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In principio erat Lactococcus lactis

Coelho Pinto, Joao Paulo

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Document Version

Publisher's PDF, also known as Version of record

Publication date:

2015

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Coelho Pinto, J. P. (2015). *In principio erat Lactococcus lactis: Towards a membrane protein overproducer host.* ([Groningen] ed.). University of Groningen.

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CHAPTER 1

GENERAL INTRODUCTION[§]

[§] Part of this chapter was published in:

João P. C. Pinto, Oscar P. Kuipers, and Jan Kok. “Responses of Lactic Acid Bacteria to Cell Envelope Stresses”. In *Stress Responses of Lactic Acid Bacteria*, edited by Effie Tsakalidou and Konstantinos Papadimitriou, 145–161. Springer US, 2011.

1 - Overview

The existence of an enclosure that delimits cells from their environment is central in the biology of all living organisms. The complexity of these surfaces has evolved enormously, from putatively simple amphiphile-stabilized interfaces to the current broad range of multi-layered structures enveloping modern organisms (1, 2). A key, mostly functional, constituent of these structures is the broad range of proteins that are imbedded in them. The importance of membrane proteins is immediately asserted, for example, through the observation that generally more than 20% of all ORFs in any given genome are predicted to code integral membrane proteins (3).

Despite their importance, studying proteins that localize in the cell envelope faces two constraints. First, they are biochemically special and often unstable and difficult to handle. Second, they are usually difficult to obtain in their native form since a marginal increase of their content in the cell envelope generally destabilizes the membrane structure, therefore affecting cellular viability.

Interestingly, as shown in this thesis, overproduction of integral membrane or secreted proteins elicits cell envelope stress responses similar to the ones that assist their survival in the natural context. A good understanding of cell envelope structures and cell envelope-specific stress responses is thus of great utility as to the understanding of the bottlenecks affecting the production of membrane proteins. An introduction on these topics, with special focus on lactic acid bacteria, is offered below.

Overall, given the importance of membrane proteins, the scope of this work was to help characterize the limitations on the production of these proteins in *Lactococcus lactis* and use that knowledge to design improved membrane protein producers.

2 - The cell envelope of lactic acid bacteria

The structure of the cell envelope of eubacteria comes in two major flavours: one that surrounds the Gram-positive bacteria and that of Gram-negatives (4). The clade of Lactic Acid Bacteria (LAB) encompasses a broad range of microorganisms that possess the cell envelope arrangement typical of Gram-positive bacteria. Still, the differences in cell envelope architecture and composition between members of this group of bacteria are quite significant, greatly influencing key features such as bacteria-host interactions for the pathogenic LAB (5), or texture formation for the ones used in dairy industry (6). Cell envelope variations have been used to classify LAB taxonomically, due to the influence on their shape (LAB are divided into three morphological groups, cocci, bacilli and bifidi (7); for reviews on shape determination see for example (8, 9)) or serologically (10).

Generically, the cell envelope of LAB is composed of a cytoplasmic membrane, spaced from the peptidoglycan sacculus by a periplasm. The cytoplasmic membrane contains proteins and other constituents such as glycolipids, while the sacculus is usually decorated with proteins, teichoic acids (that can reach the cytoplasmic membrane), polysaccharides and, often, a paracrystalline S-layer of proteins (Figure 1). Each of the cell envelope elements is briefly described below. For more extensive reviews on the cell envelope of LAB, see for example (7, 11).

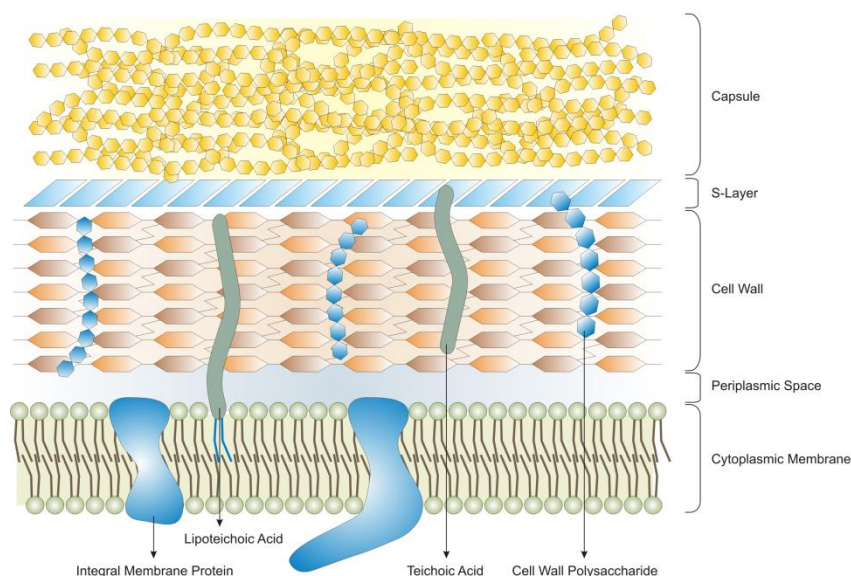


Figure 1 – A simplified scheme of the typical organization of the cell envelope of LAB. The cytoplasmic membrane contains (integral) membrane proteins. The cell wall is essentially composed of peptidoglycan intermeshed with teichoic acids, lipoteichoic acids (which are lipid-anchored to the membrane) and cell wall polysaccharides. Some LAB may also contain a proteinaceous S-layer and/or a capsule made of polysaccharides. Pili and flagella, which are not depicted in this figure, can also be found in some LAB.

2.1 - The cytoplasmic membrane

The cytoplasmic membrane is essentially composed of a phospholipid bilayer. Its hydrophobic nature makes it a permeability barrier, blocking the diffusion of water and water-soluble molecules. The cytoplasmic membrane is thought to be a very active and crowded region of the cell, with a significant degree of differentiation, both functionally and structurally (12). It is also rich in fatty acids, of which the composition is modulated according to temperature, to keep the membrane optimally fluid (13).

The existence of a membrane barrier can also be viewed as a bottleneck to the cell when the goal is to translocate molecules in and out of the cell. To do so takes considerable amounts of resources and frequently requires ingenious mechanisms for transport. Insertion of membrane proteins into or translocation of secreted proteins across the cytoplasmic membrane requires complex translocation/secretion machineries (14, 15). In fact, the complexity of these export systems partially accounts for the limited success of producing recombinant integral membrane or secreted proteins (16, 17). Interestingly, LAB, and *Lactococcus lactis* in particular, seem to have a number of properties that might make them better suited than some other protein production hosts, even when the goal is to produce eukaryotic non-glycosylated membrane proteins (18, 19).

2.2 - The sacculus

The peptidoglycan (also known as murein) sacculus of LAB has the typical Gram-positive thick multi-layered structure, composed of inextensible sugar chains cross-linked by flexible peptide bridges, that is responsible for the withholding of the crystal violet dyes during the Gram staining assay (4, 7). The glycan threads are made of the two alternating amino sugars *N*-acetylglucosamine and *N*-acetylmuramic acid, connected by cross-links of peptide chains of usually five alternating L- and D-amino acids (7). This tough structure, resembling an exoskeleton, is required not only to shield cells from mechanical aggressions but also to reduce the risk of cell rupture as a consequence of the high internal osmotic pressure in the cell. The density of the peptidoglycan lattice also disables free protein diffusion. Large proteins are passively dragged towards the outside of the cell at the pace of the inside-to-outside renewal of the peptidoglycan (20, 21). Although the peptidoglycan layer is regarded as an inactive, passive structure, it has been shown to play important pro-inflammatory roles and can function as an endotoxin in some cases (22).

2.3 - Teichoic acids

Teichoic acids are polysaccharides of glycerol phosphate or ribitol phosphate linked via phosphodiester bonds. They are found exclusively in the cell envelope of Gram-positive bacteria, where they are intermeshed with the peptidoglycan. They are of vital importance and can account for over 50% of the dry weight of the cell wall. Their polyanionic nature gives the cell wall a net negative charge (7, 23). Teichoic acids vary greatly in structure and composition, depending on the bacterial strain and growth conditions. Lipoteichoic acids are a special class of teichoic acids that are lipid-anchored to the cytoplasmic membrane. They regulate autolysis and help holding together the two main functional structures of the cell envelope: the sacculus and the cytoplasmic membrane (7, 23).

2.4 - The S-layer

Surface layers (S-layers), composed of proteins or glycoproteins, are usually single molecule-thick, planar, crystalline lattices that are commonly found in all prokaryotes. They are associated to the external side of the peptidoglycan in Gram-positive bacteria where they add protection to low pH environments and lytic enzymes (24, 25). S-layers have also been described in LAB, such as in lactobacilli, where the proteins are usually smaller than those present in S-layers of other bacteria and have predicted higher pI values (26).

2.5 - Cell wall polysaccharides and capsular polysaccharides

Cell wall polysaccharides, also known as neutral polysaccharides (as opposed to the anionic polysaccharide teichoic acid), are complex molecules that vary in the nature of the sugar monomers (rhamnose is usually found as a constituent in LAB) and in structure (7). The capsule is a thick structure typically composed of polysaccharides and is situated outside the cell wall. It is a virulence factor in pathogenic bacteria: it gives them a slimy coat preventing phagocytosis (5). Recently, a pellicle of polysaccharides (distinct from a thick capsule) was found to exist outside the cell wall of *L. lactis* (27). This pellicle also has the ability to disable phagocytosis and putatively assists in the separation of daughter cells at the septum. It should be underlined that differences in the polysaccharides of the cell wall and capsule are often at the basis of the different bacterial serotypes (5, 10).

2.6 - Other elements of the cell envelope

Although not extensively studied, pili and flagella are also found in LAB. Pili are filamentous structures that extend from the cell envelope and are central in adhesion of bacteria e.g., to host tissues (28–30). The presence of human-mucus binding proteins in the pili of *Lactobacillus rhamnosus* GG explains the enhanced interaction of this widely used probiotic strain with host tissue during colonization of the gut (31, 32). Pili have recently also been identified in *L. lactis* and shown to be involved in cell aggregation and biofilm formation (33).

LAB are generally regarded as non-motile but some lactobacilli are motile thanks to the presence of flagella (34, 35). Flagella are involved in both cellular motility and protein export, including secretion of virulence factors. They cross the entire cell envelope and, in Gram-positive bacteria, contain two basal body rings that act as mechanical bearings, one in the peptidoglycan and one in the cytoplasmic membrane (36).

3 - Membrane Proteins

Due to their key functional importance, and in consideration of the scope of this thesis, a particular focus will now be offered on membrane proteins. As mentioned above, it has been

estimated that 20 to 30% of all ORF's are predicted to encode membrane proteins (3, 37). Furthermore, the complexity of the biology of the cell is highly correlated to their existence since the fraction of membrane proteins encoded in a given genome tends to increase with the complexity of the organism (3). Their pivotal role in the biology of the cell in humans is additionally demonstrated through the estimate that membrane proteins are the direct targets of 60% of all drugs (38–40).

Despite their relevance, membrane proteins have historically been infrequently the subjects of *e.g.*, structural biology publications due to several technical constrictions. The first, and perhaps most limiting of these bottlenecks is the production and purification of appropriate quantities of membrane proteins in their native form. Mostly after the turn of the millennium, both a growing concern regarding this non proportional lack of knowledge and a sense of opportunities for significant discoveries triggered the start of many initiatives and international consortia working towards solving many bottlenecks affecting the study of these molecules (41). Since then, many improvements have been made regarding bioinformatics analysis, selection of protein targets, selection and optimization of expression systems, purification, crystallization, biochemical and/or structural analysis (41).

The bacterium *Escherichia coli* has commonly been regarded as the standard prokaryotic protein production host but, since membrane proteins differ with respect to structure, sugar decoration, lipid requirements and folding-factors, a broad set of hosts may need to be screened (17). As a general rule, membrane protein production is frequently higher in homologous production systems (*i.e.*, when the production host is the same as the organism from which the membrane protein gene originates) or in closely related hosts (17, 42). One ingenious way to circumvent this compatibility issue has been the development of cell-free expression systems (43).

The number of unique membrane protein structures has been rising exponentially and is now more than 470 (as of April 2014), which despite the above mentioned progresses still represents less than 1% of all deposited structures (44–46).

3.1 - General Functions, Structures and Topologies of Membrane Proteins

Membrane proteins can be broadly separated into two main types: the integral membrane proteins, which are permanently attached to the lipid bilayer, and the peripheral membrane proteins, which are only temporarily attached to membrane or a permanent element of the latter (47).

Integral membrane proteins that do not span the entire membrane, and are thus only attached to the membrane from one side, are monotopic. These proteins are generally enzymes. Integral membrane proteins that span the entire membrane at least once are called “integral polytopic proteins” or “transmembrane proteins”. The tertiary structure of the

proteins segments that span the lipid bilayer are based either on alpha-helical or beta-barrel structures. Membrane proteins with a low number of transmembrane segments are more common than those with a high number (3). Functionally, transmembrane proteins can be enzymes, receptors, cell adhesion molecules or transporters, such as channels/pores, electrochemical potential-driven transporters, primary active transporters (notably the ABC transporters), group translocators or transport electron carriers (48).

Peripheral membrane proteins can easily be dissociated from the membrane due to the weak nature of their attachment. They can be enzymes, electron carriers, regulatory proteins or subunits of integral membrane proteins such as transporters or receptors (47).

Additionally, pore-forming proteins/peptides are also a class of molecules with great affinity to the lipid bilayer. These usually soluble molecules produce ring-like structures in the membrane via alpha-helical or beta-sheet elements, thus yielding unregulated pores that can eventually be lethal to the target cell (49).

3.2 - Insertion of Membrane Proteins into the Cytoplasmic Membrane

In order to play their active role, proteins are not only required to be functional, they also must be where they are needed. This is particularly critical for membrane proteins since 1) the cytoplasmic membrane comprises only a minute fraction of the volume of the cell, 2) membrane proteins become irreversibly misfolded when they are not correctly accommodated in a lipid bilayer. Several strategies exist to insert membrane proteins into the cytoplasmic membrane. Some key components of these pathways are shared with protein secretion, which is not completely unsurprising since the ribosomal nascent peptide (or newly synthesized protein) of either a secreted or a membrane protein needs to first get to or cross the cytoplasmic membrane.

Six secretion systems (Type I – VI) have been identified in Gram-negative bacteria (50). Protein secretion in Gram-positive bacteria is simpler in the sense that the proteins need to cross only one lipid bilayer. In the latter organisms, most membrane and secreted proteins use the SecYEG translocon, a protein-conducting channel that is homologous to part of the Type-II and Type-IV systems in Gram-negative bacteria and to Sec61 in Eukaryotes. These membrane or secreted proteins are translocated over or inserted into the membrane, respectively, still in an unfolded state. Additionally, some Gram-positive bacteria possess the Twin-Arginine Translocation (TAT) pathway that enables folded proteins to cross the membrane (50). Also, an increasing number of examples of dedicated protein/peptide transporters have been uncovered, such as those for some toxins and bacteriocins (51).

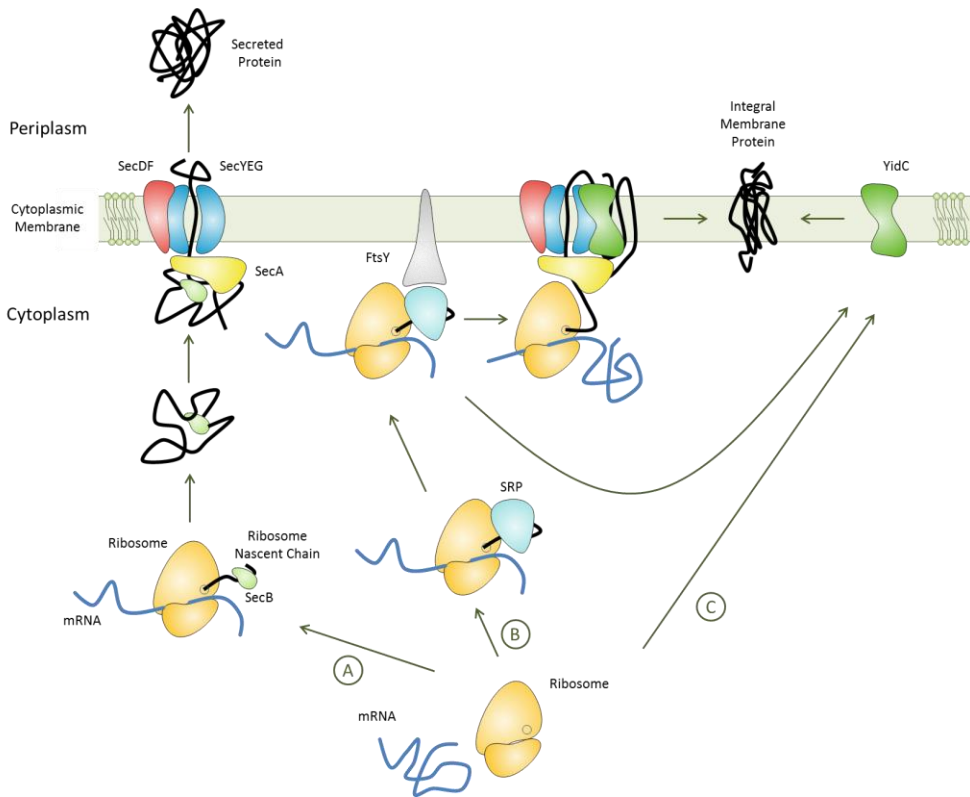


Figure 2 – Schematic overview of secretion of proteins and insertion of membrane proteins into the cytoplasmic membrane. Secretory pre-proteins are post-translationally targeted to the translocon SecYEG (blue) by SecB (light green), which keeps the pre-protein in a translocation competent state until the SecB-pre-protein complex reaches the translocon (A). Secretion is also dependent on SecA (yellow) to provide the motor force that pushes the pre-protein through the translocon. SecDF (red) is an auxiliary protein that can assist the translocation of secreted proteins and insertion of membrane proteins into the membrane. Once at the periplasmic side, the signal sequence of the pre-protein is cleaved off by a signal peptidase (not shown). Membrane proteins are inserted into the membrane mostly through the signal recognition particle (SRP; light blue) pathway (B). SRP binds to the ribosome nascent chain of the membrane protein and hampers translation until the complex reaches the translocon. This transfer is enabled by FtsY (grey), a membrane-anchored protein that captures the ribosome-mRNA-nascent chain-SRP complex close to the cytoplasmic membrane. During the insertion of membrane proteins, SecA is necessary for the translocation of large periplasmic loops. The lateral displacement of transmembrane segments, from SecYEG into the membrane, is facilitated by YidC (green). Interestingly, YidC can also insert some membrane proteins into the cytoplasmic membrane in a SecYEG-independent manner (C).

Despite the joint use of secreted and membrane proteins of the translocon SecYEG, quite some differences exist between the two pathways as a whole. Whereas proteins designed to be secreted are generally post-translationally targeted to SecYEG with the help of the chaperon SecB, membrane proteins are inserted into the membrane in a co-translational manner as the entire ribosome-mRNA-nascent-protein-chain complexes are targeted to the membrane by the signal recognition particle (SRP; a complex of a protein (Ffh) and an RNA molecule) and its receptor FtsY (50, 52, 53) (Figure 2).

Although secretion depends on SecA, only the translocation of large periplasmic loops seems to benefit from its action as a motor protein to drive those segments across SecYEG. It has been proposed that YidC mediates the lateral movement of transmembrane segments from SecYEG into the lipid bilayer. Also, YidC plays a role in the biogenesis of a subset of membrane proteins that are inserted into the lipid bilayer in a SecYEG-independent manner. Although not yet clear, SecDF is also thought to play a role in the biogenesis of membrane proteins (50, 52, 53) (Figure 2). Its function is nevertheless non-essential and some bacteria, such as *Lactococcus lactis*, do not have a homologue of these two proteins.

With respect to the topic of this research, if one wants to overproduce membrane proteins in *Lactococcus lactis*, or indeed in any other bacterial host, one has to take into account that overproduction of membrane proteins feeds back into and could interfere with proper functioning of the above-mentioned insertion pathways. In other words, it might thwart the proper functional insertion of (essential) homologous membrane proteins and might overwhelm protein quality control strategies, all of which could be detrimental to bacterial survival. Thus, bacteria have devised mechanisms to monitor the quality and integrity of their cytoplasmic membrane, or cell envelopes as a whole, and quickly respond to stresses that interfere with proper membrane/cell envelope functioning. This reinforces the idea that understanding cell envelope-specific stress responses is central to characterizing the bottlenecks affecting the production of membrane proteins. A review on cell envelope stress types and responses by LAB is offered below.

4 - Cell Envelope Stresses

LAB are faced in their diverse natural habitats and in the various industrial processes, in which they are employed, with many different sorts and sources of stressors. Several of these affect the integrity of the cell envelope and are presented in the following section. The responses that LAB developed to counteract these forms of aggression are presented in section 5 of this general introduction.

4.1 - Chemical Stresses

The cell wall is a major target of many antibiotics that either inhibit an enzymatic step of its biosynthesis or capture one of its precursors (54). Antibiotics are secondary metabolites that give their producers a competitive edge by inhibiting the growth of competitors. On the other hand, the evolution of these molecules has been paralleled in those targeted competitors by the development of stress responses and efficient resistance mechanisms.

Synthesis of the peptidoglycan represents a particularly critical point in this microbiological warfare. It is the target of several classes of antibiotics, such as the β -lactam penicillin and its analogues. This class of antibiotics disables the activity of a group of transpeptidases collectively known as penicillin-binding proteins (PBPs). The β -lactams do so by mimicking the terminus of the pentapeptide side chain of the peptidoglycan precursor and covalently binding to and blocking the active site of the PBPs. Thus, β -lactam antibiotics inhibit the peptidoglycan chains from being cross-linked (55, 56). Resistance to these antibiotics is generally not driven by a cellular response but is the result of selective pressure. In β -lactam resistant strains, the antibiotic-targeted PBP has often been replaced by a low-affinity derivative, through horizontal gene transfer, changing the relative expression profile of the ones already coded in the genome, or by a mutation of an existing PBP (55, 57). Alternative resistance mechanisms include producing β -lactamases that inactivate the antibiotic (58, 59) or extrusion of the antibiotic with efflux pumps (60).

Another important step in peptidoglycan biosynthesis concerns the transport of the pentapeptide disaccharide precursors across the cytoplasmic membrane. Undecaprenol, a lipid-soluble molecule, functions as a carrier molecule by binding to the peptidoglycan precursors synthesized in the cytoplasm, thus producing a molecule designated lipid II, and then translocating them to the periplasm. The physical properties of lipid II allow the molecule to move across the cytoplasmic membrane but the rate at which this happens *in vivo* suggests that the movement is enzyme catalysed (61). This process, labeled the lipid II-cycle due to the importance of the intermediate, is essential and the target of several antibiotics and vancomycin. For a more in-depth review on lipid II see for example (62).

In total 8 of the 14 conserved enzymatic steps in the biosynthesis of peptidoglycan have been shown to be targets of antibiotics (63) and, despite the appearance of ever more resistant strains, cell wall synthesis remains a primary target for the development of novel antibiotics (63, 64).

LAB exhibit antimicrobial activity as they produce weak organic acids. In addition, many species and strains synthesize bacteriocins (65). These antimicrobial peptides are classified into three major groups: Class I for the lantibiotics (small, heat-stable peptides containing lanthionine rings), Class II includes small heat-stable peptides (containing an N-terminal consensus sequence) and Class III for the large, heat-labile proteins. Class I and II

bacteriocins are pore-forming molecules that cause permeabilization of the membrane, while the ones from Class III are generally murein hydrolases (66).

Due to its greater exposure compared to other cellular regions or structures, the cell envelope is a prime target for many other biocides, although most do not exclusively harm that structure. Ortho-phthalaldehyde (OPA) and glutaraldehyde (GTA) are two non-oxidizing highly reactive di-aldehyde molecules that are used as disinfectants and primarily affect the cell surface by protein cross-linking (67). Sodium hypochlorite, commonly known as bleach, and other oxidizing agents can affect the cytoplasmic membrane and the cell wall to an extent as to induce loss of structure and function, and consequently cell lysis and death. Many organic compounds and detergents are able to denature proteins and destabilize the cytoplasmic membrane, leading to its disruption. For a comprehensive review on biocides and their cellular targets in bacteria, see for example (68).

As a result of their metabolism, but also while passing through the gastrointestinal tract, LAB have to withstand acidic environments and therefore have developed responses to cope with acid stress.

4.2 - Physical Stresses

LAB may face adverse situations that result from changes in external physical parameters. These invariably affect the cell envelope, the first line of defence of bacteria, although they are not commonly referred to as cell envelope stresses. Changes in temperature influence the fluidity of the cytoplasmic membrane. One main structural function of the cell envelope is to counteract the high internal osmotic pressure. LAB have response mechanisms to execute that function and maintain the essential balance between internal and external osmotic pressures when moisture and solute concentrations vary. Industrial applications of LAB usually force cells to endure mechanical and shear stresses, which result in damage inflicted on the cell envelope.

Applications of LAB such as *L. lactis* in the production of recombinant secreted or integral membrane proteins (18, 19) partially sequesters the translocation/secretion machinery and may lead to physical crowding of proteins in the cytoplasmic membrane (69, 70).

5 - Cell Envelope-Stress Responses

Bacteria have developed mechanisms to directly monitor agents and parameters of stress, or indirectly by assessing the integrity of the cell envelope. Inputs for the response that lay downstream of the aggression, such as common indirect effects, allow producing broad responses, enabling the cells to resist a wide range of stresses. Nevertheless, these non-specific responses may not always result in an increased resistance to specific sources of

stress. Many forms of resistance, notably to antibiotics, are not a product of a real-time cellular response strategy but rather an adaptation that results from natural or artificial selection (71, 72). Also, a clear distinction should be made between an immediate response and a non-permanent (mutation-independent) cellular adaptation to a different situation. For example, *L. lactis* cells, when treated with increasing amounts of the bacteriocin nisin, can become 75 times more resistant to the antimicrobial peptide than the unadapted strain (73). This adaptation is not permanent and cells regain nisin sensitivity once the pressure is removed. Although the underlying response mechanisms are not known, DNA microarray data indicates that this nisin resistance results from a broad rearrangement of gene expression modulating cell wall thickness/density (*galE* and *pbp2A*) and charge (*dltD*), acidity outside the membrane (*arcAC1C2DT2*), membrane fluidity (*fabDG1G2Z1Z2*) and, putatively, the capacity to export nisin from the cells (73).

Responses to cell envelope stress can be divided into two main categories: those mediated by two-component systems (TCS) (74) and those mediated by extracytoplasmic function (ECF) sigma factors (75). The sensor-regulator devices of these two classes are analogous in their architecture and consist of two proteins: a membrane-anchored sensor (the histidine kinase or anti-sigma factor) that becomes active only under stress conditions and activates a cytoplasmic transcriptional regulator (the response regulator or ECF). Contrary to ECF sigma factors, TCSs are widespread and conserved in LAB (74). They are best classified according to the genes they regulate, i.e. whether they aim to generally maintain the integrity of the cell envelope or activate specific detoxification modules. Another type of stress response relies on one-component systems. They are putatively widely distributed in prokaryotes but have not been extensively studied. All these classes of stress responses and their presence in LAB are described below.

5.1 - Extracytoplasmic Function Sigma Factors

ECF sigma factors are members of the sigma-70 family of proteins, which recognize the conserved “AAC” motif in the -35 region of a promoter. Their genes are usually co-transcribed with that of their cognate anti-sigma factor (75). Most bacilli have many ECF sigma factors but they seem absent in most LAB (76). To the best of our knowledge, only one ECF (SigV from *Enterococcus faecalis*) has been characterized and shown to play a role in the cell envelope stress response of an LAB. SigV, which had been previously associated with a response to heat, acid and ethanol stresses (77), also plays a central role in lysozyme resistance and virulence of this bacterium (78). This unusual resistance of *E. faecalis* seems to be specific to lysozyme and does not protect against other antimicrobials such as nisin. The mechanism of resistance is still not known but is independent of OatA (an O-acetyl transferase) and DltA (involved in the D-alanylation of lipoteichoic acids), two proteins that also contribute to the resistance of this bacterium to lysozyme. This mechanism of resistance

increases the virulence of *E. faecalis* since a *sigV* mutant displays a reduced potential to colonize host tissues (78).

Additionally, *lmg2447*, which encodes a putative extracytoplasmic-function anti-sigma factor in *L. lactis*, has been identified as a resistance factor to the cell wall-active bacteriocin Lcn972. However, the mechanism of this resistance is poorly understood since its putative cognate sigma factor, the ECF sigX, is non-functional in *L. lactis* (79).

5.2 - Two-Component Systems that Maintain the Integrity of the Cell Envelope

Like ECF sigma factors, some of the TCSs respond to a wide range of cell envelope stresses and activate the expression of genes that maintain general aspects of cell envelope integrity. They are frequently associated with other (general) stress responses, growth and competence development, supporting the idea that they have a more general role in maintaining the general homeostasis of the cell.

The TCS LiaRS is present in all LAB except the lactobacilli (76). It invariably responds to perturbations in the integrity of the cell envelope but the genes it regulates vary greatly among different species, perhaps representing a divergent evolution that resulted from different sources of and different susceptibilities to ecological aggressions. The *liaRS* genes are usually preceded by *liaF*, which encodes a strong inhibitor of LiaR (80).

In *Bacillus subtilis*, where it was originally characterized, LiaRS is strongly induced by vancomycin and bacitracin but only the promoters of *liaI* and *yhcY* were found to be regulated by LiaR (80). LiaRS-dependent gene expression is repressed in growing cells by the transition state regulator AbrB. The master regulator of sporulation, Spo0A, alleviates repression as cells enter the transition phase of growth (81). In bacilli, *liaFRS* is always part of a larger operon, either *liaIHGFRS* (e.g., in *B. subtilis*) or *liaHFRS* (e.g., in *B. cereus*). Interestingly, deletion of none of the *lia* genes modifies the sensitivity to the known inducers of LiaRS in *B. subtilis* (76).

In *Staphylococcus aureus*, *VraSR* (another widely studied homologue of LiaRS) is strongly induced by a wide range of cell wall antibiotics; a *vraSR* deletion strain shows increased susceptibility to these inducers (82, 83).

In LAB, LiaRS-like TCS-mediated responses have been characterized in *L. lactis* (84), *Streptococcus mutans* (85), *Streptococcus pneumoniae* (86) and *E. faecalis* (87). In all of these organisms only *liaFRS* are conserved, and not the other genes of the related but more extended operons in bacilli. The LiaRS regulon is larger in LAB than in *B. subtilis*, indicating a different and more central role of LiaRS in the response against cell envelope stresses (76). In all cases, LiaRS-like TCSs are positively auto-regulated and respond to cell wall antibiotics that interfere with the lipid II-cycle.

In *L. lactis*, the LiaRS-like TCS CesSR affects sensitivity to salts and osmotic pressure (88) and is induced by vancomycin, bacitracin and the two LAB bacteriocins Lcn972 and plantaricin C (84). Disruption of CesR only results in an increased susceptibility to those inducers that interfere with the lipid II-cycle: bacitracin and plantaricin C. The *L. lactis* $\Delta cesR$ mutant is also more sensitive to nisin, despite the inability of this antimicrobial peptide to induce CesSR (84). Interestingly, a nisin-adapted/resistant *L. lactis* strain, obtained by growing cells in the presence of increasing amounts of the antimicrobial, overexpresses CesSR and its regulon (73). This strain is 75-fold more resistant to nisin and is also three times more resistant to Lcn972, although a similar decreased susceptibility to Lcn972 was also found in the *cesR* deletion strain (84). Most CesSR-regulated genes encode putative membrane proteins, supporting the idea that the response is specific to stresses that operate at the level of the cellular envelope (84).

Notably, *lmrA* and *rmaB*, coding a multidrug ABC transporter and a transcriptional regulator of the MarR family, respectively, are members of the CesSR regulon. Llmg2163 (a putative transcriptional regulator as it contains a PspC domain) (89), Llmg2194 and Llmg0165 have protective roles in *L. lactis* even against unrelated and more general perturbations like temperature, pH and salt stresses. Overexpression of *llmg2163* and *llmg2164* in *L. lactis* increased resistance of the organism to Lcn972 (90). One of the most prominent targets of CesSR is SpxB, a protein that, by binding to RNA polymerase, induces the expression of *oatA* (91). The activity of the encoded peptidoglycan *O*-acetylase OatA influences the level of peptidoglycan acetylation and therefore the sensitivity to peptidoglycan hydrolysis. The CesSR-dependent expression of *spxB* thus efficiently responds to cell envelope stress evoked by treating *L. lactis* cells with hen egg white lysozyme (91). CesSR was also induced when *L. lactis* was forced to overproduce recombinant membrane proteins (69, 70) (this thesis, chapters 2 and 3). The response may result from an overcrowding of the cytoplasmic membrane with membrane proteins or may simply be caused by an overuse of the translocation/secretion machinery, as the same CesSR response was observed during the production the secreted protein AmyQ (data not published). This response might mimic normal periods in growth when production of endogenous membrane proteins is high since CesSR also regulates members of the translocation machinery, such as FtsH and OxaA2. Disrupting *cesSR* hampers growth of *L. lactis* when cells are induced to produce membrane proteins. On the other hand, co-overproduction of CesSR resulted in an improvement of up to over 3-fold in the production yield of membrane proteins (70).

In *S. pneumoniae*, LiaFSR is activated by vancomycin (92), murein hydrolases and lipid II-interacting antibiotics (86). In this organism, fratricide (by which competent cells lyse non-competent sister cells) was shown to be assisted by the secretion of cell wall hydrolases such as CbpD, LytA, and LytC (93). Lysis of competent cells is partially prevented through a response coordinated by LiaFSR, after sensing the damage inflicted to the cell wall. Among

the genes that LiaR regulates, PcpC (Spr0351) and Spr0810 are particularly important in preventing damage from lysis (86).

In *S. mutans*, LiaS regulates the levels of GbpC, a cell surface-associated protein that facilitates biofilm formation in environments such as the human oral cavity (94). Additionally, LiaSR was shown to regulate 174 genes, some of which are involved in membrane protein synthesis and peptidoglycan biosynthesis (95). Lipid II cycle-interfering antibiotics and other chemicals that disrupt the integrity of the cytoplasmic membrane activate *S. mutans* LiaSR and a disruption of *liaSR* makes the organism more susceptible to those compounds (85).

In *E. faecalis*, inactivation of the LiaR homologue RR03 (EF2911) increased the susceptibility of this organism to bacitracin (87). Another TCS from *E. faecalis*, CroRS (for “ceftriaxone resistance”; or RR05-HK05) is required for β -lactam resistance and its disruption causes a 4000-fold increase in the sensitivity to the cephalosporin ceftriaxone (96). *croRS* is induced by narrow and broad-spectrum cephalosporins, imipenem, ampicillin, oxacillin, amdinocillin and inhibitors of peptidoglycan synthesis such as phosphomycin, D-cycloserine, vancomycin, moenomycin, ramoplanin, and bacitracin (96). CroRS regulates *glnQHMP* (97) and *salB* (previously *sagA*), which encodes a putative cell wall-attached protein that is important in cell division and resistance to multiple stresses (98, 99).

The CiaRH TCS of *S. pneumoniae*, initially identified in a screen for cefotaxime resistant mutants (100), is activated by vancomycin and penicillin (92, 101) and is involved in virulence (102), competence and resistance to lysis (103). Expression of the *dlt* operon and *htrA* is controlled by *S. pneumoniae* CiaRH (104, 105). In *L. lactis*, expression of *htrA* is triggered during the overproduction and secretion of recombinant proteins (69, 70) (this thesis, chapters 2 and 3) while in *S. pneumoniae*, HtrA affects the activity of pneumocin MN, a two-peptide bacteriocin (106). In *S. mutans*, inactivation of *ciaH* diminishes production of mutacin and affects processes such as competence development and biofilm formation (107).

5.3 - Two-Component Systems that Activate Specific Detoxification Modules

Some TCSs regulate the transcription of genes that directly confer resistance to the antimicrobial that activates the TCS. These TCSs and the resistance element are usually encoded by neighbouring genes and are typically found in contexts of high genetic mobility like plasmids or transposable elements (108).

BceRS, a non-autoregulated TCS, specifically responds to bacitracin. In *B. subtilis*, BceS is not sufficient and requires BceAB, the corresponding bacitracin ABC transporter, to sense bacitracin itself (109). MbrABCD from *S. mutans*, a BceRSAB-like system, is also responsible

for bacitracin resistance and mutations in any of the respective genes leads to an increased susceptibility to bacitracin of over 100 times (110).

VanRS, a TCS that is found in certain vancomycin-resistant enterococci such as some *E. faecalis* and *Enterococcus faecium* isolates, senses the antibiotic and triggers expression of genes conferring resistance to vancomycin and, depending on the genes it regulates, teicoplanin (111). Invariably, VanR activates transcription of *vanHAX*, which encode enzymes that reprogram the synthesis of the cell wall by changing the terminal amino acid residues of the peptidoglycan precursors from D-alanyl-D-alanine to D-alanyl-D-lactate or D-alanyl-D-serine (112, 113).

5.4 - One-component systems

Despite being less-well characterized than TCSs, signal transduction systems consisting of only a single protein containing both a sensory and a DNA binding domain are now thought to be more widespread in bacteria than TCSs (114). Their involvement in the response to cell envelope stresses starts to be revealed. An example is found in *E. faecalis* in which a unique form of acquired bacitracin resistance is mediated by BcrAB, an ABC transporter that putatively exports bacitracin (115, 116). The expression of the *bcrABD* operon is regulated by BcrR, a membrane-bound bacitracin sensor and DNA-binding protein (115, 117). This sensor/regulator seems to be active and induce transcription of *bcrABD* only in the presence of bacitracin (115).

Other one-component systems, characterized by signalling proteins with a eukaryotic-type Ser/Thr kinase domain, have also been implicated in the resistance to cell envelope stresses. PrkC modulates antimicrobial resistance in *E. faecalis* (118), PknB modulates biofilm formation and resistance towards the envelope stress caused by H₂O₂ in *S. mutans* (119), while StkP of *S. pneumonia* is important for the resistance to various stresses and controls the transcription of, among others, genes that encode proteins involved in cell wall metabolism (120, 121).

6 - Scope of this Thesis

The work presented in this thesis aimed at helping establish *Lactococcus lactis* as standard option for the production of membrane proteins. Towards that end, several strategies were employed.

Chapter 2 presents the characterization at the transcriptome and proteome levels of the response that occurs when the production of membrane proteins is induced in *L. lactis*. Data is presented for both an inactive endogenous protein, whose activity had been disabled through genetic engineering, and two eukaryote membrane proteins.

Chapter 3 continues the characterization of the response to overproduction of yet another membrane protein in *L. lactis* in order to further validate the response. The knowhow obtained from the work described in both chapters was used to construct *L. lactis* strains that displayed a significantly improved capability to produce membrane proteins.

One key feature in the response of *L. lactis* to the overproduction of membrane proteins is the upregulation of the CesSR regulon. In **Chapter 4** a further characterization of this regulon is presented.

Understanding which aspects of the response are directly associated with the overproduction of membrane proteins and which are merely indirect consequences, for example of an impaired growth rate, it is crucial to extract information that helps engineering *L. lactis* to that end. In **Chapter 5** a study on the transcriptome, in time throughout the growth curve, is presented. The information thus collected can serve, among others, as a reference to filter the direct response from those that are growth-rate related.

Chapter 6 describes several contributions to the genetic toolkit of *L. lactis*. The first part of this chapter refers to the construction and use of tools that facilitate genetic engineering of *L. lactis* and help to further developing this lactic acid bacterium, *e.g.* as a host for the production of (heterologous) membrane proteins.

Most of the work described in this thesis focuses on the comprehension of the response that the overproduction of membrane proteins elicits in *L. lactis*, as a way to determine putative yield bottlenecks associated with it. Notwithstanding this, other strategies could also have been employed. Particularly, rational design of strains or random mutagenesis with selection for improved producers could also be applied. The second half of **Chapter 6** describes the implementation of Genome Array Footprinting (GAF) in *L. lactis*. Such a methodology can be used to identify, for example, overrepresented mutants in a population that display a differential feature, such as a better-than-average ability to produce membrane proteins.

Chapter 7 integrates all the above results in a general discussion. An evaluation on the impact of these research strategies and proposals for future work are also presented.

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