CHAPTER I

Introduction

INTRODUCTION

1. General introduction

Lactic acid bacteria (LAB) are Gram-positive bacteria that play an important role in many food and feed fermentations. Since many centuries LAB have served to provide an effective form of natural preservation. In addition, they strongly determine the flavour, texture and, frequently, the nutritional value of food and feed products. The eminent economic importance of these micro-organisms has led to an increased interest from both the industry and the scientific world and to an enormous boost in the fields of fundamental as well as applied LAB research. During the last decade major advances have been made in the unravelling of the genetic and molecular basis of several important properties of LAB. The potential of LAB to preserve foods and feeds has been shown to be due to their ability to produce various substances with antimicrobial activity. Examples of such substances are organic acids, diacetyl, hydrogen peroxide, reuterin (ß-hydroxypropionaldehyde), and bacteriocins (116). The latter compounds, have been defined by Tagg *et al.* in 1976 (118), as proteinaceous compounds that are bactericidal to strains closely related to the producer strain. Genetic and biochemical knowledge gained during the last few years has made it clear that this definition of bacteriocins applies to most bacteriocins. However, a number of exceptions exists; some bacteriocins are active against species more distantly related to the bacteriocin producer.

Biochemical and genetic studies allow to divide the bacteriocins of LAB into three distinct classes (75,95):

(I) Lantibiotics, small membrane-active peptides (<5 kDa) containing the unusual amino acids lanthionine, ß-methyl lanthionine and dehydrated residues.

(II) Small heat-stable, non lanthionine-containing, membrane active peptides (<10 kDa) characterized by a Gly-Gly $^{-1}\blacktriangledown^{+1}$ Xaa processing site in the precursor. The mature bacteriocins are predicted to form (amphiphatic) helices with varying amounts of hydrophobicity. They are moderately (100 °C) to highly (121 °C) heat stable.

(III) Large heat-labile proteins (>30 kDa).

The bactericidal properties of LAB bacteriocins in combination with the fact that many bacteriocin producing LAB are present in a variety of naturally fermented food and feed products, have led to a great interest in the potential of these bacteria as “biopreservatives” that could, at least partially, replace chemical preservatives (116). Due to the different inhibitory spectra of bacteriocins from LAB, they may find applications in food preservation in two different ways: they could either be used to inhibit the growth of specific undesired bacteria closely related to the bacteriocin producer (narrow spectrum bacteriocins) or they could be applied to inhibit a whole range of spoilage and pathogenic bacteria (wide spectrum

bacteriocins). Bacteriocins of LAB have already been tested for their biopreservation potential in food systems and the results of these studies seem quite promising (for an overview see reference 116). However, the only (LAB) bacteriocin that is licensed to date for use as a food additive (in over 45 countries) is nisin (20).

To be able to exploit the full potential of bacteriocins in food preservation, fundamental knowledge is needed not only about their inhibitory spectra, but also concerning their mode of action, their secretion and the immunity against these molecules.

Lactococcin A (LcnA) is a Class II bacteriocin produced by *Lactococcus lactis*. During the last several years its mode of action and immunity have been studied (see below). In this thesis, the secretion and maturation machinery of LcnA was investigated. In the following, a general overview will be given of protein secretion in prokaryotes and of methods to determine the topology of membrane proteins. The lactococcins A, B and M/N will be discussed in more detail (for additional literature on bacteriocins of LAB, the reader is referred to the following reviews: 19,66,76,95,118).

1.1. Protein secretion in prokaryotes

It has been estimated that approximately 20 % of the polypeptides synthesized by bacteria are located partially or completely outside of the cytoplasm (101). The systems for the secretion of these proteins of prokaryotes can be divided into three main classes (107):

1) The Type I secretion system. In this system the substrate is secreted directly from the cytoplasm to the environment by a specialized machinery consisting of two cytoplasmic membrane proteins and, in Gram-negative bacteria, an additional outer membrane protein. One of the two cytoplasmic membrane proteins of this secretion system belongs to the family of ABC transporters (55), while the other is referred to as an accessory protein. A subfamily of these accessory proteins is the recently defined MFP family (see below; 25).
2) Type II secretion systems: the *sec*-dependent, general secretion pathway (GSP; 101). The secretion of proteins by this pathway is believed to be a two step process. In the first step, the proteins are translocated across the cytoplasmic membrane by general (Sec) proteins that form the general export pathway (GEP) (reviewed in reference 21). In Gram-negative bacteria the proteins then have to pass the outer membrane in a second step. The secretory proteins in the outer membrane involved in this step may vary depending on the protein substrate (see below).
3) Type III secretion systems are also referred to as contact-mediated secretion systems. They involve a whole set of proteins that are distinct from those of the other two types of secretory pathways (see below; 37,111).

There is a substantial difference between protein secretion in Gram-positive and Gram-negative bacteria: the latter have an additional outer membrane that has to be traversed before a protein is released to the outside of the cell.

1.1.1. Type I secretion systems

This system, which is *sec*-independent, is comprised of a member of the ABC (ATP-binding cassette) transporter family (reviewed in references 2,10,32,52,53), an accessory membrane protein and, in Gram-negative bacteria, an outer membrane protein. The gene encoding the accessory protein is always linked to the gene encoding the ABC protein, while that encoding the outer membrane factor can be either closely linked to, or be physically quite distant from, the other two genes (43,82,135). The Type I secretion systems are specific for a particular protein or a family of closely related proteins and, hence, are often called "dedicated" transport systems (44,133).

ABC transporters of both prokaryotic and eukaryotic organisms are involved in the import or export of a great variety of substrates such as inorganic ions, sugars, amino acids, peptides and proteins (52). A typical ABC transporter consists of a hydrophobic integral membrane domain and two ATP-binding domains. These can be either present on separate polypeptides or fused into a multidomain protein. The hydrophobic integral membrane domain of the majority of ABC transporter proteins is predicted to consist of "two times six" transmembrane segments (TMSs; 52). This has been experimentally confirmed for several examples (e.g. 18,84,99,137). There are, however, examples of ABC transporters that seem to deviate from this "two times six" TMS motif (13,40,48,56,70,72,141, this thesis Chapter IV).

The main similarity between the accessory proteins is that they all are predicted to have one transmembrane segment in their N-terminus and that the (major) C-terminal part of the protein is facing the extracellular side of the cytoplasmic membrane. This membrane topology has been experimentally confirmed in several cases (108,114, this thesis Chapter II). The function of the accessory proteins in Gram-positive bacteria is unknown. It is hypothesized that these proteins of Gram-negative bacteria may connect the inner and outer membranes to facilitate the passage of substrates and, hence, the name **M**embrane **F**usion **P**rotein (MFP) has been postulated (25). It has indeed been shown that CvaA, a member of this MFP family involved in the secretion of the bacteriocin colicin V from *Escherichia coli*, interacts with both CvaB, the ABC transporter in the cytoplasmic membrane and TolC, a protein present in the outer membrane (64). A schematic presentation of Type I secretion systems is shown in Fig. 1.

The mechanism of Type I secretion is still unclear. The best characterized of these systems are those for haemolysin (HlyA) and colicin V (ColV) from *E. coli*. No periplasmic intermediates of substrates secreted by Type I systems could be identified by biochemical means (77), implying that secretion takes place directly from the cytoplasm to the extracellular environment. Interestingly, by using a very sensitive (*in vivo*) activity assay, Zhang, *et al.* (140) were able to show that ColV can access the periplasmic space and is subsequently capable of inserting into the cytoplasmic membrane. However, biochemical methods or overlayer activity assays failed to detect this ColV in the periplasmic space.

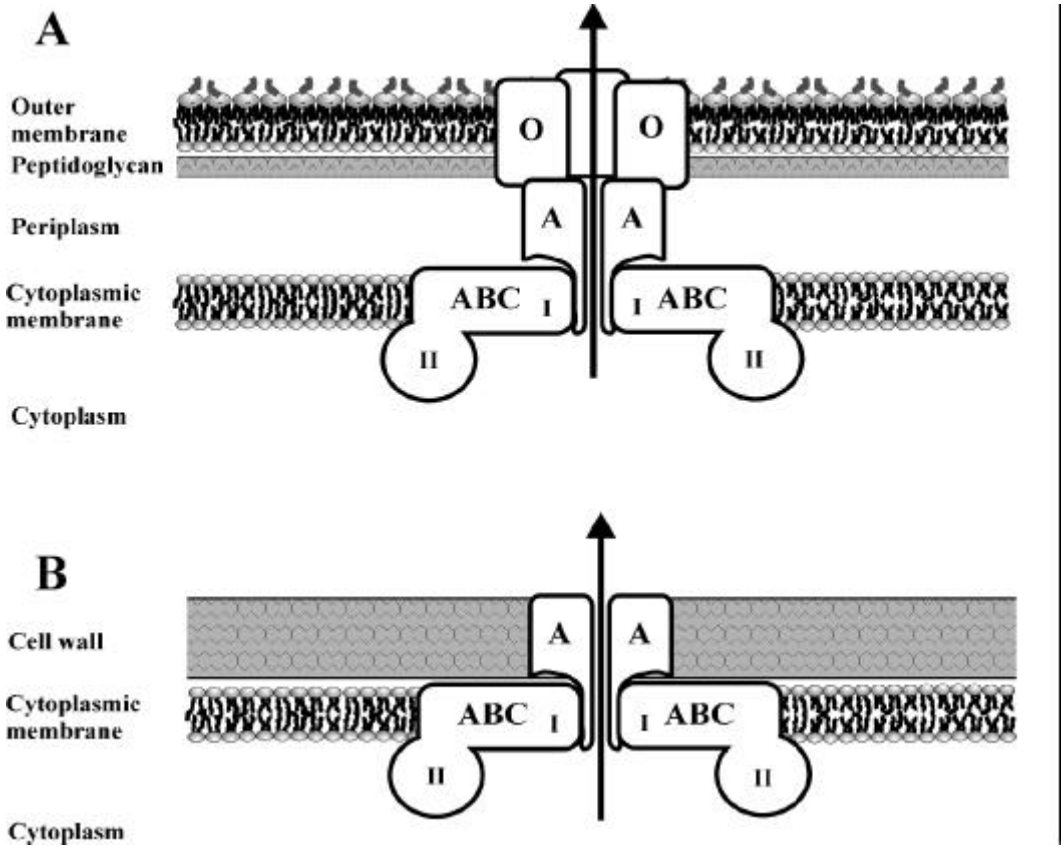

Fig. 1. Schematic representation of a typical Type I protein secretion system in Gram-negative (A) and Gram-positive (B) bacteria. The membrane protein belonging to the ABC transporter family (ABC) consists of two domains: a hydrophobic N-terminal domain (I) that is in most cases predicted to span the cytoplasmic membrane with (two times) six TMSs, and a C-terminal domain (II) containing the ATP-binding motifs. The accessory protein (A) is believed to interact with this ABC transporter protein and an outer membrane protein (O) in the Gram-negative system, thus, forming a translocation channel through both membranes by which the substrate can be directly transported from the cell interior to the extracellular environment. The function of the accessory protein in Gram-positive bacteria is as yet unknown. Based on experimental data and homologies between several ABC transporters, it is believed that two ATP-binding domains are required in each transporter complex. The integral membrane part (I) of the ABC transporter protein and the nucleotide binding moiety (II) can either be contained in one protein or can be synthesized as two separate proteins. Also, examples of ABC transporter proteins are known in which both domain I and domain II are twice represented in one single protein.

HlyA was shown to be localized in the cytoplasm, but it is also present in the cytoplasmic membrane in the absence of its dedicated Type I secretion system. This observation suggests that HlyA could interact with its secretion machinery from the lipid phase. In current models for ABC exporters, involved in multidrug resistance, substrates are, indeed, thought to interact with the substrate binding site of the ABC transporter protein from the lipid phase (reviewed in

reference 12). These models are known as the “hydrophobic vacuum cleaner model” (11) and the “flippase model” (54).
Bacterial ABC transporters have been extensively reviewed by Fath and Kolter (32), while Binet *et al*. specifically focused on those from Gram-negative origin (9).

1.1.2. Type II secretion systems

Most secreted proteins reach their final destination by Type II secretion systems. These systems are capable of secreting a large variety of proteins, which are synthesized as pre(pro)polypeptides. After translation the (mature part of the) protein is bound by general chaperones (e.g. PI, GroEL, DnaK) or by specific chaperones, the (export) targeting factors (SecB, signal recognition particle) in order to keep the protein in an export-competent state and to target it to the translocation channel formed by the (cytoplasmic) membrane proteins SecY, SecD, SecE, SecF, SecG and YajC (reviewed in reference 28). The peripheral membrane protein SecA is able to bind to this latter integral translocation complex and has affinity for both signal peptides and SecB (17,50,83). In addition, SecA has been shown to have affinity for the mature parts of preproteins (83). The driving force for translocation is provided by SecA and the proton motive force (PMF), once protein translocation is initiated by SecA. The motor function of SecA depends on its ability to bind and hydrolyze ATP, which causes major conformational changes in this protein (22,125). Recently, models have been proposed in which SecA drives protein secretion through cycles of preprotein binding, membrane insertion, preprotein release and deinsertion from the membrane (29,30).

During translocation the signal peptide is removed by a specific protease, the signal peptidase. The exported proteins often remain membrane associated after translocation until their folding is complete. Folding is assisted by specific factors like those involved in disulfide bond formation and isomerization (e.g. PDI, DsbA), peptidylprolyl-*cis-trans*-isomerases (rotamases), and specific molecular chaperones (e.g. PapD) or endochaperones that are involved in post-translocational proteolytic processing (101,112).

Protein secretion via the Type II system requires a second step in Gram-negative bacteria: the translocation across the outer membrane. This translocation step is highly specific and involves, for each secreted (group of) protein(s) produced by a particular bacterium, a secretion machinery composed of up to 13 or 14 specific proteins (101).
The Type II secretion system has been reviewed in great detail by Pugsley (101), Wandersman (134), Duong *et al.* (28) and, specifically for *Bacillus*, by Simonen and Palva (112).

1.1.3. Type III secretion systems

Since the discovery of the first Type III secretion system in 1993, other such systems have been identified in several plant and animal bacterial pathogens (for review see reference 37). Examples of such pathogens (and their in virulence involved proteins secreted by Type III systems) are *Yersinia* spp (*Yersinia* outer proteins, Yops), virulent *Shigella flexneri* (invasion

protein antigens, Ipas), *Salmonella typhimurium* (Sips), *Salmonella typhimurium* (InvJ), enteropathogenic *E. coli* (EspB), *Erwinia amylovora* (Hrps), *Pseudomonas syringae* (Hrps), *Pseudomonas solanacearum* (PopA1) and *Xanthomonas spp* (HrpX).

Type III secretion systems are characterized by (a) the absence of a typical, cleavable *sec*-dependent signal sequence in secreted substrates, (b) the requirement of accessory proteins for secretion, (c) the export of proteins through both the inner and outer bacterial membranes and (d) the requirement of activating signals to initiate secretion (78). Although most of the putative components of this system have been identified, little is known about their function or organization in the bacterial envelope.

The best characterized Type III secretion system to date is that operative in pathogenic *Yersinia* spp. (also called Ysc type III secretion system, reviewed in reference 111). The secreted Yop proteins are not made as precursors and they can be classified in three functional groups. First, the effector Yops (e.g. YopE, YopH) are the proteins actually injected into the eukaryotic cell, leading to cell death. The second group consists of YopB and YopD, proteins required for the efficient translocation of YopE and YopH into the mammalian cell. The last function is fulfilled by YopN. It is believed to function like a “cork” that closes the Ysc secretion apparatus in the cytoplasmic membrane of the pathogen. Twelve proteins that are secreted by the Type III pathway have been identified thus far in *Yersinia* spp. (reviewed in reference 37). Cytoplasmic chaperones (different from those of the Type II secretion pathway) play an important role in the secretion of the Yop proteins. For some of these chaperones it has been shown that they prevented the degradation of the proteins destined for secretion (36,91). In addition, it has been proposed that the chaperones maintain the secreted proteins in a secretion-competent conformation (106,136).

The IpaB protein of *S. flexneri* was secreted by *Y. pseudotuberculosis* under the same conditions required for Yop secretion. Also, YopE could be secreted by *S. typhimurium* (106). These observations suggest that, although some of the individual components of the secretion systems are not interchangeable, the molecular principles governing secretion are similar.

The Type III system seems to be less common than the other two types of secretion systems and, to our knowledge, so far no examples have been described in Gram-positive bacteria.

1.2. Secretion signals

The best studied secretion system is the general secretory pathway (reviewed in reference 101). The N-terminus of the precursor of the protein secreted through this pathway

is composed of a stretch of 15 to 30 amino acids that is known as the signal peptide. Signal peptides share the following common features (131): they have a long hydrophobic region (H domain) that is usually preceded by one or more positively charged residues in a short, generally hydrophilic region (the N-domain) and followed by a typical processing site. No two signal peptides from different presecretory proteins have exactly the same sequence. In general, the typical signal peptides of proteins of Gram-positive bacteria are longer than those of proteins from Gram-negative species. The signal in proteins that are to traverse the outer membrane of Gram-negative bacteria is not yet fully defined.

The secretion signal of several proteins secreted via the Type I secretion system has been located in the C-terminus (59,61,71,109,115). These proteins are not processed during secretion. However, several bacteriocins utilizing a Type I secretion system (see section 2.4), have been shown to contain a secretion signal in the N-terminus of the prebacteriocin (33,42,124). This N-terminal part (or leader) of the prebacteriocin is removed during secretion. Besides the leader, it seems that also the bacteriocin molecule itself contains structural information important for (efficient) secretion (42, this thesis Chapter V).

The secretion signal of the YOP products that use the Type III secretion pathway is encoded in the very beginning of each of the *yop* genes (16). The amino acid sequences at the N-termini of the 12 known Yop proteins do not share any common features. Detailed analysis of Yop protein secretion indicates that, most probably, the secretion signal is not contained in the Yop amino acid sequence itself, but rather in the Yop messenger RNA (4). The specific signal for Yop toxin recognition by the YopB/D complex, that is believed to form a translocation channel in the eukaryotic membrane, is not known.

1.3. Membrane protein topology

To better understand the mechanisms of transport of various substrates across the cytoplasmic membrane and to predict interactions between substrates and/or the components of the secretion machineries, the topology of various membrane proteins involved in transport processes has been studied. The most refined method is the determination of the three-dimensional structure of the protein of interest by crystallography and X-ray diffraction. This method is highly dependent on the proteins of interest to form proper crystals. Unfortunately, most membrane proteins are difficult to purify in their native states and to crystallize. Another problem is that, *in vivo,* membrane proteins adopt their proper topology in the membrane, implying that the crystallization should also take place in the presence of membranes (or a membrane-mimicking hydrophobic environment). Alternatively, if crystallization is done in an aqueous environment, it is necessary to determine whether the topology is indeed the same as that of the protein *in vivo.*

Computer programs have been developed that predict the two-dimensional topology of membrane proteins based on the hydrophobic character of parts of the protein and, for several of these programs, the distribution of positive charges over the protein (according to the "positive inside" rule; 132). Although these programs have become more and more refined, experimental evidence is still required to verify the predicted models (see Chapter IV of this thesis). To this purpose, various biochemical and genetic methods have been developed that will be discussed now.

1.3.1. The use of antibodies

This method has the advantage that the protein of interest can be investigated in its native form in its natural environment. If it is known which epitope is recognized by a specific antibody, the localization of the epitope in the protein of interest can be determined by examining the accessibility of the epitope to the antibody. To this end, right side out (r.s.o.) and inside out (i.s.o.) vesicles prepared from cells expressing the protein of interest can be used (128). Alternatively, the accessibility of the epitope to the antibody can be compared between permeabilized and intact cells.

The disadvantage of this approach, especially in the case of multitopic membrane proteins, is that it requires a whole set of specific antibodies, making the method very laborious and time-consuming. A genetic method to tackle these problems is to introduce foreign epitopes, specifically recognized by existing antibodies, at certain positions in the protein of interest (67). In certain cases, the epitopes may no longer be recognized by the antibodies after their insertion in the protein of interest. This may be due to shielding of the epitope by local structures in the protein or by other proteins or lipids that may interact under native conditions with the protein under study (90). Moreover, not all insertion sites are permissive if examination of the protein in its "native" form (as judged from the preservation of biological activity) is desired (90). As the epitope insertion procedure depends upon the availability of convenient restriction enzyme sites or two-step PCR strategies, it is not suited for a random approach to study membrane protein topology. It has been used mainly for eukaryotic proteins (3) or outer membrane proteins (96; see also this reference for an extensive overview of the advantages and limitations of the method).

1.3.2. Protease accessibility studies

This approach which is based on the accessibility of certain amino acid stretches within a protein of interest, makes use of proteases. The proteases are either specific, like trypsin, or less specific, such as proteinase K (72). The method is simple, but its resolution is rather low when non-specific proteases are used or when the precise recognition site of the protease in the protein under study is unknown, as it is often based on the estimation of sizes of breakdown products after protease treatment. Moreover, all potential cleavage sites are not necessarily

cleaved *in vivo*. Like for the failure to detect, in certain cases, epitopes in a protein of interest, this may be due to steric hinderance (70).

A genetic method that has been developed to use protease accessibility in a more refined way, introduces specific protease cleavage sites in the protein of interest (81,104).

1.3.3. Reporter proteins

Another genetic approach to obtain information concerning the topology of a membrane protein is the construction of fusions of the protein of interest to a reporter protein of which the enzymatic activity depends on its localization in the cell (87). These fusion proteins are generated at the genetic level by constructing hybrids of the respective genes. Examples of reporter proteins used for topological studies are ß-galactosidase (LacZ), alkaline phosphatase (PhoA), ß-lactamase (ßla), maltose binding protein (MBP), all derived from *E. coli*, and histidinol dehydrogenase (HD), arginine permease (AP) and invertase (Inv) from the yeast *Saccharomyces cerevisiae* (for examples see references 14,38,39,45,49,92,110). In most cases the chimeric proteins are generated by fusing N-terminal parts of the protein under study to the N-terminus of a reporter protein but, alternatively, the reporter protein can be inserted into the protein of interest in a sandwich-like manner (31).

This method of generating sets of reporter protein fusions is most frequently used for the determination of the membrane topology of proteins of prokaryotic origin. Although there are examples of eukaryotic membrane proteins of which the topology has been determined in *E. coli* using this approach (8,38,40,142), complications may arise from the necessity to express the eukaryotic membrane proteins in a prokaryotic host (when using LacZ, PhoA, ßla or MBP): due to different codon usage in eukaryotic genes the proteins may be poorly expressed and, in addition, it has to be confirmed that the topology in the prokaryotic host is the same as in the eukaryotic host.

The reporter protein fusion approach is relatively simple to use, and both random and directed fusions can be generated. Its resolution as to the localization of amino acid stretches in the protein of interest relative to the cytoplasmic membrane is rather high. The most commonly used reporter proteins are LacZ and PhoA, two enzymes that show complementary properties (86): ß-galactosidase activity is only observed if the LacZ moiety of the fusion protein is located in the cytoplasm, whereas alkaline phosphatase is active when the PhoA moiety is present in the periplasm of *E. coli* (see Fig. 2). LacZ is active as a tetramer that is formed *in vivo* in the cytoplasm. For PhoA to be active, the formation of two internal disulfide bridges is required, which is mediated by the protein DsbA located in the periplasm of *E. coli* (6).

1.3.4. Other methods

Other techniques to investigate the membrane topology of proteins include the engineering of disulfide bridges (26), glycosylation scanning (applicable to glycosylated

eukaryotic membrane proteins (60) and site-directed chemical labeling of cysteine residues (74). However, these methods are rather limited in their application due to the specific characteristics imposed upon the protein under study.

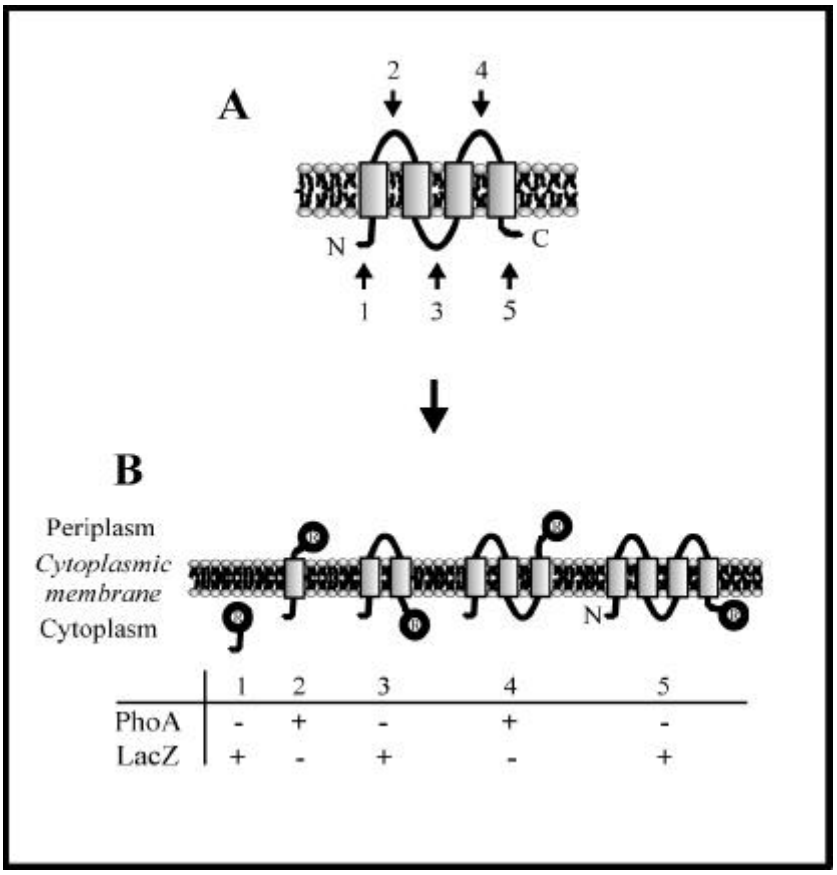

Fig. 2. Schematic representation of the use of the reporter enzymes ß-galactosidase (LacZ) and alkaline phosphatase (PhoA) in the determination of the membrane topology of a protein of interest in *E. coli*. Panel (A) gives a model of a membrane protein with four transmembrane segments (TMSs). By fusing N-terminal parts of this protein of various lengths (1 to 5) to one of the two reporter enzymes (R), chimeras are created that are shown in the lower panel (B). Due to the membrane topology-determining properties of TMSs and the position of the reporter protein fusion to the protein under study, the reporter enzyme moiety is found either in the cytoplasm or the periplasm. PhoA is only active after translocation into the periplasm of *E. coli*, whereas LacZ is only active when it has a cytoplasmic location and is able to form tetramers. Thus, the respective enzymatic activities of the chimeras indicate whether the amino acid to which the reporter protein has been fused is located in the cytoplasm or periplasm. The expected PhoA and LacZ activities of the chimeras 1 to 5 are indicated in the table of panel B; -: low enzymatic activity, +: high enzymatic activity.

2. Lactococcins

2.1. Genetic organization

Van Belkum *et al.* characterized the bacteriocinogenic plasmid p9B4-6 and showed that it carried three bacteriocin operons. Each operon encodes a different bacteriocin (lactococcin) with either low (LcnM/N), high (LcnA) or an intermediate antimicrobial activity (LcnB) (119-121). LcnA and LcnB are bacteriocins encoded by their corresponding structural genes *lcnA* and *lcnB*, while LcnM/N is a two-peptide bacteriocin encoded by the genes *lcnM* and *lcnN*. In all three cases an additional gene is present in the same operon, the product of which confers immunity upon the producer cell to the corresponding bacteriocin. Two operons, each carrying a copy of *lcnC* and *lcnD*, are located elsewhere on the same plasmid. Together, *lcnC* and *lcnD* encode the bacteriocin secretion apparatus.

2.2. Mode of lactococcin action and immunity

The lactococcins LcnA and LcnB act by permeabilizing the cytoplasmic membrane of sensitive cells, probably by the formation of (non-selective) pores (122,126). The C-terminal part of LcnA contains a hypothetical transmembrane helix, while in the C-terminus of LcnB a putative amphiphatic α-helix can be identified. Both of these structures are proposed to play a role in pore formation. The mode of action of lactococcin M/N has not yet been determined. Another two-peptide bacteriocin isolated from *L. lactis*, lactococcin G, has been shown to act by the formation of channels selective for efflux of potassium ions and (in)flux of sodium and other monovalent cations (93,94,98).

The only immunity protein that has been investigated in detail is LciA, conferring immunity to LcnA (97,122,128). LciA is thought to interact with the receptor for LcnA. Although LcnA is still able to bind to the receptor in the presence of the immunity protein, it is unable to form pores in the cytoplasmic membrane (for a detailed description of the mode of lactococcin action and immunity, the reader is referred to the review of Venema *et al.;* 130).

2.3. Lactococcin synthesis

All three lactococcins, like all other representatives of Class II bacteriocins from LAB, are synthesized as precursor proteins. The common motif in these precursors is a conserved glycine doublet (position -1 and -2) in the N-terminal extension, or leader peptide, behind which the prebacteriocin is processed into its mature form. Moreover, they share similarity in their primary structure, including hydrophobic residues at positions -4, -7, -12 and -15 as well as hydrophilic residues at positions -8, -9 and -11 (34,51). The leader peptide and processing site do not follow the rules of Von Heijne for signal peptides required for *sec*-dependent protein export (131), indicating that for the secretion of these bacteriocins a pathway other than the general secretion pathway is utilized.

2.4. Lactococcin secretion and maturation

The secretion and maturation of all three lactococcins depends on the two proteins LcnC and LcnD that, together, form a Type I secretion system (see 1.1.1). The genes of these two proteins were identified in an operon upstream of the bacteriocin operons on plasmid p9B4-6, but homologues of *lcnC* and *lcnD* were also shown to be encoded by the chromosome of *L. lactis* IL1403, a strain that does not produce bacteriocin activity (127). LcnC and LcnD are membrane proteins essential for bacteriocin activity (117). LcnC belongs to the family of ABC transporters, while LcnD is the accessory protein. For bacteriocins of other LAB as well as for colicin V from *E. coli* and cytolysin from *Enterococcus faecalis* similar secretion systems have been described (5,24,32,41,103,105).

LcnC differs from the earlier described "classical" ABC transporter proteins in that it has an extended N-terminal domain responsible for the processing of the prebacteriocin into its mature form (see Chapter IV). The N-terminal domains of the ABC transporter proteins LcnC, LagD (both involved in lactococcin secretion) and PedD (required for pediocin translocation in *Pediococcus acidilactici*) show amino acid homologies including a completely conserved cysteine residue. This conserved domain is also present in other ABC proteins involved in the secretion of other bacteriocins (51). An alignment of the N-terminal parts of several of these ABC transporters is shown in Fig. 3. Data base searches revealed that also other ABC transporters, such as HlyB (secretion of *E. coli* α-haemolysin; 67), AppB (externalization of *Actinobacillus pleuropneumonia* haemolysin; 15), LktB (*Actinobacillus actinomycetumcomitans* leukotoxin translocation; 47), CyaB (*Bordetella pertussis* cyclolysin secretion; 43), ComA (secretion of the *Streptococcus pneumoniae* competence factor; 62), HetC (early regulation of heterocyst differentiation in *Anabaena* sp.; 73), and a hypothetical protein from *Synechocystis* sp. PPC6803 (function unknown; 68) contain similar N-terminal extensions.

Lactococcin G has been shown in *in vitro* experiments to be processed by the N-terminal domain of its dedicated ABC transporter (LagD; 51). To this end, the 150 N-terminal amino acids of LagD were expressed with a C-terminal extension of six histidine residues (His-tag) and 22 vector-encoded amino acids. Similarly, precursor and mature lactococcin Gα were overproduced with N-terminal His-tags, either with or without an enterokinase cleavage site between the His-tag and the lactococcin Gα moiety. Mature lactococcin Gß was also overproduced with an N-terminal His-tag and enterokinase cleavage site. After purification of the various His-tagged proteins, processing of lactococcin Gα by the N-terminal domain of LagD was observed using SDS-PAGE and by the emergence of

Fig. 3. Alignment of the N-terminal domain of LcnC (including the first TMS) with the equivalent domains of other ABC transporter proteins present in LAB. These proteins are implicated in the transport of pediocin PA-1 (PedD; 88), lactococcin Gα and Gβ (LagD; 51), plantaricin A (PlnG; 23), sakacin B (SapT; 5), sakacin P (SspT; 58), carnobacterium B2 (CbnT; 102), divergicin 750 (DvnT; 57), mesentericin Y105 (MesD; 35), leucocin A (LcaC; 123), cytolysin of *Enterococcus* (CylB; 41), streptococcin A-FF22 (ScnT; 65) and lactococcin DR (LcnDR3; 105). Below the sequences the similarity is indicated: (*), identical amino acid; (.), conserved amino acid.

Underneath the alignment of the ABC transporter domains from LAB, an alignment is shown with domains from non-LAB ABC transporters involved in the secretion of colicin V of *E. coli* (CvaB; 32) and sublancin 168 of *B. subtilis* (SunT; 79).

antimicrobial activity. The authors concluded that LagD is a member of a new family of proteolytic enzymes. Based on homology alignments, this family was assigned to the (family of) cysteine proteases (51). Independently, pediocin PA-1 from *Pediococcus acidilactici* PAC1.0 has been shown to be processed by its ABC transporter protein PedD: when prepediocin was produced in the heterologous host *E. coli* together with the first 172 amino acids (lacking any putative TMS) or 192 residues (including the first putative TMS) of PedD, processing of prepediocin was observed as judged from zymographic analysis of crude cell extracts (129).
When prelactococcin A was overproduced in *L. lactis* in the presence of the first 164 amino acids of LcnC, processing was observed (this thesis Chapter IV).

Apart from the presence of the N-terminal processing domain, another aspect in which LcnC seems to deviate from the classical ABC transporter proteins is its membrane topology. Instead of the typical six TMSs spanning the cytoplasmic membrane, experimental data indicate that it spans this membrane only by four TMSs (see Chapter IV).
As mentioned above (1.1.1), other ABC transporter proteins have been shown to deviate from the "two times six" TMSs paradigm (52). Moreover it becomes increasingly clear that some (integral) membrane proteins can adopt more than one topology (1,27,85,100) and that during substrate translocation considerable conformational changes can take place in transporter proteins (80,89,138). Interestingly, one ABC transporter protein, P-glycoprotein (Pgp), has been proposed to exist in more than one (fixed) membrane topology. Pgp, a homodimeric protein, has been postulated to exist in at least two forms: in one conformation "two times four" TMSs span the membrane, whereas in the other "two times six" TMSs are present (69,139). It has been proposed that these two topologies could represent different functional states of the molecule (139,141). Experimental data generated during the last few years concerning the topology of Pgp have been conflicting. Several groups, using various techniques, have proposed six different models with the number of TMSs ranging between seven to twelve (7,46,69,84,113,139). By using immunocytochemistry and proteolysis/ membrane protection assays, Zhang *et al.* have presented compelling evidence that PgP can exist in a "two times four" TMSs conformation. In this study, Pgp was, in contrast to the previous studies, unaltered. However, the authors could not rule out that other conformations of Pgp may exist (141). For the other ABC transporter proteins mentioned in Fig. 3, no experimental data concerning their membrane topologies are available.

The membrane topology of LcnD conforms to that of the general models for accessory proteins of Type I transport systems: they are bitopic proteins with a short N-terminal part residing in the cytoplasm, one TMS and the C-terminal part, representing the majority of the protein, at the extracellular side of the cytoplasmic membrane. A remarkable feature of the *lcnD* gene is the presence of an internal alternative translation start site, giving rise to the expression of an N-terminally truncated version of LcnD (LcnD*), which lacks the N-terminal

TMS and is present in the cytoplasm of *L. lactis* (M. Varcamonti, personal communication). This is also the case for CvaA, the accessory protein in the secretion of colicin V. The N-terminally truncated accessory protein CvaA* is not essential for colicin V secretion, but its absence leads to decreased colicin V activity. In the presence of CvaA*, CvaA is more stable and it has been proposed that this stabilizing effect enhances colicin V secretion. A model has been presented in which CvaA* is thought to interact with the secretion machinery from the cytoplasmic side of the membrane (63). These data are integrated in the model shown in Fig. 4 for the secretion machinery of the lactococcins.

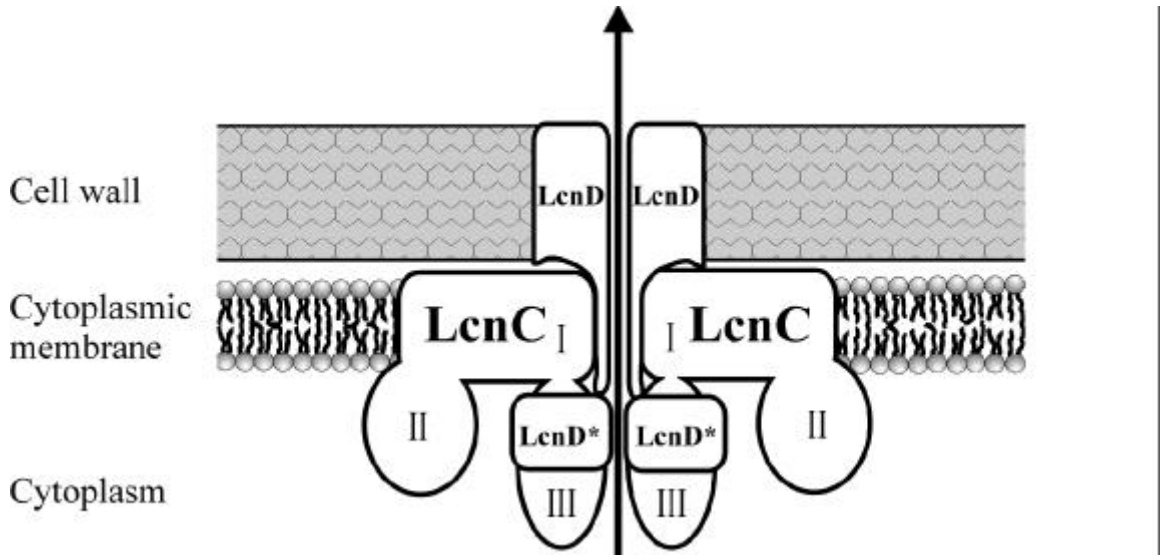

Fig. 4. A schematic representation of the secretion and maturation system for lactococcins. In addition to the earlier shown model for a typical Type I secretion system of Gram-positive bacteria (Fig. 1B), two additional features have been included in this model: an extra N-terminal domain (III), involved in the processing of the prebacteriocin into its mature form, and another protein component (LcnD*) in the complex. LcnD* is formed by an alternative translation start in the *lcnD* gene.

3. Outline of this thesis

This thesis describes the analysis of the lactococcin secretion apparatus of *L. lactis*. Two membrane proteins, LcnC and LcnD, have been shown to be essential. These two proteins form a Type I (*sec*-independent) transport system. LcnC belongs to the family of ABC transporter proteins, while LcnD is an accessory protein. In Chapter II, the membrane topology of LcnD is analyzed by constructing translational fusions of N-terminal parts of LcnD to the reporter enzymes ß-galactosidase (LacZ) and alkaline phosphatase (PhoA) lacking its own secretion signal. These studies indicate that LcnD contains one transmembrane segment (TMS) in its N-terminus and that approximately 20 N-terminal amino acids of LcnD reside in the cytoplasm while the (major) C-terminal part is located at the extracellular side of the cytoplasmic membrane.

To facilitate the generation of protein fusions to the reporter enzymes LacZ and PhoA, a wide-host-range fusion vector system consisting of the two plasmids pFUSLC and pFUSPC, respectively, has been developed (Chapter III). During the construction of this system the *phoA* gene has been mutagenized, resulting in a translation product (PhoA*) that retained the enzymatic properties of PhoA. The plasmid carrying *phoA** was better maintained in *L. lactis* than that specifying native PhoA. Another set of fusions of LcnD with LacZ and PhoA* has been constructed to test the system. This study proves the usefulness of the plasmid system and confirms the membrane topology model of LcnD established in Chapter II.

The fusion vector system has been used in Chapter IV to examine the membrane topology of LcnC. Instead of the commonly observed six TMSs in ABC transporter proteins, LcnC seems to span the cytoplasmic membrane with only four TMSs. Moreover, it was shown that the N-terminal cytoplasmic domain of LcnC is involved in the processing of preLcnA into its mature form *in vivo* in the natural host.

In Chapter V, the effects of mutations on the antimicrobial activity of LcnA are described. Moreover, the cellular localization of (pre)LcnA derivatives was investigated by means of epitope tagging. The data show that preLcnA is mainly present in the cytoplasmic membrane, either in the presence or absence of LcnC/D, indicating an intrinsic property of the prebacteriocin to insert into the cytoplasmic membrane. In addition, the results suggest that the information for the targeting of preLcnA to the membrane is located in the putative α-helix in the C-terminal part of (pre)LcnA and, in the presence of the secretion machinery, in the leader peptide. Moreover, the results suggest that both these domains are also involved in (efficient) secretion.

In Chapter VI, the topological information obtained for LcnD was used to investigate whether LcnD could be used to express epitopes at the extracellular side of the cytoplasmic membrane of *L. lactis*. N-terminal parts of the pp65 protein from cytomegalovirus (CMV)

were fused to the N-terminal 80 amino acids of LcnD. Proteinase K accessibility studies using monoclonal antibodies raised against the N-terminal part of pp65 were used to show that the viral epitope was expressed in *L. lactis* at the extracellular side of the cytoplasmic membrane.

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