *N*-deacetylation and *O*-acetylation patterns at both GlcNAc and MurNAc, *N*-glycolylation of MurNAc residues, the modification of MurNAc to muramic *δ*-lactam, or formation of 1,6-anhydro MurNAc. The extent of these substitutions is species-dependent and has been extensively reported in previous literature works [45].

N-deacetylated sugars in the PG layer of *Bacillus* strains have been reported extensively for both residues [46][47][48], and more recently also for Streptococcal bacteria (e.g., *S. pneumoniae*, *S. pyogenes*, *S. iniae*) [49][50][51]. Deacetylation most likely occurs on the mature peptidoglycan since deacetylated precursors have not yet been reported in species containing PG deacetylases, and the latter enzymes have precise extra-cytoplasmic localization [50].

It is well known that *N*-deacetylation plays a key role in the protection of certain bacteria from host antimicrobial proteins such as lysozymes, which catalyzes the breakage of the β-(1→4)-linkage between GlcNAc and MurNAc leading to hydrolyzed PG layers and bacterial lysis [52]. This was shown to be related to the increased positive charge of the PG layer which decreased the efficiency of the secondary (non-enzymatic) mechanism of action of lysozymes [53], where these proteins cross the cell wall and bind to negatively charged bacterial membranes leading to its permeabilization. The lack of *N*-acetyl groups has been shown to provide a poor substrate for lysozyme binding, while upon chemical *N*-acetylation of the substrate lysozyme activity seems to be restored [50][54][55][56].

N-glycolylated muramic acids in PG, first described in *Mycobacterium smegmatis* [57], are used in bacterial taxonomy to classify *Actinomycetales*, a class of bacteria from which important antibiotics are isolated (e.g., streptomycin, actinomycin, streptothricin) [58]. Such a modification is widely present in the Mycolata taxon, a group of bacteria containing mycolic acids in their cell walls (e.g., *Mycobacterium, Rhodococcus*, *Tsukamurella*, *Gordonia*, *Nocardia*, *Skermania, Dietzia*) [59][60][61]. The modification is introduced during the synthesis of the UDP-precursors and generally is present only on a fraction of the muramic acid residues. Therefore, bacteria containing *N*-glycolylated muramic acids also contain MurNAc residues in the PG layer. The role of the *N*-glycoloyl group as a substituent has been the object of speculation but is still to be clarified. It has been proposed that the extra hydroxyl would engage in hydrogen bonding within the cell envelope with stabilizing effects [62].

Intramolecular amide bond formation between the amino group and the carbonyl of the lactyl group in MurNAc generates the muramic acid δ-lactam modification which appears to be quite abundant in *Bacillus* species and *Clostridium sporogenes* spores. For this modification to occur, the lactyl group must not have any peptide attached and the MurNAc residue must be *N*-deacetylated. This process is generally handled by just two enzymes. In *B. subtilis* for instance, an amidase CwlD removes the peptide that is linked to MurNAc and the peptidoglycan MurNAc *N*-deacetylase PdaA cleaves the acetyl group of the sugar residue [63]. Their role seems to be related to the spores' ability to complete the germination process to produce viable cells. The muramic acid δ-lactam modification is employed by germination-specific hydrolases as a marker to recognize the spore's PG [64][65][66].

The *O*-acetylation in PG appears to be uniquely present at the MurNAc residues [67][68]. First reported in *Micrococcus luteus* and *S. faecalis*, it is present in both gram-positive and gram-negative bacteria (e.g., *B. cereus*, *Staphylococcus aureus*, *Enterococcus hirae*, and *S. pneumoniae* for the gram-positive; *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *H. pylori*, and *Proteus mirabilis* for the gram-negative ones). As a structural variation, *O*-acetylation is more prevalent than *N*-deacetylation and the extent of it varies between <20% and 70% depending on the species and strain that is being considered [69][70][71]. The source of acetyl groups for the *O*-acetylation has been a matter of discussion over the years. It has been proposed to be extracted from the N-2 position of GlcNAc or MurNAc [72]. But there are cases where acetylation in MurNAc6Ac is present in fully *N*-acetylated PG (e.g., *P. mirabilis*); alternatively, it was suggested to be obtained from peptidoglycan turnover products [69]. More recent theories have proposed acetyl-coenzyme A (CoA) or acetyl phosphate to be the source [73].

O-acetylation of PG has an important role in the ability of bacteria to produce disease. The first experimental evidence suggested this substitution to be responsible for the increased resistance towards hen egg-white lysozyme hydrolytic activity [68]. Further shreds of evidence showed the same resistance to egg white lysozyme on 14 strains of *P. mirabilis* containing acetylated PG, and further species lacking *O*-acetylation patterns were shown to be more sensitive to exogenous lysozymes [38][73][74]. Therefore, *O*-acetylation is a contributing factor offering resistance against antimicrobial proteins. Interestingly, the degree of *O*-acetylation seems to be negatively affected by β-lactam antibiotics that target proteins that are related to transpeptidation reactions in the synthesis of peptidoglycans. *O*-acetylation of peptidoglycan appears to play a not-yet resolved role in the cross-linking reaction [75][76][77].

2.2. Cell Wall Polysaccharides: Structure and Functions

Gram-positive and gram-negative bacteria produce extracellular polysaccharides which can either be covalently bound to the cell envelope or surface proteins or be a slime that is weakly attached to the bacterial cell wall. Being present at the interface between microbes and mammalian hosts–other than representing recognition elements for pathogens–these glycoconjugates often contain antigenic moieties that trigger an immunogenic response in hosts. For this reason, these glycans and their biosynthetic pathways have experienced increased attention from the scientific community during the last decades.

As a result of Red Queen evolutionary dynamics [78], the structures of bacterial glycans present the highest variability compared to other non-bacterial classes of glycans and cannot, therefore, be easily generalized. As previously reported, the term “Red Queen” effect takes inspiration from Lewis Carroll’s quote “it takes all the running you can do, to keep in the same place” borrowed to explain the glycan structural variation that contribute to the bacterial diversity in nature. As above mentioned, bacteria have historically been divided based on the outcome of the gram staining procedure. In gram-negative bacteria, glycans are mostly present in the form of lipopolysaccharides (LPSs).

LPS is generally divided into three sections: the lipid A, the core oligosaccharide (inner and outer core), and the O-antigen polysaccharide. The lipid A (or endotoxin) is the innermost and hydrophobic region of the lipopolysaccharide and possesses a conserved structure that is composed of two β-(1→6)-linked glucosamine residues often phosphorylated at the reducing end and non-reducing end residues in positions 1 and 4′, respectively. Acyl groups are condensed at the hydroxyl and amine groups in a variable number [79][80]. Due to this common architecture, most of the lipid A moieties are detected at picomolar levels by receptors that are present on immune system macrophages and endothelial animal cells [80], and a multitude of enzymes that are linked to the lipid A biosynthesis (e.g., LpxC) have over the years been validated as a target for newly developed antibiotics.

Discovered in the late 1800s and isolated from *E. coli*, it was confirmed that LPS was responsible for the pathogenic (endotoxic) properties, which correlated to pathological outcomes (e.g., fever and septic shock). Lipid A is part of pathogen-associated molecular patterns (PAMPs), i.e., molecules that can activate innate immune systems when they are recognized by toll-like receptors (TLRs) or other pattern recognition receptors (PRRs).

Roughly 10^6 lipid A residues are present in a single cell of *Escherichia coli* [81]. The fatty acids are somehow variable in structure and chain length but only one type of fatty acid is amide-linked, i.e., (*R*)-3-hydroxy fatty acids. The length of these fatty acids is 10–20 carbon atoms per chain and if the third carbon atom carries a hydroxyl group it is most often substituted by an additional fatty acid in at least one of the sugar residues [82][83][84][85].

Some species have been shown to contain a keto group in place of the hydroxyl in the *N*-acyl fatty acids [86][87]. Besides that, (*R*)-3-hydroxy fatty acids are predominant also for the ester-bound fatty acids, but these are still more variable and species-dependent. These are fatty acids from myristic and lauric acids and their hydroxylated derivatives, to even more unique structures such as *cis*-11-octadecenoic acid, 3-hydroxy-5-dodecenoic acid, and iso-2,3-dihydroxytetradecanoic acid [88].

The lipid A is covalently linked to heteropolysaccharides via a core region consisting of two parts, viz., first an inner core containing one to four units of Kdo (3-deoxy-d-manno-oct-2-ulosonic acid) [89], as well as l-*glycero*-d-*manno*-heptose residues, which are found in many inner cores. Additional charged elements such as phosphate and uronic acids may be present in the inner core structure providing a binding site for divalent cations (Mg^{2+} and Ca^{2+}) with the effect of stabilizing the outer membrane [90].

The second portion of the core, referred to as the outer core is slightly more variable but structural modifications are still limited if compared to the O-antigen. This last complex repeating unit determines the serological and antigenic properties of the LPS and possesses a high structural variability. This diversity is reflected in the wide range of residues composing the repeating unit, which includes uronic acids, amino sugars, methylated and acetylated derivatives, deoxy sugars, but also non-sugar moieties such as amino acids and phosphate groups. The variability is further extended by the linkage diversity between the different residues in the branched and non-branched repeating units. The number of sugars in each repeating unit is typically four or five, although up to seven sugar residues have been reported for *E. coli* [91]. The total number of residues in a single chain can add up to several hundred, as for some bacterial species up to one hundred repeating units have been shown to be the outcome of the O-antigen biosynthesis. Hence, even a single bacterial species can account for hundreds of different O-antigen structures. *E. coli* is one of the most characterized species in terms of serotypes having–to date–197 reported O-antigens [91].

Over the years databases have been created to collect the different O-antigen structures of various bacterial species from a structural and genetic point of view [92][93][94]. Gram-positive bacteria contain a wide range of glycans on their cell wall. Similarly to glycans from gram-negative bacteria, these are important structural elements with significant patho/physiological relevance, other than representing key surface units for cellular recognition and signaling. Some of the most common and well-studied glycans from gram-positive bacteria are the cell wall teichoic acids (WTAs), which consist of copolymers of glycerol phosphate or ribitol phosphate and carbohydrates that are linked together by phosphodiester bridges.

These polyanionic glycopolymers play critical roles in the cell with functions that include cell morphology and division, autolytic activity, antibiotic resistance, metal ion homeostasis, phage-mediated horizontal gene transfer,

and protection of bacteria from host defense peptides and antimicrobial peptides [95][96]. For their importance, these structures and their biosynthetic pathways represent attractive targets for the design of antibiotics and vaccines.

The WTA polymers can account for up to 60% of the cell wall mass containing between 40 to 60 polyol repeats. Structurally, WTA can be divided into two parts: the main chain polymer and a linkage disaccharide that acts as a bridge between the main chain and the peptidoglycan [95][97][98].

The disaccharide unit is highly conserved across species and its structure is β-d-ManNAc-(1→4)-α-d-GlcNAc-1-*P*, where *P* represents a phosphodiester group that is linked to the hydroxyl group at position 6 of *N*-acetyl muramic acid of the PG layer. The *N*-acetyl-β-d-mannosamine residue is substituted in position O4 with one to two glycerol 3-phosphate units [99][100].

The most common WTA repeating units contain ribitol 5-phosphate (RboP) or glycerol 3-phosphate (GroP), but there is great variability in the WTA monomer structure with more unusual moieties [101][102][103]. Additional structural diversity may arise from the presence of substituents that are attached to the hydroxyl groups of the polyol repeating units, e.g., d-alanine, monosaccharides such as Glc or GlcNAc, or oligosaccharides [104].

The presence of these substituents is important in bacterial defense against a host and antibiotics. The d-alanylation, for instance, has been proven to protect against host-defense mechanisms [105][106]. A reduction or complete absence of d-alanine residues was shown to be correlated with an increased susceptibility to phagocytes, neutrophils, lysostaphin, and lysozymes, together with glycopeptide antibiotics and cationic antimicrobial peptides [107][108][109][110][111]. These structural changes may thus be correlated to the overall change in the charge of the membrane [110]. While the specific removal of β-d-GlcNAc modification has been reported to increase sensitivity to β-lactam antibiotics [112].

The second most characterized cell-wall glycopolymer in gram-positive species are the lipoteichoic acids (LTAs), which, similar to WTA, are zwitterionic polymers, but with a simpler structure compared to WTA. LTA typically consists of a polyglycerolphosphate (PGP) chain that is linked to the bacterial membrane via a glycolipid anchor [113]. As in WTA, the backbone of LTA is modified with alanine or glycosyl residues. Their structures also contain an initial linkage unit which is mostly represented by a disaccharide that is linked to diacylglycerol (DAG). In *S. aureus* and *B. subtilis*, the disaccharide unit is *β*-d-Glc(1→6)-*β*-d-Glc.

LTA can coexist with WTA in the same bacteria and their functions are generally similar. In bacteria containing only LTA moieties, the lack of these may lead–in addition to the same roles that are observed for WTA–to morphological defects such as the increased size of the bacteria or temperature-sensitive growth phenotypes [114][115]. Besides their location in the membrane, where LTA are tethered to the membrane via van der Waals forces and WTA are covalently attached, the LTA generally do not exceed in length past the peptidoglycan membrane while WTA extends further out into the extracellular space.

While WTA and LTA structures are possibly the most common and well-known membrane glycans in gram-positive bacteria, the cell walls of many gram-positive bacteria of the genera *Streptococcus*, *Enterococcus* and *Lactococcus* lack these polyanionic structures and have evolved glycopolymers that are characterized by the presence of rhamnose. These rhamnose-containing CWPs (RhaCWPs) are of particular interest due to the total absence of l-rhamnose in humans, which makes their biosynthetic pathway an attractive therapeutic target. Indeed, l-rhamnose is very often essential for bacterial virulence or viability [116].

First reported by Rebecca Lancefield in the 1930s, these structures have historically been used in the serological classification of streptococci, which initially were used to define Group A–E streptococci and later expanded to more than 20 serogroups [117][118][119].

Over the years, the classification became superfluous since it was found to be unable to differentiate between different species. It is still commonly accepted that a single streptococcal species can express different Group antigens (e.g., *Streptococcus dysgalactiae* subsp. *equisimilis* strains) [120]. Similarly, to WTA, the RhaCWP comprises up to 60% of the dry cell wall mass. They are mainly localized on the outermost surface of the cell wall, but they can also be found intercalated in the peptidoglycan net [121][122][123].

Streptococcal species seem to lack orthologues of the genes that are associated with the expression of WTA (e.g., TagB, TagD, TagF) [124], while Lactococcal and Enterococcal species were reported to contain orthologues for WTA and both WTA and LTA biosynthesis, respectively, thus having a more heterogeneous expression of glycans on their surface [125][126][127][128][129]. The main constituents of the RhaCWP are rhamnose with variable combinations of Glc, GlcNAc, Gal, GalNAc, and phosphate groups, that are differently linked [130]. Even though the structure of the polymers may vary significantly across species, some exceptions show structural similarities, such as GAC and GCC. These different motifs reflect the discriminatory ability of the Lancefield scheme that is based on the diverse structural elements that are present in the different groups. Variations of these structures have been reported in different works and serve as homologs of WTA in terms of biological functions [131].

The first studies on the biological functions of RhaCWP indicated a strong relationship between these and the structural identity of the cell wall [132]. The lack of RhaCWP expression was shown to lead to overgrowth and cell division abnormalities [132][133][134][135]. Inhibition of the UDP-GlcNAc:lipid phosphate transferases in *S. galactiae* [134] and *S. pyogenes* [136] was shown to cause important morphological alterations such as a reduced level of cross-linked PG, increased chain length, and mislocalization of PG hydrolases.

RhaCWP also represents an important phage receptor in many species, as demonstrated in several studies, forming epitopes in conjunction with side-chains of the polymer (e.g., GlcNAc, Glc) [137][138][139]. Additional roles are associated with the impact on virulence of the bacteria, modulated by structural modification on the RhaCWP, still maintaining unaltered bacterial physiology [127][136][140] and cell wall morphology [136]. Furthermore, knowledge of the biosynthetic pathways of RhaCWP facilitates the identification of attractive targets for developing new

antimicrobial agents with limited risk of side effects due to off-target actions, since humans completely lack l-rhamnose in their system [141][142][143].

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