Production and secretion of heterologous proteins by *Lactococcus lactis*

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Production and secretion of heterologous proteins by *Lactococcus lactis*.

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in het openbaar te verdedigen
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Cover shows a schematic representation of plasmid pNZ10α5, enclosing an iodine stained starch plate with *E. coli* and *L. lactis* strains, containing various expression and secretion plasmids.

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Stellingen

1. Om een overtuigend bewijs te leveren dat verlaging van hydrophobiciteit van het signaalpeptide in een vermindering van secretie-efficiëntie resulteert, hadden in de studies van Borchert en Nagarajan ten minste signaalpeptiden van dezelfde lengte moeten worden vergeleken.

   Borchert, T. V. and V. Nagarajan. 1991. Effect of signal sequence alterations on export of levansucrase in *Bacillus subtilis*. J. Bacteriol. 173: 276-282.

2. Sibakov en coauteurs wekken ten onrechte de schijn het eerste lipo-protein signaalpeptide in *Lactococcus* geïdentificeerd te hebben.

   Sibakov, M., T. Koivula, A. Von Wright, and I. Palva. 1991. Secretion of TEM ß-lactamase with signal sequences isolated from the chromosome of *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. 57: 341-348.
   Vos, P., M. Van Asseldonk, F. Van Jeveren, R. Siezen, G. Simons and W.M. De Vos. 1989. A maturation protein is essential for production of active forms of *Lactococcus lactis* SK11 serine proteinase located in or secreted from the cell envelope. J. Bacteriol. 171: 2795-2802.
   Haandrikman, A. J., J. Kok, H. Laan, S. Soemitro, A. Ledeboer, W. N. Konings and G. Venema. 1989. Identification of a gene required for maturation of an extracellular lactococcal serine proteinase. J. Bacteriol. 171: 2789-2794.

3. Het gegeven dat in het humane genoomprojekt elke nucleotide gemiddeld negen maal bepaald wordt, geeft aan dat de grote hoeveelheid financiële middelen voor dit project meer de inspanning dan de creativiteit van de onderzoekers stimuleren.

4. Het grote aantal publikaties over verbetering van een bepaalde methode, voor bijvoorbeeld sequencing van ds-DNA of PCR-produkten, geeft aan dat de kwaliteit van de meeste van deze methoden vaak te wensen over laat.

5. Politieke partijen die een ongenuanceerd terughoudende of afwijzende opstelling innemen ten opzichte van de biotechnologie laten zich ten onrechte progressief noemen.

6. Indien soortskruisingen in de plantenveredeling voor het eerst in deze tijd toegepast zouden worden, zouden verschillende groeperingen, waaronder de in stelling 5 genoemde politieke partijen, hier zeer waarschijnlijk tegen ageren.

7. Indien het gebruik van walkmans op de fiets verboden zou worden vanwege het gevaar voor ongelukken, zou met dezelfde reden ook een verbod op "car-audio" moeten worden ingesteld.

8. Rokers beginnen als doorzetters.

9. Het gebruik van de auto om kroost naar school te vervoeren wijst in veel gevallen op weinig interesse in het toekomstig leefmilieu van eigen, en de veiligheid van andere kinderen.

10. Genetisch inzicht in de effecten van beten van vampiers en weerwolven zou kunnen leiden tot toepassing van deze beten in gentherapie.

    King, S. 1982. Bezeten stad.

11. Observatie van concertbezoekers zou de indruk kunnen wekken dat het humane heavy metal resistentie gen op het Y-chromosoom ligt.

Stellingen behorende bij het proefschrift "Production and secretion of heterologous proteins by *Lactococcus lactis*" van Martien van Asseldonk
Wageningen, 18 februari 1994

VOORWOORD

Dit proefschrift vormt het besluit van een roerige periode uit mijn leven. Naast het opzetten en uitvoeren van een stuk wetenschappelijk onderzoek en dit daarna als een goedlopend verhaal in een boekvorm te brengen, werd ik tot twee maal toe vader, leerde ik alles over voedselallergieën, en ondervond ik dat het menselijk lichaam flexibeler is dan een auto. Gelukkig stond ik er in deze periode niet alleen voor. Ik wil op deze pagina dan ook iedereen die op wat voor manier een steun voor me is geweest bedanken.

Guus, na een aftastende periode, waarin we allebei even eigenwijs waren, kwamen we in de laatste periode tot zeer vruchtbare discussies, die dan ook tot de leukste hoofdstukken hebben geleid. Gelukkig is aan onze samenwerking met de afsluiting van deze periode geen einde gekomen en ik verheug me dan ook op nog vele toekomstige discussies op Keygene.

Willem, niet alle extra invalshoeken van het onderzoek die jij aandroeg zijn uiteindelijk onderzocht, deels door een vooringenomen mening van Guus en mij, deels door een gebrek aan tijd. Toch heb je door altijd op het juiste moment een kritische noot te plaatsen of een compliment te geven, een belangrijke invloed op het onderzoek gehad.

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Ik wil vooral mijn paranimfen niet vergeten: Peter, mijn wapenbroeder in deze periode. Het was leuk om samen te delen in successen, en steun aan elkaar te verlenen als het wat minder ging. Pieter, ook na mijn stageperiode kon ik bij je terecht voor de fijne kneepjes van de moleculaire biologie. Het vertrouwen dat je altijd in me hebt gehad heeft me meer dan eens door een pessimistische periode heengeholpen.

Karin, bedankt, omdat je er altijd was als ik het weer eens niet zag zitten, het zat was, ermee ging stoppen, of nog liever bij Kok-Ede ging werken.

Am I stepping into the twilight zone,
This is a madhouse
Feels like being cloned.

Golden Earring

CONTENTS

The studies described in this thesis have been performed at the Netherlands Institute for Dairy Research (NIZO) and have been supported by the Mesdag foundation (Leeuwarden, The Netherlands), the Cooperative Rennet and Dye Factory (CSKF) (Leeuwarden, The Netherlands) and Dutch the Programme Committee on Industrial Biotechnology.

CHAPTER I

GENERAL INTRODUCTION

Before the Storm (Queensrijche)

1. General Introduction.

Lactococcus lactis is a member of the lactic acid bacteria, which are known to play a major role in the production of fermented foods. Lactococci are utilized in the fermentation of vegetables (Daeschel et al., 1987), but are primarily employed in the dairy industry for the production of products such as cheese, butter, and buttermilk. The main role of lactococci during the manufacture of these dairy products is the conversion of the milk sugar lactose into the food-preserving lactic acid. Additionally, lactococci accomplish the degradation of the milk protein casein, resulting in the specific texture and flavour of the product. The conversion of citrate by *L. lactis* subsp. *lactis* biovar. *diacetylactis* species results in the production of diacetyl, which is an important butter aroma component.

Apart from these traditional applications, lactococci are attractive organisms for the production of commercially valuable products. The increasing knowledge of the physiology, and the profound experience in fermentation technology of *L. lactis*, make these bacteria promising candidates for the production of (a) metabolites such as lactic acid or diacetyl, (b) homologous proteins, such as proteinases, peptidases or bacteriocins, and (c) heterologous proteins of interest for the food industry, such as prochymosin or lysozyme. During the last ten years intensive research on the genetics of *L. lactis* has led to an increased understanding of the important characteristics of these strains, such as lactose and sucrose fermentation (De Vos et al., 1990; Van Rooijen et al., 1991; Rauch and De Vos, 1992) casein degradation (Kok, 1990), citrate utilization (David et al., 1990), bacteriophage resistance (Klaenhammer, 1987; Hill, 1993) and bacteriocin production (Klaenhammer, 1988; Klaenhammer, 1993). These investigations also provided insight into the more fundamental aspects of lactococcal gene expression, such as transcription and translation signals (de Vos, 1987; De Vos and Simons, 1993; Van de Guchte et al., 1992a). Furthermore, *L. lactis*, being a gram-positive organism with low extracellular proteolytic activity, is able to secrete proteins into the growth medium (Simons et al., 1990a). *L. lactis* is considered to be a "GRAS" (generally regarded as safe) organism, and its use in the manufacturing of food products could facilitate the acceptance of heterologous or engineered proteins, produced by these bacteria.

2. Gene expression in bacteria.

As in all living organisms, the production of proteins in bacteria is depending on gene expression. Production of a protein by cells is controlled by transcription of the gene encoding the protein, and translation of its mRNA. Several features are important in these two processes. The stability of the mRNA molecules, the secondary structure of the mRNA and codon usage can influence the rate of translation. Moreover, initiation of transcription and translation plays a major role. The latter two aspects will be briefly reviewed.

2.1 Transcriptional initiation signals.

Most bacterial transcriptional initiation signals consist of two well-conserved regions at position -35 and the -10 of the transcriptional start. In *Escherichia coli* the sigma factor σ^{70} is involved in the transcription of the majority of genes, recognizing the -35 consensus (TTGACA) and the -10 consensus (TATAAT) sequences (Hoopes and McClure, 1987). In the transcription of some specific regulated sets of genes, like heat shock genes and genes involved in fixation and assimilation of nitrogen, other sigma factors (σ^{32} and σ^{60}, respectively) are involved, recognizing other consensus sequences (Hoopes and McClure, 1987).

In *Bacillus subtilis* 9 different sigma factors are known to be involved in expression of genes during the different growth phases of this organism (Moran, 1989). Nevertheless, σ^{43} is involved in transcription of most of the characterized genes. This sigma factor exhibits considerable sequence similarity with σ^{70} of *E. coli* (Gitt, 1985) and recognizes vegetative promoter sequences with the same features as the *E. coli* σ^{70} promoters (Stephens, 1984). Besides the -35 and -10 promoter region, the region upstream of these specific promoter sequences is also involved in transcription initiation. An AT-rich region upstream of the -35 hexamer contributes significantly to promoter strength as has been shown for gram-negative organisms such as *E. coli* (Lamond and Travers, 1983), and gram-positive organisms such as *B. subtilis* (Weickert, 1989) and *Staphylococcus aureus* (Mahmood and Khan, 1990).

Recently, the *rpoD* gene, encoding the principal σ factor of *L. lactis* has been cloned and characterised (Araya et al., 1993). It showed a significant homology with σ^{70} of *E. coli* and σ^{43} of *B. subtilis*. Several lactococcal promoters originating from plasmid-located genes, chromosomal genes or lactococcal phages have been characterized. Analysis of the promoter sequences thus far revealed a consensus

which also consists of the -35 (TTGACA) and -10 (TATAAT) region, resembling the consensus of vegetative *E. coli* and *B. subtilis* promoters (De Vos, 1987; De Vos and Simons, 1993; Koivula et al., 1991; Lakshmidevi et al., 1990; Van de Guchte et al., 1992a; Van Der Vossen et al., 1987). In addition, in more than 40% of the lactococcal promoters, the -10 sequence is immediately preceded by TGN (De Vos and Simons, 1993; Van der Vossen et al., 1987). As in other bacteria, the region upstream of the -35 sequence also contributes to efficient gene expression in *L. lactis* (Van Rooijen et al., 1992).

Despite the conservation of promoters between the mentioned species, there exists a significant difference in efficiency of transcription of heterologous promoters in different hosts. Most *B. subtilis* and *L. lactis* promoters are functioning in *E. coli*, but in contrast, *E. coli* promoters are poorly transcribed in gram-positive organisms (Goebel et al., 1979; Kreft et al., 1983).

2.2 Translational initiation signals.

The bacterial translational initiation signal, designated the ribosome binding site (RBS), constitutes the translational start codon and the Shine Dalgarno (SD) sequence which is complementary to the 3'-end of 16S rRNA (Shine and Dalgarno, 1974). In *E. coli* and *B. subtilis*, this sequence is usually located within 15 nucleotides upstream of the start codon (Gold et al., 1981; Hager and Rabinowitz, 1985). More than 90% of the sequenced bacterial genes contain AUG as initiation codon. In *E. coli*, 8% of the genes start with GUG and 1% with UUG (Gren, 1984). In contrast, in *B. subtilis* the UUG start codon is more common than the GUG codon (Hager and Rabinowitz, 1985). The SD sequence only diverges slightly among different bacterial species. The extent of complementarity of the SD sequence with the 16S rRNA sequence contributes to the efficiency of translation (Hartz et al., 1991; Ringquist et al., 1992). Furthermore, the translation rate is influenced by the spacing between the SD sequence and the start codon, the sequence of the start codon and the secondary structure in the translational initiation site (Hartz et al., 1991). In *E. coli*, the complementarity to 16S rRNA is of crucial importance when translation initiates from a weak start codon such as UUG. (Weyens et al., 1988). The SD region of *B. subtilis* genes usually displays a higher degree of complementarity to the 16S rRNA sequence than gram-negative SD sequences (Hager and Rabinowitz, 1985). It is assumed that this difference contributes to the very low expression of genes from gram-negative

organisms in gram-positives (Gold et al., 1981; McLaughlin et al., 1981).

Statistical and genetic analysis of RBSs revealed that the optimal spacing between the SD sequence and the initiation codon is 7-9 bp in *E. coli* (Hager and Rabinowitz, 1985; Munson et al., 1984; Ringquist et al., 1992; Vellanoweth and Rabinowitz, 1992) and in *B. subtilis* (Vellanoweth and Rabinowitz, 1992). Analysis of the effect of extending the spacing in the RBS on translation efficiency have demonstrated that the range in allowed spacing is much broader than that observed for naturally occuring RBSs, both in *E. coli* (Ringquist et al., 1992; Vellanoweth and Rabinowitz, 1992), and in *B. subtilis* (Vellanoweth and Rabinowitz, 1992). Moreover, *B. subtilis* is relatively more efficient than *E. coli* in recognizing translational initiation sites with a larger than optimal spacing between SD sequence and start codon (Vellanoweth and Rabinowitz, 1992).

L. lactis translational start signals are similar to those of *B. subtilis* and *E. coli* (Van de Guchte et al., 1992a). For *L. lactis* the 3' terminal sequence of the 16S rRNA is 3'-UUUCCUCCA-5', (Chiaruttini and Milet, 1993). In contrast to RBSs of other gram-positives (Hager and Rabinowitz, 1985), the degree of complementarity of the SD region with the 16S rRNA sequence is on the average only slightly higher than in *E. coli* (De Vos and Simons, 1993, Van de Guchte et al., 1992a). Predicted free energy values for *L. lactis* RBSs vary between -36 kJ/mol for the *repA* gene of pSH71 (De Vos, 1987) to -75 kJ/mol for the *nisZ* gene (Mulders et al., 1991), with a mean value of -48.2 kJ/mol. The variability in spacing between the SD sequence and start codon in *L. lactis* seems higher than in *E. coli* and *B. subtilis*. Spacing between SD sequences and putative start codons as long as 15 (for a putative translocase of IS904; Rauch et al., 1990) or 16 (for the *dnaJ* gene; Van Asseldonk et al., 1993) nucleotides have been postulated in lactococcal translational initiation sites.

3. Protein secretion.

Many proteins synthesized by bacteria are secreted across the cytoplasmic membrane to perform their function. In gram-negative organisms these proteins are transported into the periplasm, to the outer membrane, or to the extracellular medium, whereas in gram-positive organisms proteins are exported into the medium external from the cells. (Freudl, 1992; Pugsley, 1993). The exported proteins are of many classes, like hydrolytic enzymes (e.g. amylases, nucleases, proteinases, esterases and cellulases), antimicrobial proteins (e. g. bacteriocins), structural proteins (e. g. flagellar

proteins) or protective proteins (e.g. penicillase or ß-lactamase).

The secretion of proteins in bacteria has been the subject of many fundamental studies. *E. coli* mutants, disturbed in secretion, have played a major role in the discovery of different genes involved in the transport of proteins (Schatz and Beckwith, 1990). In the last 5 years, the cloning of these genes and the biochemical characterization of their gene products, has provided considerable insight into the molecular mechanism of secretion in *E. coli* (Hartl et al. 1990; Pugsley, 1993; Wickner et al., 1991). Recently, studies have been initiated on the mechanism of secretion of proteins in other prokaryotes (Freudl, 1992; Simonen and Palva, 1993). These studies revealed similarities as well as differences between the secretion of proteins in different species. Besides scientific curiosity in the mechanism of export of proteins, there exists great interest in the employment of protein secretion in the production of heterologous proteins.

3.1 Features of exported proteins.

Most exported proteins are synthesized as a precursor polypeptide, consisting of a signal peptide, a mature part, and in some cases a propeptide.

The amino-terminal extension, called the signal peptide is a very well-conserved feature of exported proteins (Pugsley and Schwartz, 1985; Von Heijne, 1985). The properties of the signal peptide are universal in prokaryotes and eukaryotes. It consists of 15-35 amino acid residues, which can be arranged into three characteristic regions (Fig. 1A): (i) The N-region: the amino-terminal 2-8 amino acids of signal peptides containing at least one positive charged residue; (ii) The H-region: a region of 8-15 amino acids forming a hydrophobic core; (iii) The C-region: this carboxy terminal region, consisting of 4-6 uncharged amino acids, contains the target site for the signal peptidase. (Dalbey, 1991; Von Heijne 1985). The consensus sequence of this target site, follows the so called -1, -3 rule as proposed by Von Heijne (1986). However, a considerable variation between the different sites in the various bacterial and eukaryotic species has been observed (Von Heyne and Abrahmsén, 1989); While residue -2 can be any amino acid, residue -3 is preferentially a small neutral (alanine, glycine, serine, threonine, cysteine) or larger aliphatic (isoleucine, leucine, valine) residue. The small neutral amino acid residues (alanine, glycine, serine, threonine, cysteine), glutamine or proline residues are often found at site -1 (Von Heyne, 1986). During secretion the signal peptide is cleaved off at the C-terminal side of residue -1.

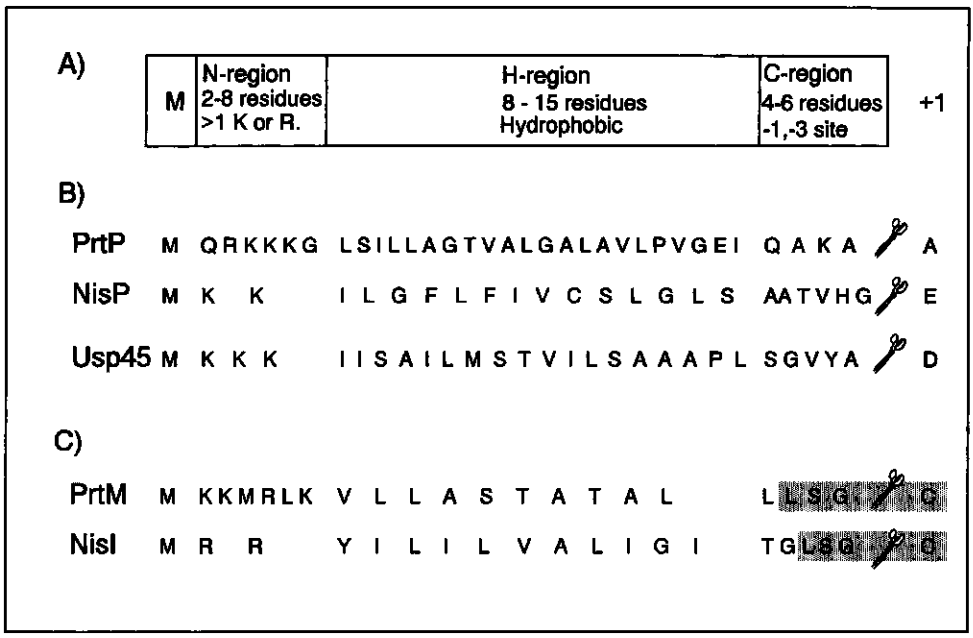

Fig 1 A: Schematic presentation of the N-, H-, and C-, region of a signal peptide. The first box, marked with an M, represents the first methionine residue. +1 represents the first amino acid of the propeptide, or mature protein. B) and C) Representation of the arrangement of the amino acids in the N-, H-, and C- region of the characterized lactococcal signal peptides of secreted proteins (B) and lipoproteins (C). PrtP: SK11 proteinase; NisP: nisine leader proteinase; Usp45: unidentified secreted protein. PrtM: SK11 proteinase maturation protein; NisI: Nisin immunity protein; The cleavage site is indicated with scissors. The lipoprotein signal peptide cleavage site is shaded.

A specific signal peptide cleavage site is present in exported lipoproteins. This cleavage site is a more conserved sequence in bacteria and consists of a leucine at position -3, a serine, valine or alanine residue at -2 and an alanine or glycine residue at -1. At position +1 a cysteine residue is always present (Pugsley and Schwartz, 1985). Before cleavage this residue is modified into a glyceride-fatty-acid-modified cysteine and is required for the interaction of the protein with the cellular membrane.

A pro-peptide is present between the signal (pre-)peptide and the mature protein of many secreted proteins from bacteria and eukaryotes. This pro-peptide has no explicit function in secretion, but mainly functions as an intermolecular chaperone in the folding of the mature protein (Ikemura et al., 1987; Ikemura and Inouye, 1988). The length of the pro-peptide can vary from less than 10 amino acids as in the precursor of the *B. subtilis* α-amylase (Sasamoto et al., 1989), to more than 200 as for the neutral protease of *B. stearothermophilus* (Vasantha et al., 1984). Cleavage of

propeptides can occur autocatalytically (e.g. chymosin; Barkholt Pedersen et al., 1979; or subtilisin; Ikemura and Inouye, 1988) or catalyzed by extracellular proteases (e.g. *B. subtilis* α-amylase; Takase et al., 1988). Processing of the *L. lactis* proteinase-precursor requires PrtM, a lipoprotein that functions as an extracellular chaperone (Haandrikman et al., 1989; Vos et al., 1989b).

The properties of the mature protein play a important role in the export of proteins. This has been well established, for instance by studies with hybrid precursors consisting of the cytoplasmic protein ß-galactosidase fused to various signal peptides (Moreno et al. 1980; Silhavy et al., 1985). In *E. coli* as well as in *B. subtilis* these fusions did not result in export of the ß-galactosidase, while no particular part of this protein could be assigned as export-blocking sequence (Lee et al., 1989). In addition, mutations in the mature maltose binding protein resulted in a loss of export (Duplay and Hofnung, 1988). No correlation has been found yet between the amino acid composition or distribution, or secondary structure of mature secreted proteins and the export competency of a polypeptide chain.

The combination of signal peptide and mature protein affects the secretion efficiency. Exchange of signal peptides between different extracellular proteins resulted in notable differences in export efficiency, both in *E. coli* and in *B. subtilis* (Schein et al., 1986; Smith et al., 1988). In *E. coli*, exchange of the H- and C- region of the *E. coli* alkaline phosphatase signal peptide with corresponding regions of M13 major coat protein failed to direct secretion of alkaline phosphatase, whereas exchange of the H- and C- regions of the maltose binding protein and OmpA resulted in secretion of this enzyme (Laforet et al., 1989). *B. subtilis* failed to secrete the *E. coli* outer membrane proteins OmpA and OmpF when fused to the *B. amyloliquefaciens* α-amylase signal peptide (Puohiniemi et al., 1992). Moreover, in *B. subtilis*, this α-amylase signal was functional in secreting *B. licheniformis* penicillinase, but the use of the penicillinase signal peptide fused to mature α-amylase, resulted in a very inefficient secretion of α-amylase. (Himeno et al., 1986).

3.2 Protein secretion in *E. coli.*

The export of proteins in *E. coli* has been extensively studied by means of genetic and biochemical approaches. These studies resulted in the following model of signal-peptide dependent secretion. The nascent polypeptide chain, containing the signal sequence is rendered in an unfolded, secretion-competent state by various

molecular chaperones. Besides these chaperones, a membrane-bound protein complex, forming the translocase (Wickner et al., 1991) is involved in translocation of the pre-proteins across the inner cell membrane. During this translocation the signal peptide is cleaved off.

Analysis of *E. coli* mutants, disturbed in secretion, revealed a set of genes, which are of crucial importance in the secretion of most proteins. In this set of genes, several *sec* genes and some genes encoding molecular chaperones are necessary for the stabilization of the unfolded conformation of the precursor (Lecker et al., 1989). These molecular chaperones in *E. coli* are identified as the GroEL and the GroES (Kusukawa et al., 1989; Lecker et al., 1989) proteins, the trigger factor (Crooke and Wickner, 1987; Lecker et al., 1989) and SecB (Kusters et al., 1989; Lecker et al., 1989). Besides its role in maintaining the unfolded state of several precursors, SecB is also believed to prevent the pre-protein from association with non-productive membrane sites (Hartl et al., 1990). Furthermore, SecB is known to target the pre-protein prePhoE to the SecA protein for translocation (Hartl et al., 1990; Tomassen et al., 1992). The SecA protein, which is located on the interior surface of the cytoplasmic membrane can associate with the integral proteins SecE and SecY. This association enhances its affinity for the SecB/preprotein complex. After release of SecB from the complex, SecA renders the precursor in its export-competent state (Hartl et al., 1990). Translocation of the precursor is associated with ATP hydrolysis, which is probably used to release the precursor from the SecA protein. The function of SecE/Y in export is still under investigation. These membrane associated compounds of the secretion machinery may act as receptor for precursors of secreted proteins or they may be involved in the formation of transport channels (Driessen, 1992). Protein conducting channels have been reported for *E. coli*. However, it remains unclear whether these channels consist of proteins (Simon and Blobel, 1992). No function had been assigned yet to the membrane proteins SecD and SecF. They possess large periplasmic domains (Gardel et al., 1990) and apparently function in a late step of translocation (Tai et al., 1992). Recently it has been postulatyed that SecD is involved in the release of the translocated mature protein from the cytoplasmic membrane (Matsuyama et al., 1993). After translocation of the secreted protein across the membrane, signal peptidases (Dalbey, 1991) are responsible for the removal of the signal peptide, after which the mature protein can fold into its soluble native conformation. Two signal peptidases, SpaseI and SpaseII, specific for the two described types of signal

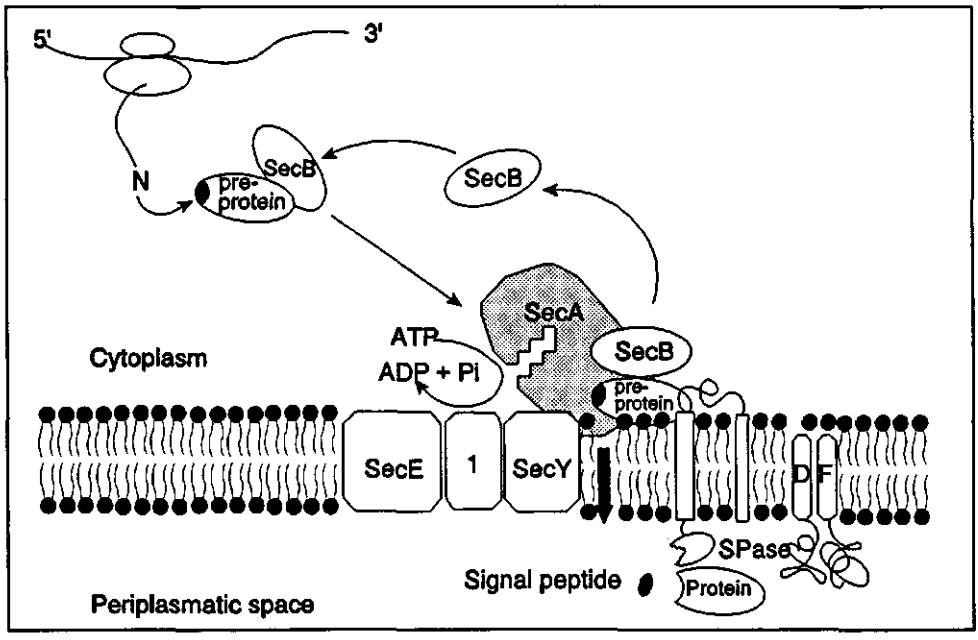

Fig 2: Schematic representation of the several Sec-proteins and signal peptidase I and their role in the translocation of a pre-protein, derived from the model as proposed by (Hartl et al., 1990). D: SecD, F: SecF. 1 represents a polypeptide of unknown fuction that co-purifies with SecE and SecY subunits.

peptides, have been characterized from *E. coli*. SPaseI is necessary for the release of the protein into the periplasm (Dalbey and Wickner, 1985; Wolfe et al., 1982). SPaseII is involved in the processing of the signal peptide of exported lipoproteins (Tokonuga et al., 1982; Yamada et al., 1984), and results in inner-membrane bound proteins. The described signal-peptide-dependent pathway is schematically represented in Fig. 2.

Besides the described pathway, used for the transport of proteins into the periplasm or to the outer membrane of gram-negative bacteria, alternative routes are used for the secretion of proteins, such as hemolysin (Koronakis et al., 1991), colicin V (Gilson et al., 1990), pullanase (d'Enfert et al., 1989), and several proteinases (Létoffé et al., 1990; Létoffé et al., 1991), into the extracellular medium (for a review see: Pugsley et al., 1990).

3.3 Protein secretion in gram-positive bacteria.

The knowledge of the mechanism of secretion in gram-positive bacteria is less

detailed than that of *E. coli.*

3.3.1 Protein secretion in *B. subtilis*

The cloning of *B. subtilis* genes, that are homologous to *secA* (Overhoff et al., 1991; Sadaie et al., 1991) and *secY* (Nakamura et al., 1990a; Suh et al., 1990) of *E. coli*, indicates that a Sec-dependent pathway exists in *B. subtilis*. Both genes are shown to be functionally involved in protein export (Nakamura et al., 1990b; Sadaie et al., 1991). However, the cloning of the *B. subtilis* Spase I gene, (Van Dijl et al., 1992) revealed significant differences, both in protein sequence and in affinity for different signal peptides, between the *E. coli* and the *B. subtilis* SPasel. Furthermore, signal peptides of gram-positive and gram-negative organisms display a significant difference in length and composition (Von Heyne and Abrahmsén, 1989). In general, gram-positive organisms require longer signal peptides, with more N-terminal charge, a larger hydrophobic core and an extended C-region. These observations suggest that the secretion apparatus in gram-positive organisms has other requirements for certain structural features of the signal peptide than that in gram-negative bacteria.

Mutants of *B. subtilis* and *S. aureus* displaying reduced or increased levels of secreted proteins have been isolated. Several genes of *B. subtilis* involved in regulating the production of extracellular enzymes have been cloned (*e.g. senN* (Wong et al., 1988), *degQ* (Kunst et al., 1974), *tenI* and *tenA*, (Pang et al., 1991), as well as genes from *S. aureus*. (e.g. *exp* (Morfeldt et al., 1988). Besides these regulatory genes, another gene, *prsA*, involved in protein export from *B. subtilis*, has been isolated. It is proposed to encode an extracellular molecular chaperone, involved in a late stage of protein export (Kontinen et al., 1991). The product of this gene is a membrane-associated lipoprotein and shows homology to the lactococcal PrtM protein. The PrsA protein is indispensable for viability and essential for proteins secretion in *B. subtilis*. Overproduction of PrsA resulted in an enhanced secretion of α-amylase and protease in strains that produced high levels of these exoenzymes, suggesting that the PrsA protein could be a rate-limiting component in the export machinery of *B. subtilis* (Kontinen and Sarvas, 1993).

In *B. subtilis,* signal-peptide-independent export has also been demonstrated, as is the case of the bacteriocin subtilin. The subtilin operon contains the *spaT* gene, encoding a protein homologous to HlyB, the *E. coli* hemolysin transport protein. It has been suggested that this gene encodes a translocator, necessary for subtilin secretion

(Chung et al., 1992).

3.3.2 Protein secretion in *L. lactis*.

Several extracellular proteins have been characterized in *L. lactis* (Table 1; Simons et al., 1990a). The gene encoding the lactococcal extracellular proteinase, PrtP, a key enzyme in casein degradation, has been characterized (Kok et al., 1988; Vos et al., 1989a). The signal peptide cleavage site of this protein is proposed to be located after Ala^{33}. The precursor contains a 150-residue propeptide and the mature protein is attached to the cell envelope by a C-terminal membrane anchor.

A second protease, NisP, involved in processing of the nisin precursor is also attached to the cell envelope with a C-terminal membrane anchor. The signal peptide cleavage site is predicted to be located after Gly^{22} (Van de Meer et al., 1993).

Furthermore, the *usp45* gene encoding the most abundant extracellular protein of *L. lactis*, has been cloned and characterized (Van Asseldonk et al., 1990). The deduced amino acid sequence of the protein revealed no significant homology with any protein of known function. Moreover, the protein displayed no enzymatic activity as tested so far. The signal peptide of this protein consists of 27 amino acids (Van Asseldonk et al., 1993).

The *prtM* gene, encodes a maturation protein, necessary for the processing of the proteinase into an active conformation. PrtM is a membrane-associated lipoprotein with a 23-residue lipoprotein signal peptide (Haandrikman et al., 1989; Haandrikman et al., 1991; Vos et al., 1989b).

Recently the *nisI* from the *nis* operon has been characterized (Kuipers et al., 1993). It is predicted to encode an extracellular protein involved in nisin immunity, with a lipoprotein signal peptide of 19 residues.

The sequence charactersistics of the signal peptides of the described proteins are shown in Fig 1B.

Several bacteriocins of *L. lactis*, such as nisin and several lactococcins, are also secreted to the extracellular medium. Although the lantibiotic nisinA and nisinZ are synthesized as a precursor, their leader peptides cannot be considered as signal peptides (Buchman et al., 1988; Mulders et al., 1991). The sequence of these leader peptides is not in accordance with the Von Heyne rules. Furthermore, the precursor of nisin is translocated without removal of its leader peptide (Van de Meer et al., 1993). The genes of three other extracellular lactococcal bacteriocins, lactococcin A

TABLE 1: Secreted proteins of *L. lactis.*

Protein	Function	Leader length (a)	Signal peptide normal	Signal peptide lipo	ref
PrtP	Proteinase	33 (-)	+		Vos et al., 1989a
NisP	Proteinase	23 (-)	+		Van de Meer et al., 1993
Usp45	Unknown	27 (+)	+		Van Asseldonk et al., 1990
PrtM	Maturation	23 (+)		+	Vos et al., 1989b
NisI	Immunity	19 (-)		+	Kuipers et al., 1993
NisA	Bacteriocin	23 (+)	-		Buchman et al., 1988
NisZ	Bacteriocin	23 (+)	-		Mulders et al., 1991
LcnA	Bacteriocin	21 (+)	-		Van Belkum et al., 1991
LcnB	Bacteriocin	21 (-)	-		Van Belkum et al., 1992
LcnMa	Bacteriocin	21 (-)	-		Van Belkum et al., 1991

(a):Postulated (-) or determined (+)

(Holo et al., 1991; Stoddard et al., 1992; Van Belkum et al., 1991) lactococcin B (Van Belkum et al., 1992) and lactococcin M (Van Belkum et al., 1991) have also been characterized. Lactococcin A is produced as a precursor, but its 21 residue leader does not resemble signal peptides (Holo et al., 1992). The primary sequence of lactococcin B and M, indicates that they are also secreted in a signal-peptide-independent manner (Van Belkum et al., 1991; Van Belkum et al., 1992).

The secretion mechanism of proteins by *L. lactis* is unidentified yet, but the cloning of a *secY* homologue from this organism is suggestive for a signal peptide-dependent pathway (Koivula et al., 1991). The transport mechanism of the above-mentioned bacteriocins is still unclarified, but the *nisT* and *lcnC* genes are very likely responsible for the transport of nisin and lactococcin A, respectively (Engelke et al., 1992; Kuipers et al.,1993; Stoddard et al., 1992). Homology of their gene products with ATP-dependent translocators as SpaT and HlyB, which are involved in subtilin and hemolysin transport, suggests a conservation between gram-positive and gram-negative organisms in signal-peptide-independent protein export (Engelke et al., 1992; Kuipers et al.,1993; Stoddard et al., 1992).

4. Heterologous gene expression in *L. lactis*.

The extensive research on the genetics of *L. lactis* in the last decade has led to the development of several cloning vector systems and efficient transformation systems (De Vos, 1987; De Vos and Simons, 1993; Kok, 1991). Furthermore, various

expression and secretion signals are available now (De Vos and Simons, 1993). These genetic tools are of crucial importance for the investigation of heterologous gene expression in *L. lactis*. Various heterologous genes have been expressed in *L. lactis* and the secretion of heterologous proteins has been accomplished as summarized in section 4.1 and 4.2 and in Table 2.

4.1 Intracellular proteins

4.1.1 Antibiotic resistance markers.

Dominant selection markers are essential in the development of a reliable gene cloning system. Several genes conferring resistance against chloramphenicol, erythromycin, kanamycin and tetracyclin, derived from *B. pumilus*, *S. aureus*, and *Streptococcus faecalis*, are used in various lactococcal cloning vectors (De Vos, 1987; De Vos and Simons, 1993; Kok, 1991; Platteeuw et al., 1993a). All these markers have been expressed under control of their own regulatory sequences. Furthermore, several promoter-probe-vectors have been constructed based on the promoter-less chloramphenicol resistance conferring genes *cat86* or *cat194* (Bojovic et al., 1991; Koivula et al., 1991; Lakshmidevi et al., 1990; Van der Vossen et al., 1987).

4.1.2 *E. coli* ß-galactosidase

Initial studies on heterologous gene expression in *L. lactis* have been performed using the *E. coli lacZ* gene, encoding ß-galactosidase. The expression signals of the *L. lactis* SK11 *prtP* gene, and the DNA encoding the first 9 amino acids of the proteinase, were used in an in frame fusion with the *E. coli lacZ* gene. High levels of ß-galactosidase activity were detected in *L. lactis* containing this gene fusion. The expression of this heterologous lactose-degrading enzyme by *L. lactis* resulted in lactose fermentation by these bacteria (De Vos and Simons, 1988).

Furthermore, fusions of the *lacZ* gene to the P32 promoter and translational initiation signals have been used in studies on the efficiency of translational coupling in *L. lactis* (Van de Guchte et al., 1991). These studies revealed that translational coupling could result in up to 3-fold higher heterologous gene expression in *L. lactis*.

4.1.3 Lysozyme

The antimicrobial activity of hen egg lysozyme is of commercial interest as a

food preservative. One of the randomly isolated lactococcal chromosomal promoters, P32, has been used in expression vectors for the production of hen egg lysozyme (Van de Guchte et al., 1989). A low level of expression could be observed, but no activity was detected. In contrast, expression of the phage T4 lysozyme and phage lambda lysozyme in *L. lactis*, using the same P32 expression signals, resulted in the detection of lysozyme activity in cell lysates (Van de Guchte et al., 1992b).

4.1.4 *C. acetobutylicum* ß-galactosidase.

The ß-galactosidase gene of *C. acetobutylicum* has been cloned, and transformed to *L. lactis*. This resulted in expression of the gene under control of its own regulatory sequences. ß-galactosidase activity was obtained in *L. lactis*. However, no metabolization of lactose was reported. (Pillidge and Pearce, 1991).

4.1.5 *E. coli* ß-glucuronidase

The *E. coli gusA* gene has been used as a reporter gene for promoter screening in *L. lactis*. Furthermore the *gusA* gene has been used to quantitate the activity of several characterized lactococcal promoters in *L. lactis*. All tested promoters were functional in producing ß-glucuronidase in *L. lactis*. The *usp45* promoter resulted in the highest levels of ß-glucuronidase activity (Platteeuw et al., 1993b).

4.1.6 *Vibrio fischeri* luciferase

Several *Lactobacillus casei* chromosomal promoter elements have been used to express the *lux* gene from *V. fischeri* in several lactic acid bacteria. These studies showed that the expression signals from *Lb. casei* were functional in the expression of the *lux* gene in *L. lactis* (Ahmad and Stewart, 1992).

Furthermore, the *luxAB* genes were used as reporter genes to investigate the regulation of expression of the *lacABCDFEGX*, and the *lacR* promoter (Eaton et al., 1993).

4.2 Extracellular proteins

The homologous signal peptides from Usp45 and PrtP have proven to be functional in directing secretion of heterologous proteins in *L. lactis*. Furthermore, random isolated export elements from *L. lactis* and some heterologous signal peptides can be used to accomplish secretion in *L. lactis*.

TABLE 2: Heterologous proteins produced by *L. lactis*.

Protein	gene	Source	activity	expression signals	reference
Intracellular					
ß-galactosidase	*lacZ*	*E. coli*	+	*prtP*	De Vos and Simons, 1988
ß-galactosidase	*lacZ*	*E. coli*	+	P32	Van de Guchte et al., 1991
egg white lysozyme	HEL	Hen	-	P32	Van de Guchte et al., 1989
T4 lysozyme	T4 *e*	*E. coli* phage T4	+	P32	Van de Guchte et al., 1992b
λ lysozyme	λ *R*	*E. coli* phage λ	+	P32	Van de Guchte et al., 1992b
ß-glucoronidase	*gusA*	*E. coli*	+	e.g. *usp45, lac*	Platteeuw et al., 1993b
ß-galactosidase	*lac*	*C. acetobutylicum*	+	*lac*	Pillidge and Pearce, 1991
Luciferase	*lux*	*V. fischeri*	+	random *Lb. casei*	Ahmad and Stewart, 1992
Prochymosin	PC	Bovine	-	*prtP*	Simons et al., 1992
Secreted				**Expression/secretion signal**	
Prochymosin	PC	Bovine	-	*prtP*	Simons et al., 1992
Neutral protease	*nprE*	*B. subtilis*	+	P32/*nprE*	Van de Guchte et al., 1990
ß-lactamase	*ß-lam*	*E. coli*	+	random	Sibakov et al., 1991
α-amylase	*amyL*	*B. licheniformis*	+	random	Pérez-Martínez et al., 1992
α-amylase	*amyS*	*B. stearothermophilus*	+	*prtP*	Simons et al., 1990b
α-amylase	*amyS*	*B. stearothermophilus*	+	*usp45*	Van Asseldonk et al., 1993b
α-amylase	*amyS*	*B. stearothermophilus*	+	*dnaJ/usp45*	Van Asseldonk et al., 1993a
α-galactosidase	*α-gal*	*C. tetragonoloba*	+	P59, *prtP,prtM/amyL*	Haandrikman et al., 1990
Streptodornase	*sdc*	*S. equisimilis*	+	*sdc*	Wolinowska et al., 1991

4.2.1 Bovine prochymosin

Chymosin establishes the initial cleavage of k-casein and hence is an important enzyme in the process of cheesemaking. Various expression and secretion cassettes have been constructed for the production of prochymosin in *L. lactis* (Simons et al., 1988; Simons et al., 1992). These cassettes contained the *prtP* promoter sequence, ribosome binding site and various parts of the coding sequence of the SK11 proteinase fused to prochymosin. A fusion with the coding sequence for the first 7 amino acids of PrtP, resulted in intracellular accumulation of prochymosin (Simons *et* al., 1992). This intracellular fraction could not be activated at low pH. Fusion of the coding sequences of prochymosin and the first 33 or the first 62 amino terminal residues of PrtP directed the synthesis and secretion of prochymosin. Although the putative signal peptide of PrtP consists of the first 33 amino acids, the secretion efficiency could be increased when the first 62 amino acids were used. This may be due to positively charged residues present at the amino-terminal end of prochymosin. The presence of this charged region immediately after the signal peptide cleavage site

is most likely to obstruct efficient secretion of the protein.

4.2.2 *B. subtilis* neutral protease.

The *B. subtilis* neutral protease is known to degrade casein and hence can be used to accellerate the cheese ripening. The *nprE* gene encoding this protein, has been introduced into *L. lactis* on the lactococcal cloning vector pGKV210. No protease expression could be detected in cellular or supernatant fractions of *L. lactis* strains harboring this gene. However, when the *nprE* transcription signals were replaced by the lactococcal promoter P32, a significant amount of neutral protease was secreted by *L. lactis*. Determination of intracellular accumulation of the precursor of the protein was not reported. These data show that the *nprE* promoter is not functional in *L. lactis*, but that the heterologous signal peptide of the protease is recognized by the secretion machinery of *L. lactis* (Van de Guchte et al., 1990).

4.2.3 *E. coli* TEM ß-lactamase.

Signal-peptide-probe vectors have been constructed for *L. lactis* employing the *E. coli* TEM *bla* gene as a reporter gene. Random cloning of lactococcal chromosomal DNA fragments resulted in the isolation of several DNA sequences which directed the secretion of ß-lactamase in *L. lactis*. (Sibakov et al., 1991; Pérez-Martinez et al., 1992). The production of this enzyme was increased when a strong promoter was used for expression (Sibakov et al., 1991). Furthermore, an additional increase in ß-lactamase production could be obtained using the lysozyme-sensitive *L. lactis* strain, V207. Unfortunately, efficiency of secretion was not reported.

4.2.4 *B. licheniformis* α-amylase

Besides the ß-lactamase gene, the *B. licheniformis amyL* gene has been used for the selection of export functions from lactococcal DNA. Several isolated export signals resulted in production and secretion of active α-amylase in *L. lactis*. Up to ninety percent of the activity was located in the extracellular medium. These studies showed that lactococci are able to secrete this enzyme when the proper expression and secretion signals were used (Pérez-Martinez et al., 1992).

4.2.5 *B. stearothermophilus* α-amylase

The *amyS* gene of *B. stearothermophilus* has been used to test the applicability

of the expression and secretion signals of the *prtP* and the *usp45* gene for heterologous gene expression. Several fusions have been constructed encoding the PrtP or Usp45 putative signal peptides fused to the mature α-amylase. In *L. lactis* these fusions resulted in secretion of active α-amylase into the culture supernatant (Simons et al., 1990b; Van Asseldonk et al., 1993a; Van Asseldonk et al., 1993b).

4.2.6 *Cyamopsis tetragonoloba* α-galactosidase

A fusion between the *B. subtilis* α-amylase signal sequence and the mature α-galactosidase gene from *C. tetragonoloba* has been used as a reporter gene in a vector developed for the analysis for divergent expression signals (Haandrikman et al., 1990). A translational fusion of the expression signals of *prtP*, *prtM* or the chromosomal promoter P59 and the α-amylase-α-gal fusion resulted in significant α-galactosidase activity in the culture supernatant. These results show that the *B. subtilis* α-amylase signal peptide is sufficient to direct the secretion of the eukaryotic α-galactosidase in *L. lactis*.

4.2.7 *Streptococcus equisimilis* streptodornase

Introduction of the *sdc* gene, encoding the nuclease streptodornase of *S. equisimilis*, on a lactococcal cloning vector, resulted in extracellular DNase activity, suggesting that the *sdc* expression and secretion signals are functional in *L. lactis*. However, the secretion of streptodornase is not efficient since a considerable amount of DNase activity remained intracellular (Wolinowska et al., 1991) .

5. Outline of this thesis

To investigate whether *L. lactis* is an attractive organism for the production of heterologous proteins, a model system for heterologous gene expression has been developed based on the *B. stearothermophilus amyS* as a reporter gene. This system has been used to analyze various features of expression and secretion signals of *L. lactis*, and their capacity to direct heterologous protein production.

In **Chapter two** the identification of extracellular proteins in the culture supernatant of *L. lactis* is described. Furthermore, the cloning in *E. coli* of the *usp45* gene, encoding the major secreted lactococcal protein, Usp45, and the sequence and transcriptional analysis of this gene is reported.

Chapter three describes the construction of vectors based on the expression and secretion signals of the *usp45* gene for the secretion of the homologous PrtP and the heterologous AmyS. This chapter provides insight in the secretion capacity of *L. lactis*. In addition, the role of the signal peptide length in secretion in *L. lactis* and *E. coli* is investigated.

In **Chapter four** attempts to improve the developed expression system based on the expression and secretion signals of *usp45* and the *B. stearothermophilus amyS*, are described. The influence of the region upstream of the promoter region, the gene dose, and gene location on heterologous expression in *L. lactis* are investigated.

Chapter five represents a pilot study on the ribosome binding site of the *usp45* gene. The role of the two putative SD sequences in gene expression is investigated by site-directed mutagenisis using the α-amylase expression vector.

In **Chapter six** the cloning, sequence and transcriptional analysis of the lactococcal *dnaJ* gene is described. The expression signals of this gene are used to construct an inducible α-amylase secretion system in *L. lactis*.

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CHAPTER 2

CLONING, EXPRESSION IN *ESCHERICHIA COLI* AND CHARACTERIZATION OF *USP45*, A GENE ENCODING A HIGHLY SECRETED PROTEIN FROM *LACTOCOCCUS LACTIS* MG1363.

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Perfect Strangers (Deep Purple)

SUMMARY

We have cloned *usp45*, a gene encoding an extracellular protein of *Lactococcus lactis* subsp. *lactis* strain MG1363. Usp45 is secreted by every mesophilic lactococcal strain we tested so far and is chromosomally encoded. The nucleotide sequence of the *usp45* gene revealed an open reading frame of 1384 bp encoding a protein of 461 amino acids. The mature protein initiates at Asp 28 resulting in a signal peptide of 27 amino acids. The gene contains a consensus promoter sequence, but a weak ribosome binding site could be identified which is rather uncommon for gram-positive bacteria. Expression studies in *E. coli* showed efficient synthesis and secretion of the protein.

Usp45 has an unusual amino acid composition and distribution and is predicted to be structurally homologous with P54 of *Enterococcus feacium*. Up to now, no biological activity could be postulated for this highly secreted protein.

INTRODUCTION

Lactococci (*Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*) are used on a large scale as starter cultures in the manufacturing of dairy products such as cheese, butter, yoghurt and buttermilk.

Recently, the complete nucleotide sequence of the genes for two extracellular proteins e.g. proteinase *prtP* (Vos et al., 1989b, Kok et al., 1985, De Vos et al., 1989) and the bacteriocin nisin (Buchman et al, 1988) have been determined. The proteinase is synthesized as a preproprotein with a consensus ala/ala signal peptidase I cleavage site between residue 33 and 34. It has been demonstrated that this signal peptide directs the secretion of heterologous proteins in lactic acid bacteria (Simons et al, 1988). The proteinase shows significant sequence homology to a number of serine proteinases of the subtilisin family and also contains a C-terminal membrane anchor (Vos et al, 1989b). The gene for the bacteriocin nisin encodes a precursor polypeptide of 57 amino acids with a molecular weight of 7,500. A signal peptide of 23 amino acids has been deduced which deviates from the consensus signal peptide rules as postulated by Von Heijne (1985). A series of post-translational modifications involving dehydration of serine and threonine residues and crosslinking with cysteine residues are required to obtain biologically active nisin.

To gain more insight in the properties and structure of lactococcal extracellular proteins, we screened lactococcal strains for extracellular proteins. In this paper, we report the cloning of a gene encoding a 45 kDa protein which is secreted into the extracellular medium as an apparent 60 kDa protein and is produced by every lactococcal strain we tested so far.

MATERIALS AND METHODS

(a) Bacterial strains, phages and plasmids Bacterial strains used are listed in Table 1. As cloning vector for the construction of a genomic library, lambda phage 47.1 (Loenen & Brammar, 1980) was used. Further subcloning was performed in pUC19 (Vierra and Messing, 1982) and in M13mp18/mp19 (Norrander et al, 1983).

(b) Media, enzymes and chemicals Lactococci were cultured on a whey based medium: 5% whey permeate, 0,5% casiton (Difco), 1,9% ß-glycerophosphate and 0.5 % glucose. For culturing of *Lactobacillus*, *Leuconostoc* and *Streptococcus* strains the casiton was replaced by 1.5% yeast extract (Difco). Media used for *E.coli* were L-broth (Miller, 1972) and trypticase peptone (Baltimore Biological Laboratories), as broth or solidified by 1.5% agar. Supplements were added at the following concentrations: Carbenicillin (Beecham Pharmaceuticals) 100 µg/ml, X-Gal (Boehringer) 40 µg/ml, IPTG (Bethesda Research Laboratories, BRL) 25µg/ml. Restriction endonucleases and reverse transcriptase were purchased from BRL. T4 DNA ligase, polynucleotide kinase were from New England Biolabs and sequenase from United States Biochemical Corporation. [α^{32}P]dATP and [^{32}P]dATP (3000Ci/mmol.) were obtained from Amersham. Oligonucleotides were synthesized on a Biosearch Cyclone DNA synthesizer (New Brunswick Scientific Corp.).

(c) Construction and screening of a genomic library of *L.lactis* MG1363 Chromosomal DNA was extracted as follows: *L.lactis* cells were grown until E_{600} = 1.0 and protoplasted in THMS-buffer (25% sucrose, 30 mM Tris-HCl pH 8.0, 3 mM $MgCl_2$) containing 2 mg/ml lysozyme (Sigma) for 1 hour at 37°C. After centrifugation (6000g). Protoplasts were resuspended in 1/20 volume TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and extracted 3-4 times with phenol. Subsequently, the DNA was dialyzed against TE. A genomic library was constructed as described by Maniatis (1982) in

Table 1	Bacterial Strains	
Strain	**Characteristics**	**Source or Reference**
E. coli		
JM83	*ara rpsL* ▲*(lac-proAB)*	Messing (1983)
JM103	*supE thi* ▲*(lac-proAB)*	Messing (1983)
MB406	*supE recB*22 *recC*21 *sbcB*15	W. Dove, University of Wisconsin
L. lactis subsp. *lactis*		
MG1363	plasmid-free	Gasson (1983)
IL1403	plasmid-free	Chopin et al. (1984)
NIZO R1		NIZO
NZ22186	Nis^+ Suc^+	NIZO
NCDO176	var. diacetylactis	Natl. Collection of Dairy Organisms (NCDO)
subsp. *cremoris*		
SK110	Prt^+ Lac^+	De Vos & Davies, 1984
NCDO1200	Prt^+ Lac^+	NCDO
WG2	Prt^+ Lac^+	NIZO

phage lambda 47.1 using packaging mixtures from Promega. The library was plated on *E.coli* strain MB406 and replicas were made on nitrocellulose filters (Schleicher and Schuell). Filters were blocked for 1 hr in PBS-buffer (10 mM NaPi pH 7.0, 0.85% NaCl) containing 1 % BSA and incubated for at least 4 h with antibodies against the 60 kDa protein in PBS-buffer containing 0.1% BSA. The blots were subsequently incubated with Swine anti rabbit peroxidase conjugate (Swarpo) (Dakopatts). The proteins were visualized by incubation in substrate solution (40 µg 4-chloro-1-naphthol in 100 ml of 50 mM Tris-HCl pH 7.5 containing 30 µl H_2O_2).

(d) RNA extraction *L.lactis* MG1363 cells were grown at 30°C until E_{600} = 0.4-0.6. Cells were protoplasted in THMS-buffer containing 10 mg/ml lysozyme for 10 min on ice and centrifuged. RNA was extracted from the protoplasts by the hot-phenol method (Markham et al. 1984).

(e) Protein purification Extracellular proteins of *L.lactis* MG1363 were separated on SDS/PAGE (Laemmli,1970).The 60 kDa protein was excised from the gel and recovered by isotachophoresis (Öfverstedt et al, 1983).

RESULTS AND DISCUSSION

(a) Characterization of secreted proteins of lactococci.

L.lactis subsp. *lactis* and *L.lactis* subsp. *cremoris* strains as listed in Table 1 were screened for secreted proteins by analyzing culture supernatant fractions on SDS/PAGE (Laemmli, 1970). As shown in Fig. 1A, all screened strains secrete proteins, of which the most abundant one had an apparent molecular weight of 50-60 kDa. The amount of this secreted protein was estimated to be 2-4 $\mu g/ml/E_{600}$.

To investigate whether these 50-60 kDa proteins were related to each other, antibodies against the 60 kDa protein, isolated from the plasmid-free *L.lactis* strain MG1363, were raised in rabbits. Immunoblot analysis (Towbin et al, 1979) of the culture supernatant fractions showed a strong reaction between the 50-60 kDa proteins of all tested strains and the isolated antibodies (Fig. 1B) indicating an immunological relationship between these proteins. No 50-60 kDa proteins could be detected in cell extracts of the tested strains, indicating that these proteins are transported efficiently into the extracellular medium and are not associated with the cell envelope as is the case for components of the proteolytic system of lactococci (Vos et al, 1989b., Kok et al., 1985).

Extracellular proteins of *Leuconostoc paramesenteroides*, *Streptococcus thermophilus*, *Lactobacillus casei*, *Lb.acidophilus* and *Lb.bulgaricus* strains showed no reaction with the antibodies (data not shown) suggesting that this protein is specific for lactococcal subspecies and does not have a counterpart in other lactic acid bacteria.

Since every lactococcal strain we tested produces this 50-60 kDa protein and no strains defective in synthesis of this protein were available, it was not possible to postulate a function for this protein. Lactococci are known to produce extracellular proteins such as proteinases, peptidases and bacteriocines (Vos et al, 1989b, Kok et al, 1985, De Vos et al 1989, Buchman et al, 1988). However, no proteolytic activity of the 60 kDa protein of MG1363 on casein, its degradation products or on synthetic substrates could be detected, indicating that the protein has presumably no function in the proteolytic system of *L.lactis*. In addition, no antimicrobial activity against other gram- positive bacteria, assayed by the method of Fowler (1975), could be assigned to the protein.

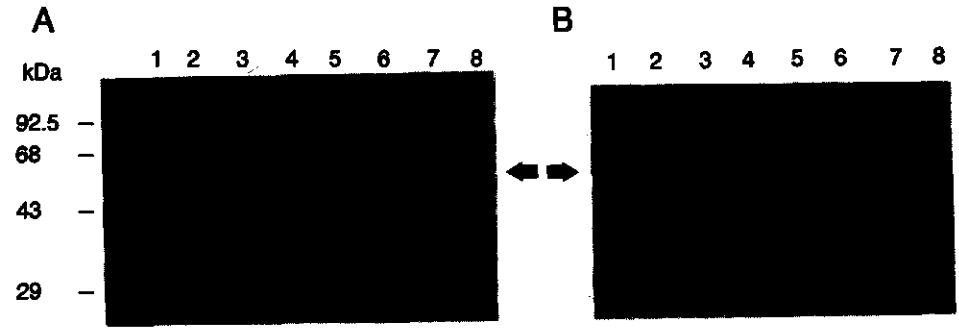

Fig. 1: Gel analysis of extracellular proteins of *L.lactis* strains. Supernatants of logarithmic growing cultures were dialyzed against water and concentrated by lyophilisation. Samples were analyzed by 10% SDS/PAGE (Leammli, 1970) and immunoblotting. (Panel A) Coomassie brilliant blue staining of 1,0 ml of supernatant; (Panel B) Immunoblot of 0.2 ml supernatant. 1, MG1363; 2, IL1403; 3, R1; 4, NZ22186; 5, NCDO176; 6, SK1128; 7, NCDO1200; 8, WG2. The position of the 50-60 kDa proteins is indicated by arrowheads. The lanes are flanked on the left side by molecular weight markers, indicated in kDa.

(b) Cloning and expression of the gene for the 60 kDa protein in *E.coli*.

Since the plasmid-free strain MG1363 is able to produce the 60 kDa protein, chromosomal DNA of this strain was used as starting material for the cloning of the gene of the 60 kDa protein. A genomic library of *L.lactis* MG1363 was constructed and screened with the isolated antibodies. A restriction enzyme digestion map from the DNA insert of one of the phages which reacted with the antibodies is shown in Fig. 2.

To verify that the gene for the 60 kDa protein was located on the insert, Southern blot analysis of the insert DNA was performed. For this purpose we determined the 10 amino-terminal amino acids of the mature protein with a gasphase sequenator (Applied Biosystems). The sequence found was Asp-Thr-Asn-Ser-Asp-Ile-Ala-Lys-Gln-Asp. Mixed oligonucleotide probes were derived against the last six determined the 10 amino-terminal amino acids of the mature protein which had the least degenerate codon usage. Hybridization studies of the digested phage DNA with these probes showed that the gene for the 60 kDa protein was found to be located on a *KpnI/EcoRI* fragment of ± 3 kb (Fig. 2A).

To show that this fragment contained the complete gene for the 60 kDa protein, it was cloned in pUC19 generating pNZ1011. Immunoblot analysis of cellextracts of *E.coli* JM83, containing pNZ1011, showed synthesis of the protein (Fig. 3, lane 2).

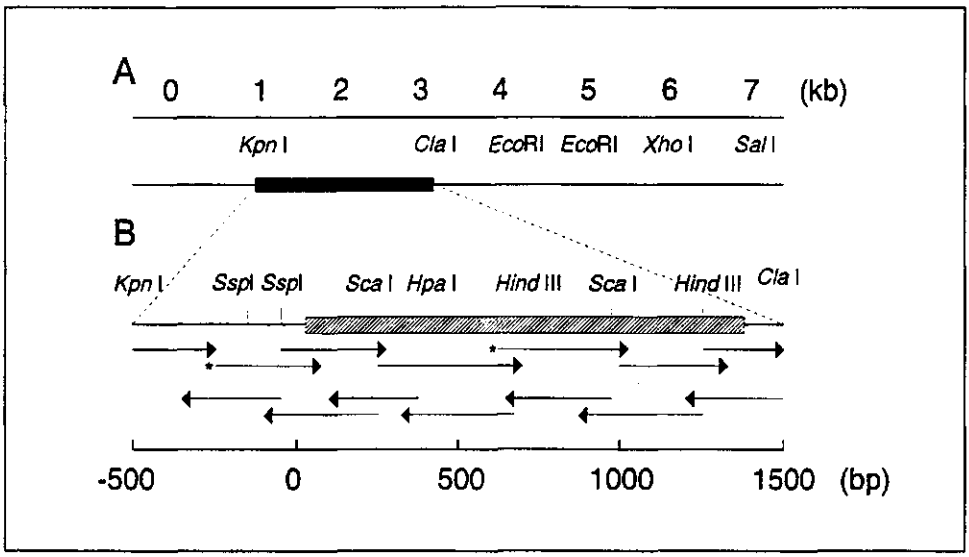

Fig 2: Restriction map and sequence strategy of the gene encoding the 60 kDa protein from MG1363. (A) Restriction map of the inserted DNA of a positive phage clone. Hybridisation with the mixed set oligonucleotides 5'-GA[C/T]-AT[A/C/T]-GCN-AA[A/G]-CA[A/G]-3'as probe, is indicated with a black bar. (B) Restriction map and sequence strategy of the *KpnI/ClaI* fragment of pNZ1011. The map shows the coding region of the gene (hatched bar). The arrows indicate the length and direction of the individual stretches sequenced. M13 primers and synthetic oligoprimers (marked with *) were used. Sequencing was performed using the dideoxy termination method (Sanger, 1977) following the sequenase protocol (Tabor & Richardson, 1987).

Moreover, a substantial amount of the 60 kDa protein could be detected in the periplasmatic fraction of the cells (lane 3). To test whether the small amount of 60 kDa protein which was still present in the stripped cells (lane 4) was due to incomplete isolation of the periplasmatic fraction, ß-lactamase assays (Miller, 1972) were performed (data not shown). The ratio of ß-lactamase activity in the periplasmatic fraction compared to stripped cells (4:1) was in complete agreement with the amounts of 60 kDa protein detected within these two fractions. Hence, we conclude that the 60 kDa protein is transported into the periplasmatic space of *E.coli* cells.

It has been demonstrated that regulatory sequences of gram-positive organisms such as Bacilli and Staphylococci can function efficiently in *E.coli* (Makaroff et all, 1983, McLaughlin et al, 1981, Graves & Rabinowitz, 1986). Our data show that the regulatory sequences of this lactococcal gene and the signal sequence of its gene product are also recognized in *E.coli*.

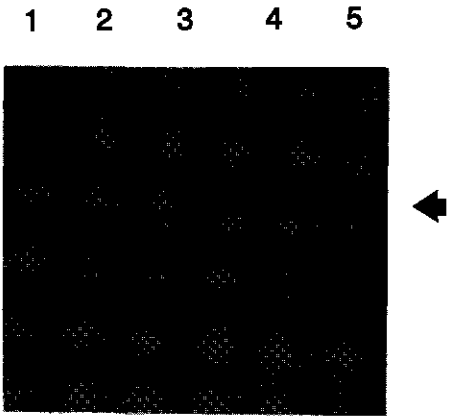

Fig. 3: Analysis of proteins encoded by *E.coli* JM83 cells harbouring pNZ1011. Cells were fractionated by osmotic shock (Neu & Heppel, 1965) resulting in a periplasmatic fraction and a stripped cells fraction. The immunoblot was probed with the antibody against the 60 kDa protein from MG1363. Lanes: (1) JM83 harbouring pUC19, unfractionated cells. (2), (3) & (4) JM83 harbouring pNZ1011. (2) unfractionated cells, (3) stripped cells, (4) periplasmatic fraction. (5) MG1363, culture supernatant. The position of the 60 kDa protein is indicated.

(c) Nucleotide sequence of the gene for the 60 kDA protein.

The nucleotide sequence of the *KpnI/ClaI* fragment from pNZ1011 was determined (Fig 4) following the sequencing strategy outlined in fig 2B.

The DNA sequence encoding the first 10 N-terminal amino acids of the mature protein was found at nucleotide position 125-154 and followed by an open reading frame encoding a mature protein of 434 amino acids. This protein has a predicted molecular mass of 44628 Da and it was designated Usp45 (Unidentified Secreted Protein-45).

Upstream of the mature N-terminus, three in frame initiation codons are located. The most likely initiation codon is ATG at positon 44, since it precedes a precursor-region which has the characteristics of a signal peptide: several basic residues followed by a hydrophobic core of 15-20 residues and a shorter uncharged region ending with an amino acid carrying a small side chain (Von Heijne, 1985). Although the highest cleavage probability by signal peptidase I is C-terminal of Ala-19 according to the von Heijne (1986) rules, the observed cleavage after Ala-27 in the sequence Val-X-Ala is also commonly found in prokaryotes, and particularly in gram-positives (Von Heijne and Abrahmsén, 1989).

The ATG start codon at position 44 is preceded by two potential Shine and Dalgarno sequences (Shine and Dalgarno, 1974) e.g. the sequence GGAGG at position 19 to 23 and AAAG at 34 to 37. The calculated free energy of the complementarity between the 3' of the 23S rRNA of *L. lactis* and the latter Shine and Dalgarno sequence is only -4.6 kcal/mol (Tinoco et al, 1973). Free energy values of complementarity between the 3' end of 16S-rRNA and Shine and Dalgarno sequences of ribosome binding sites from lactococci (De Vos, 1987) and other gram-positive organisms like Bacilli (McLaughlin et al, 1981) usually range between -10 and -15

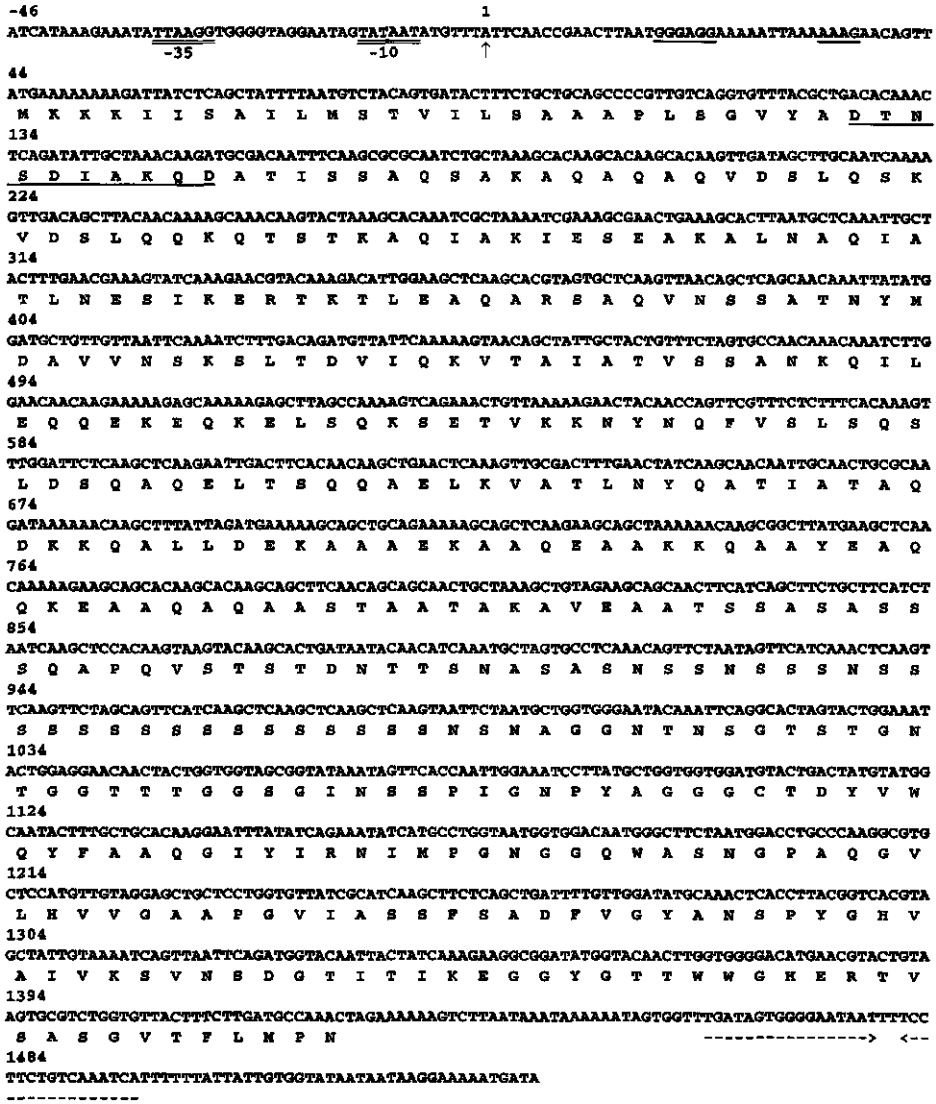

Fig 4: Nucleotide sequence of the *usp45* gene and the deduced amino acid sequence. The sequences represent the -35 and -10 promoter boxes. The start of transcription is indicated with an arrow. The two putative ribosome binding sites are also underlined.The determined N-terminal amino acids (Asp 28 - Asp 37) of the mature protein are underlined. Downstream of the gene an inverted repeat is marked whith dotted arrows representing a rho-independent terminator of transcription.

kcal/mol. For the other possibility a free energy of -14.4 kcal/mol could be calculated. However, the spacing to the ATG codon is 21 bp. This length does not fit with the Shine and Dalgarno hypothesis. Site directed mutagenesis experiments have to be

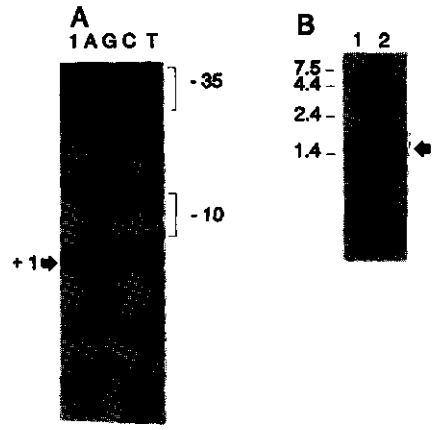

Fig. 5: RNA analysis and Northern blotting. A) Autoradiogram of the sequence gel used to analyze the primer extension products of RNA of MG1363. cDNA was generated by reverse transcriptase elongation with the oligoprimer 5'CAGCAGAAAGTATCACTG 3' (nucleotide position 98 to 80). The sequence ladder obtained by using the same primer was used as a marker. The position of the transcription initiation site and the -10 and -35 boxes are indicated.The primer extension product is indicated (arrowhead). B) Autoradiogram of a Northern blot of total RNA of MG1363 hybridized with a M13-generated probe complementary to the usp45 gene. The 1.5 kb messenger is indicated (arrow). To the left, the size of RNA markers (Bethesda Research Laboratories) are shown in kb.

performed to determine whether the weak Shine and Dalgarno sequence is used and what the contribution is of the GGAGG sequence to the initiation of translation of the *usp45* gene.

Primer extension experiments (Debarbouille and Raibaud, 1983) were performed (Fig. 5A) and showed that transcription of the *usp45* gene was initiated with an adenine residue at nucleotide position 1. A consensus -10 hexanucleotide sequence (TATAAT) was found at position -12 to -7. The most probable corresponding -35 sequence is TTAAGG (-32 to -27), although the spacing between the two is smaller (14bp) than that usually found in promoters in *E.coli* (Hawley & Mclure, 1987) and *L.lactis* (de Vos, 1987, van der Vossen et al., 1987).

Inspection of the DNA sequence showed the presence of several repetitive sequences the hexanucleotide sequence GC[A/C/G/T]CAA occurs 19 times in the gene, of which 16 times in the open reading frame, whereas the more degenerated sequence TC[A/T]AG[C/T] appears 16 times. Translation of this sequence would result in 12 serine doublets of which 7 are in tandem in one part of Usp45, resulting in an unusual amino acid sequence in the protein as discussed in **(d)**.

Downstream of the TAG stop codon of the usp45 gene an inverted repeat was found at nucleotide position 1457 to 1495, which could form a hairpin-like structure with a free energy of -8.2 kcal/mol (Tinoco et al, 1973). The formed hairpin is flanked on both sides by A or T stretches and hence could function as a rho-independent, bi-directional terminator of transcription. Northern blot analysis revealed a messenger

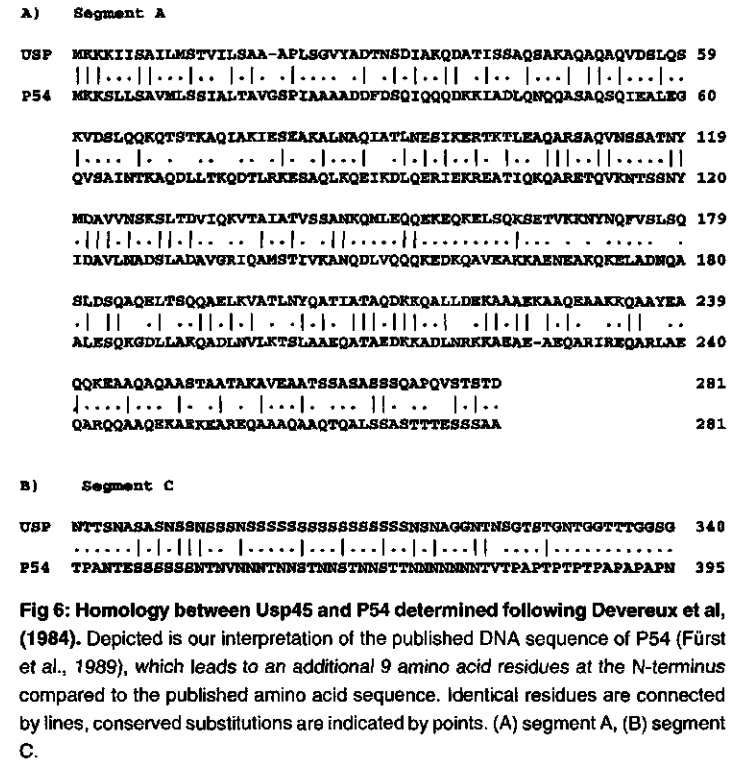

Fig 6: Homology between Usp45 and P54 determined following Devereux et al, (1984). Depicted is our interpretation of the published DNA sequence of P54 (Fürst *et al., 1989), which leads to an additional 9 amino acid residues at the N-terminus* compared to the published amino acid sequence. Identical residues are connected by lines, conserved substitutions are indicated by points. (A) segment A, (B) segment C.

RNA of 1500 nucleotides (Fig. 5B). This is in agreement with the deduced transcription termination.

(d) Amino acid sequence, homology analysis and structural predictions of Usp45

The amino acid sequence of Usp45 as shown in Fig. 4 reveals a high content of serine and alanine residues, together more than 32%. In one region of the protein, from residues 264-316, serine residues constitute 33 out of 53 residues, including a continuous stretch of 16 residues.

Data base searches for homology were performed with the SWISSPROT (release 12.0) and the NBRF (release 22.0) protein sequence libraries. The N-terminal two-thirds of the Usp45 amino acid sequence shows weak homology with myosins, keratins and streptococcal M proteins, which presumably reflects a common type of α-helical secondary structure (Cohen & Parry,1986).

Significant homology was detected with the recently published sequence of P54

Table 2.

Characteristics of the Usp45 and P54 domains.[a]

Domains	Signal peptide		SegmentA		SegmentB		SegmentC		SegmentD	
	Usp45	P54	Usp45	P54	Usp45	P54	Usp45	P54	Usp45	p54
Residues	1-27	1-28	28-281	29-281	-	282-336	282-340	337-395	341-461	396-516
Main aa	A,S,I	A,S,I	A,Q,S,K	A,Q,K,E		S,T,F	S,N,T,G	N,T,S,P	G,A,V,S	G,V,A,S
Secondary structure	α-helix		α-helix		turn/coil		turn/coil		ß-sheet	
Charge	low; positive		high; neutral		high; negative		none		low; neutral	
Hydropathy	hydrophobic		amphipathic		hydrophilic		hydrophilic		hydrophobic	
Flexibility	low		low		high		high		low	

[a] Secondary structure, hydropathy and flexibility predictions are determinated using the PCgene (version 5.01) nucleic-acid and protein sequence analysis software system (Genofit).

protein of *Enterococcus faecium* (Furst et al., 1989), as shown in Fig 6. This protein of unknown function is attached to the cell wall, and is generated from a 516 amino acid precursor. In the N-terminal 281 amino acids, including the signal peptide, 31% of the residues are identical and another 53% are conservative substitutions (Fig. 6A). Besides the homology on primary amino acid sequence level, there appears to be an overall resemblance of the two proteins on the secondary and tertiary structure level. On the basis of structural predictions and amino acid compositions, the two mature proteins can be divided into four segments with similar characteristics, as outlined in Table 1.

Segment A, immediately after the signal peptide, is predicted to be almost exclusively α-helical by the method of Garnier (Garnier et al, 1978).

Segment B is unique for P54 and is highly negatively charged due to a large excess of glutamic acid residues. It is predicted to be highly hydrophilic and flexible. A corresponding segment is absent in Usp45.

Segment C consists mainly of the residues Ser, Asn, Gly and Thr (Pro instead of Gly in P54) which are typical helix-breakers and more commonly found in turns and random coil secondary structure (Deleage and Roux, 1987). Characteristic is the long stretch of Ser and Asn residues in both proteins at the N-terminal end of this segment, as shown in Fig 6, while Gly and Pro residues are concentrated at the C-terminal end.

Segment C is also extremely hydrophilic: 80% of the residues in Usp45 contain either a hydroxyl or carboxyl group, but there are no charges in this segment.

Segment D is predicted to have largely ß-sheet secondary structure in short stretches, alternating with turns or bends. The amino acid compositions of segment D are very similar in Usp45 and P54, and contain much higher contents of aromatic and other hydrophobic residues than segments A, B and C.

(f) Conclusions

(1) Screening of various *L.lactis* strains for the production of extracellular proteins revealed a protein, designated Usp45, which is secreted in large amounts by every lactococcal strain we tested.

(2) The gene for Usp45 was located on the bacterial chromosome and was cloned into *E.coli*. It was expressed in *E.coli* under control of its own regulatory sequences and the gene product was secreted into the periplasmatic space of this organism.

(3) The nucleotide sequence of the *usp45* gene was determined and the deduced amino acid sequence showed an unusual amino acid composition and distribution of the protein.

(4) Usp45 shows homology with P54, an extracellular protein of *Enterococcus faecium*. Besides homology on the amino acid level the two have the same structural characteristics, but until now the function remains unclear.

(5) The cloning of the *usp45* gene may be an introduction for further investigation of the secretory pathway of *L.lactis* and could lead to the construction of expression and secretion vectors in these food-grade dairy micro-organisms for the production of homologous and heterologous proteins.

Acknowledgements

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CHAPTER 3

FUNCTIONAL ANALYSIS OF THE *LACTOCOCCUS LACTIS USP45* SECRETION SIGNAL IN THE SECRETION OF A HOMOLOGOUS PROTEINASE AND A HETEROLOGOUS α-AMYLASE.

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In and Out (Van Halen)

SUMMARY

The *usp45* gene encodes the major extracellular protein from *Lactococcus lactis*. The deduced 27-residue leader peptide revealed the tripartite characteristics of a signal peptide. This leader peptide directed the efficient secretion of the homologous proteinase (PrtP) in *L. lactis*, indicating that the putative signal peptide of PrtP can be replaced by the 27-residue Usp45 leader peptide. In addition, the 27-residue leader peptide could be used to secrete the *Bacillus stearothermophilus* α-amylase, encoded by the *amyS* gene. Fusion of the *usp45* promoter region and various parts of the leader sequence to the *amyS* gene, devoid of its signal sequence, showed that in *E. coli* the first 19, 20, and 27 residues of the Usp45 leader are able to direct α-amylase secretion. In *L. lactis* the shorter signal peptides did not result in secretion of α-amylase, providing experimental evidence for the hypothesis that Gram-positive bacteria require a longer signal peptide for secretion than Gram-negative organisms.

INTRODUCTION

The structural characteristics of signal peptides of procaryotic origin are highly conserved (Von Heijne and Abrahmsén 1989). The most common type of signal peptide consists of a positively charged N-terminus, a central hydrophobic core and a C-terminal cleavage region (for a review see Pugsley 1989). While the structural features are conserved between bacterial signal peptides, their sequences and lengths may vary. Examination of the variation in length of known signal peptides has led to the observation that signal peptides from Gram-positive organisms tend to be longer (mean length 29-31 residues) than signal peptides from Gram-negative bacteria (mean length 24 residues) (Von Heijne and Abrahmsén 1989). However, there exists no experimental evidence that shorter signal peptides are incapable of directing secretion in Gram-positive organisms.

Lactococci are Gram-positive microorganisms capable of secreting proteins across the cell-envelope into the culture medium (Simons et al. 1990). Until now, no detailed investigations on lactococcal signal peptides have been reported. A few extracellular proteins from lactococci have been characterized such as the cell-envelope-located proteinase (PrtP; Vos et al. 1989a) with a putative signal peptide of

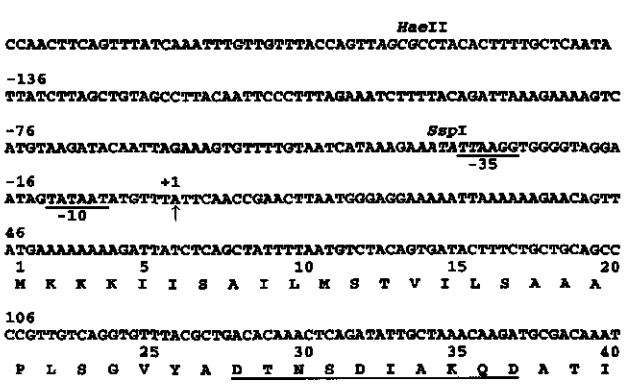

Fig. 1: The nucleotide sequence encoding the N-terminal part of Usp45, its translation and upstream sequences. The start of transcription is indicated by a vertical arrow. The -10 and -35 promoter sequences are underlined. Relevant restriction sites are indicated. Predicted signal peptidase I cleavage sites are indicated by vertical arrows. The determined N-terminus of the mature protein is underlined (Van Asseldonk et al., 1990).

33 residues and the maturation protein (PrtM; Haandrikman et al. 1991) with a 23-residue lipoprotein signal sequence. Other functional signal sequences have been isolated from *Lactococcus lactis* by using a signal peptide probe vector, which was based on the *Escherichia coli* TEM-ß-lactamase gene devoid of its signal peptide coding sequence (Sibakov et al. 1991). However, processing sites of the putative signal peptides were not determined.

Recently, we have cloned the *usp45* gene of *L. lactis* encoding the major extracellular protein of lactococci (Van Asseldonk et al. 1990). Amino-terminal analysis revealed that the mature Usp45 protein starts at the position of residue 28 in the primary sequence (Fig. 1). In the precursor three putative signal peptidase I cleavage sites were postulated after residues Ala^{19}, Ala^{20} and Ala^{27} (Fig. 1; Van Asseldonk et al. 1990).

In this paper we used the Usp45 leader peptide for the secretion of the homologous PrtP of *L. lactis* and the heterologous α-amylase of *B. stearothermophilus*. In addition, we determined the size limits of the signal peptide coding sequence in the secretion of the heterologous α-amylase.

Table 1. Plasmids used in this study.

Plasmid	Relevant genetic characteristics[a]	Source/Reference
pNZ511	pNZ121 carrying the *prtP* and *prtM* genes; Cm^R	Vos et al. 1989b
pNZ511-mut3	pNZ511 containing a G to C mutation at position 100 of the prtP, generating a *NarI* site at the end of the coding region for the putative PrtP signal peptide; Cm^R	P. Vos (unpublished results)
pNZ516	pNZ121 carrying the *prtP* gene; Cm^R	Vos et al. 1989b
pNZ582	piL153 carrying the *prtM* gene; Em^R	Vos et al. 1989b
pNZ1011	pUC19 containing a *KpnI-EcoRI* fragment carrying the *usp45* gene	Van Asseldonk et al. 1990
pNZ123	pSH71 replicon; Cm^R ; polylinker.	This study
pNZ1024	pNZ123 carrying a *SspI-ClaI* fragment from pNZ1011 carrying the *usp45* gene; Cm^R	This study
pNZ1025	pNZ123 carrying a *HaeII-ClaI* fragment from pNZ1011 containing the *usp45* gene; Cm^R	This study
pNZ1050	pNZ123 carrying nt -33 to 127 of the *usp45 gene* containing the promoter, ribosome binding site and leader peptide coding region; Cm^R	This study
pNZ1052	pNZ123 carrying the *prtP* gene under control of the *usp45* expression and secretion signals; Cm^R	This study
pNZ1054	pNZ123 carrying nt -158 to 127 of the *usp45 gene* containing the promoter, ribosome binding site and leader peptide coding region; Cm^R	This study
pNZα15	pNZ121 carrying the *amyS* gene of *B. stearothermophilus* (Nakajima et al. 1985), under controlof the *prtP* expression and secretion signals; Cm^R	Simons et al. in preparation.
pNZ10α5	pNZ123 carrying the *amyS* gene fused to nt -158 to 127 of the *usp45* gene; Cm^R	This study
pNZ10α6	pNZ123 carrying the *amyS* gene fused to nt -158 to 105 of the *usp45* gene; Cm^R	This study
pNZ10α7	pNZ123 carrying the *amyS* gene fused to nt -158 to 102 of the *usp45* gene; Cm^R	This study

a. Abbreviations: Cm, Chloramphenicol; Em, Erythromycin

MATERIALS AND METHODS.

Bacterial strains, plasmids and media. *E. coli* strain MC1061 (Casadaban et al. 1980) and *L. lactis* strain MG1363 (Gasson 1983) were used as hosts. The plasmids used are listed in Table 1. *E. coli* was grown in TY broth (Rottlander and Trautner 1970) or on TY solidified with 1.5% agar. *L. lactis* was grown in glucose M17 medium (Terzaghi and Sandine 1975) or in whey-permeate culture broth (De Vos et al. 1989). To screen for α-amylase-producing transformants, whey-permeate agar plates containing 0.5% starch were used. The antibiotics used for selection in *L. lactis* were chloramphenicol (10 μg/ml) and erythromycin (5 μg/ml). In *E. coli* 10 μg/ml of

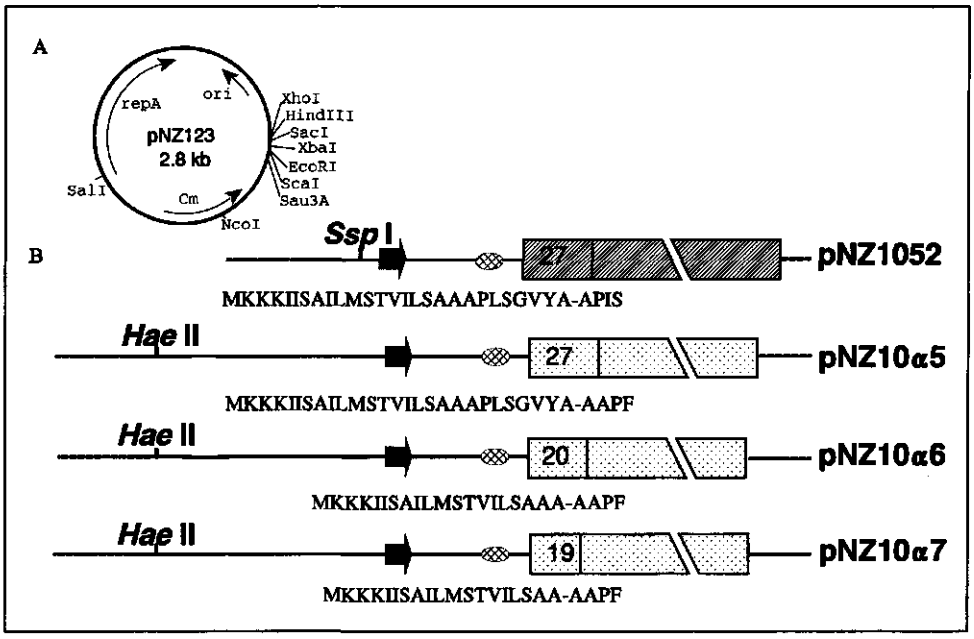

Fig. 2: Schematic representation of pNZ123 (2A) and of the various expression and secretion cassettes (2B) used in this study. The *usp45* promoter is displayed by an arrow and the *usp45* ribosome binding site by an ellipse. The number of encoded residues of the Usp45 leader peptide is shown in the white box. The amino acid sequence of the Usp45 leader peptide and the first 4 amino acids of PrtP or α-amylase is indicated. The DNA encoding the pro-proteinase is drawn as a hatched box and the mature α-amylase as a dotted box. Relevant restriction sites are indicated. The nucleotide sequence at the junctions was verified by DNA sequence analysis.

chloramphenicol was used for selection.

DNA manipulations. Plasmid DNA was isolated as described by Birnboim and Doly (1979). For *L. lactis* cells TSM buffer (30 mM Tris-HCl pH 8, 25% sucrose, 3 mM $MgCl_2$) containing 2% of lysozyme was used for 30 min. at 37°C to prepare protoplasts. Enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, Md.) or New England Biolabs (Beverly, Mass.) and were used as recommended by the suppliers. General procedures for DNA manipulations were essentially as described (Sambrook et al. 1989). For electroporation of *L. lactis*, cells were cultured, washed and recovered as described by Holo and Nes (1989) and plated on glucose M17 agar plates.

Secretion vector constructions. The lactococcal vector pNZ123 (Fig. 2A), based on

the *L. lactis* pSH71 replicon (De Vos 1987), the chloramphenicol resistance gene of pC194 and a useful polylinker sequence, was used as cloning vector for our secretion cassettes. pNZ123 is able to replicate in *E. coli* as well as in various Gram-positive organisms such as *Bacillus subtilis*, lactobacilli species and lactococci (De Vos 1987 and Chassy 1987). Subsequently, the *usp45* gene including regulatory regions were cloned into pNZ123. Plasmids pNZ1024 and pNZ1025, respectively, were constructed by inserting a 1.6 kb *Ssp*I-*Cla*I and a 1.85 kb *Hae*II-*Cla*I *usp45* fragment, isolated from pNZ1011, into the unique *Sca*I site of pNZ123. To this end the *Cla*I sites and *Hae*II site were made blunt by incubation with the Klenow fragment of DNA polymerase I.

To construct the proteinase secretion vector pNZ1052, pNZ1024 was cut with *Pst*I and *Hin*dIII and a synthetic linker was inserted to generate a *Pvu*II site downstream of the Ala^{27} codon, generating pNZ1050. Subsequently, pNZ1050 was digested with *Pvu*II and *Xho*I and ligated to a 4.6 kb *prtP* fragment. The 4.6 kb *prtP* fragment, containing the pro-proteinase sequence, was isolated from plasmid pNZ511-mut3, a derivative of the pNZ511 (Vos et al. 1989b). Plasmid pNZ511-mut3 was partially digested with *Nar*I, the recessed ends filled-in with the Klenow fragment of DNA polymerase I and followed by digestion with *Sal*I.

To construct the α-amylase secretion vector pNZ10α5, plasmid pNZ1025 was cut with *Pst*I and *Hin*dIII and a synthetic linker was inserted to generate a *Pvu*II site downstream of the Ala^{27} codon, resulting in pNZ1054. Plasmid pNZα15 was digested with *Eag*I and the recessed ends were partially filled in with dGTP using the Klenow fragment of DNA polymerase, followed by digestion with mung bean nuclease. Subsequently, it was cut with *Hin*dIII and the 2255 bp fragment was isolated and ligated into pNZ1054 which was cut with *Hin*dIII and *Pvu*II, generating pNZ10α5. A schematic drawing of pNZ123 and the various expression secretion cassettes is outlined in Fig 2.

Deletions by PCR-mutagenesis. Plasmids pNZ10α6 and pNZ10α7 were constructed by deletion mutagenesis of pNZ10α5 using the PCR-mutagenesis protocol as described by Tomic et al. (1990). The following oligonucleotides 5'GGAACCTGCAACGCAG-CTGCAGCAGAAAG-3' [oligo 1], 5'GGAACCTGCAACGCAGCTGCAGAAAGTATC-3' [oligo 2] and 5'GGAACCTGCAAAGGCTGCCGCACCGT-3' [oligo 3] were used. Oligonucleotides were synthesized on a Cyclone DNA synthesizer (Biosearch; San Rafael, Calif.).

Oligo's 1 and 3 were used for the construction of pNZ10α6 and oligo's 2 and 3 for the construction of pNZ10α7.

Preparation of cellular- and supernatant fractions of L. lactis and E. coli. *L. lactis* and *E. coli* cells were grown to an OD_{600} of 1.0 and harvested by centrifugation. Cell lysates were obtained by disrupting the cells 3 times for 3 min in a bead beater (Biospec Products; Bartlesville, Okla.) using zirconium beads (diameter 0.1 mm). A 100-fold concentrated supernatant fraction was obtained by centrifugating the culture medium in 30 K microsep tubes (Filtron; Northborough, MA).

PAGE and immunoblotting. SDS-PAGE and immunoblotting were performed as described previously (Laemmli 1970, Towbin et al. 1979). Antibodies against *B. stearothermophilus* α-amylase, purchased from Cerestar (Vilvoorde, Belgium), were raised in rabbits. Goat anti-rabbit peroxidase was used as a second antibody.

α-Amylase activity assay. Culture supernatant and cell lysates were incubated with 20 mg of amylose azure (Sigma Chemical Co; St. Louis, MO) for 1 h at 60 °C in 1 ml of α-amylase buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM $CaCl_2$). After centrifugation, absorption at 595 nm of the supernatant was measured. α-Amylase (4800 U/ml) was used as a reference. L-Lactate Dehydrogenase (LDH) activity was measured by the method as described by Hillier and Jago (1982).

RESULTS

Expression of the lactococcal proteinase under control of the usp45 signals in L. lactis.

To examine whether the expression and secretion signals of the *usp45* gene were capable of directing the expression of other lactococcal genes and secretion of their products, these signals were fused to the *prtP* gene, encoding the extracellular proteinase of *L. lactis* SK11, devoid of its putative signal peptide and C-terminal membrane anchor, resulting in plasmid pNZ1052 (Fig. 2). PrtP plays an essential role in the degradation of casein in milk by lactococci (De Vos et al. 1989a). PrtP is secreted as a pro-proteinase and for the processing of this precursor into an active enzyme, the maturation protein PrtM is needed (Vos et al. 1989b).

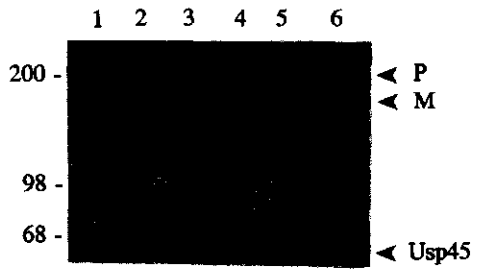

Fig. 3: SDS-PAGE of 2 ml of supernatant of *L. lactis* strains stained with Coomassie Brilliant Blue. Lane 1, Molecular weight marker; lane 2, MG1363[pNZ123]; lane 3, MG1363[pNZ516]; lane 4, MG1363[pNZ1052]; lane 5, MG1363[pNZ511]; lane 6, MG1363[pNZ1052][pNZ582]. The Mw of the marker proteins are shown in kDa. Usp45 and the mature (m) and precursor (p) forms of the proteinase are indicated with arrowheads.

Protein analysis showed that *L. lactis* harbouring pNZ1052 (denoted as MG1363[pNZ1052]) produced a protein, which was efficiently secreted into the culture medium, with an identical size to the unprocessed form of the proteinase produced by MG1363[pNZ516] (Fig. 3, lanes 3 & 4). No PrtP could be detected in the cell pellet of MG1363[pNZ1052] (results not shown). Subsequently, strain MG1363[pNZ1052] was tested for growth in milk. *L. lactis* strain MG1363 which has a Prt$^-$ phenotype (Gasson 1983) is unable to grow in milk. Production of an active proteinase is required for growth. Strain MG1363 harbouring only pNZ1052 did not grow in milk, indicating that the secreted proteinase was inactive. However, when pNZ1052 was introduced into MG1363 harbouring the compatible plasmid pNZ582 carrying the *prtM* gene, the resulting strain displayed growth in milk, indicating production of active extracellular proteinase. The growth rate of this strain was lower than that of strain *L. lactis* SK11

(results not shown), indicating that less than wild-type amounts of active proteinase were produced (Bruinenberg et al. 1992). Analysis of culture supernatant of MG1363[pNZ582][pNZ1052] confirmed this and showed efficient secretion and complete maturation of the proteinase into the processed form (Fig. 3, lane 6). In addition, Western blot analysis have demonstrated that the precursor and mature form of the proteinase produced by MG1363[pNZ1052] and MG1363[pNZ1052][pNZ582] respectively, reacted with polyclonal antibodies against PrtP (data not shown).

Expression of B. stearothermophilus α-amylase under control of the usp45 signals in L. lactis.

The *usp45* expression and secretion signals were fused to the *B. stearothermophilus amyS* gene, devoid of its signal peptide coding sequence. The resulting plasmid, designated pNZ10α5 (Fig. 2), encoded the first 27 amino acids of

Table 2. α-Amylase activities in supernatant and cell lysates of *L. lactis* and *E. coli* harbouring the various plasmids.

	Activity in U/ml		
STRAIN	*L. lactis*		*E. coli*
	SUPERNATANT	CELL LYSATE	CELL LYSATE
pNZ10α5	0.6	0.01	5.4
pNZ10α6	<0.001	<0.001	5.7
pNZ10α7	<0.001	<0.001	5.9

One α-amylase unit is defined as the amount of enzyme which will hydrolyze 10 mg of starch in 10 min. at 60 °C.

Usp45 followed by the mature α-amylase. When *L. lactis* MG1363 cells carrying this plasmid were plated on whey-based agar supplemented with starch, they displayed large halo's around their colonies after staining with iodine as illustrated in Fig. 4, indicative of α-amylase production and secretion. Culture supernatant of the described strain was tested for α-amylase activity (Table 2). Indeed, the supernatant fraction of MG1363[pNZ10α5] contained 0.6 units/ml of α-amylase activity. About 5% of the α-amylase activity was found in the intracellular fractions (Table 2; cell lysates of *L. lactis*). In addition, LDH activities were determined of the supernatant fraction and cell lysate of MG1363[pNZ10α5]. A very low LDH activity (less than 1% of the activity of a cell lysate) could be detected in the supernatant indicating that α-amylase was actively secreted and that no cell lysis or leakage had occured. Subsequently, the culture supernatant of MG1363[pNZ10α5] was analyzed on sodium dodecyl sulphate/poly-acrylamide (SDS/Paa) gels and showed production and secretion of wild type amounts of Usp45. However, no band with a molecular size of α-amylase could be identified in these coomassie brilliant blue stained gels (results not shown). On immunoblots, a band with the same migration as mature *B. stearothermophilus* α-amylase could be detected in the supernatant of MG1363[pNZ10α5] (Fig. 5, lane 3). The higher molecular weight proteins detected in lane 3 most probably represent the unprocessed form of the α-amylase and are due to the very small amount of lysis (about 1% of the total cells as determined by measuring the LDH activity).

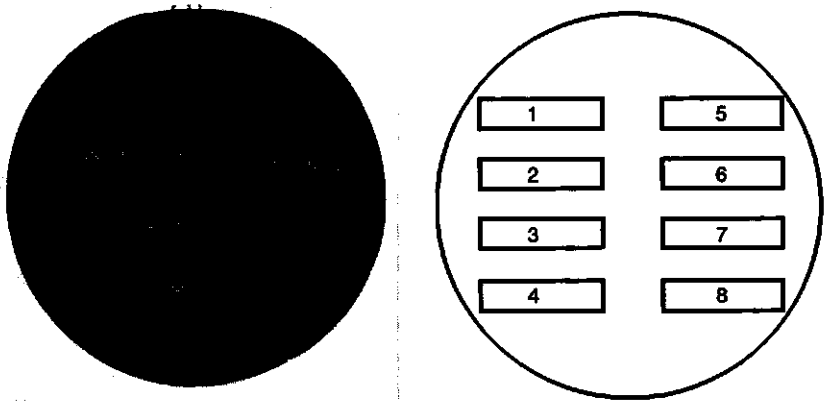

Fig. 4: Iodine staining of whey-permeate agar starch plates containing *E. coli* MC1061 and *L. lactis* MG1363 strains. 1, MC1061[pNZ123]; 2, MC1061[pNZ10α5]; 3, MC1061[pNZ10α6]; 4, MC1061[pNZ10α7]; 5, MG1363[pNZ123]; 6, MG1363[pNZ10α5]; 7, MG1363[pNZ10α6]; 8, MG1363[pNZ10α7].

Functional analysis of the Usp45 signal peptide.

The leader peptide preceding the amino-terminus of mature Usp45 contains 3 putative signal peptidase cleavage sites after Ala^{19}, Ala^{20} and Ala^{27} (Fig. 1). To test whether a shorter Usp45 leader peptide, such as residue 1-19 or 1-20 would also be functional in *L. lactis* as well as in *E. coli* in directing translocation of proteins, fusions with the *amyS* gene devoid of its signal peptide coding sequence were constructed, generating pNZ10α6 and pNZ10α7, respectively (Fig. 2B). The homologous proteinase gene is not suitable for these studies since proteinase plasmids, such as pNZ1052, are not stably maintained in *E. coli* De Vos et al. 1989). In addition, α-amylase activity can be easily assayed on plates as well as in liquid media. *E. coli* cells harbouring pNZ10α6 or pNZ10α7 formed large halo's on starch plates (Fig. 4). Activity measurements showed that active α-amylase was indeed produced by these transformants (Table 2). About equal amounts of α-amylase activities were measured in *E. coli* cell lysates of pNZ10α5, pNZ10α6 and pNZ10α7. In contrast, no halos could be detected on starch plates containing MG1363[pNZ10α6] or MG1363[pNZ10α7] (Fig. 4) and when supernatant fractions of *L. lactis* MG1363 cells harbouring either pNZ10α6 or pNZ10α7 were assayed, they showed almost no detectable extracellular

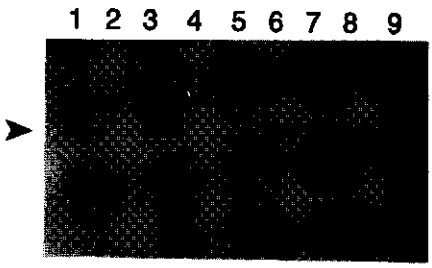

Fig. 5: Immunoblot of 1 ml of supernatant (sup) and 0.2 ml of cell lysate (cl) of *L. lactis* strains incubated with α-amylase antibodies. Lane 1, α-amylase; lane 2, MG1363[pNZ123] sup; lane 3, MG1363[pNZ10α5] sup; lane 4, MG1363[pNZ10α6] sup; lane 5, MG1363[pNZ10α7] sup; lane 6, MG1363[pNZ10α5] cl; lane 7, MG1363[pNZ10α6] cl; lane 8, MG1363[pNZ10α7] cl; lane 9, MG1363[pNZ123] cl. The position of the mature α-amylase is indicated with an arrowhead.

α-amylase activity (Table 2). The α-amylase activity was about 100-fold lower than the activity displayed by MG1363[pNZ10α5] (Table 2) suggesting that the 19 or the 20 amino-terminal residues of the Usp45 leader peptide are not capable of translocating α-amylase into the extracellular medium in *L. lactis*. As expected, no α-amylase band could be detected on immunoblots of supernatant fractions of MG1363[pNZ10α6] and MG1363[pNZ10α7] (Fig. 5, lanes 4 & 5).

Unprocessed α-amylase remains intracellularly.

In addition to the supernatant fractions, equal amounts of cell lysates of MG1363[pNZ10α5], MG1363[pNZ10α6], MG1363[pNZ10α7] and MG1363[pNZ123] were analysed on immunoblots as shown in Fig. 5, lanes 6, 7, 8 and 9. As expected,no immunoreactive band could be detected in cell lysates of MG1363[pNZ123] (lane 9). Surprisingly, in cell lysates of MG1363[pNZ10α5] (lane 6), a protein reacting with α-amylase antibodies displaying a lower mobility than mature α-amylase could be detected, suggesting that this α-amylase was not processed. The level of intracellular unprocessed α-amylase was estimated to be approximately 80% of the total α-amylase production. In cell lysates of MG1363[pNZ10α6] and MG1363[pNZ10α7] (lanes 7 and 8) also an immunoreactive band with a significantly larger molecular weight than mature α-amylase could be detected, suggesting an unprocessed and inactive form of α-amylase. The expected lower mobility, however, of the intracellular form produced by MG1363[pNZ10α5] (Fig. 5, lane 6) in comparison with those produced by MG1363[pNZ10α6] (Fig. 5, lane 7) and MG1363[pNZ10α7] (Fig. 5, lane 8), as a result of the reduced size in leader peptide, is hardly visible. In addition, denaturation/renaturation studies on SDS-PAA gels (Lacks and Springhorn 1980) were performed on cell lysates of MG1363 cells carrying the various plasmids and showed that the unprocessed form did not display activity.

To investigate whether the unprocessed α-amylase remains intracellularly, cells of MG1363[pNZ10α5], MG1363[pNZ10α6] and MG1363[pNZ10α7] were incubated with lysozyme followed by a trypsin treatment. After pelleting, the protoplasts were analysed on immunoblots. No digestion of unprocessed α-amylase after trypsin treatment of protoplasts was detected (data not shown), indicating that the unprocessed α-amylase is not translocated but remains intracellularly.

DISCUSSION

The mature part of the lactococcal Usp45 starts at Asp^{28}, suggesting a signal peptide of 27 residues (Van Asseldonk et al. 1990). We have used the coding sequence of this Usp45 signal peptide to study its effectivity in secretion of the *L. lactis* proteinase PrtP, which lacked its own putative signal peptide (residue 1-33). Furthermore, the region encoding the C-terminal anchor (Vos et al. 1989a) by which PrtP remains attached to the cell-envelope was removed. Our results with both *L. lactis* MG1363[pNZ1052] and MG1363[pNZ516] showed that the proteinase was secreted efficiently into the culture supernatant, indicating that the putative signal peptide of PrtP can be replaced by the 27-residue Usp45 leader peptide.

L. lactis strains harbouring a plasmid encoding the first 27 residues of Usp45 fused to the mature *B. stearothermophilus amyS* gene, produced α-amylase which could be detected both in the supernatant and intracellularly. The intracellular α-amylase had a significant higher apparent molecular weight than the secreted form, indicating that this product still contained the leader peptide. This unprocessed form showed no detectable activity. Accumulation of an inactive intracellular form suggests that the Usp45 leader peptide is not sufficient for efficient α-amylase secretion. The accumulation of precursor could be due to the obstruction of the secretory pathway by the precursor as is suggested by Schein et al. (1986) for the secretion of interferon-α2 by *B. subtilis*. In this case both a secreted form of IFN-α2 and an intracellular precursor form were detected. As a result the overall secretion of other extracellular proteins would also be inhibited. However, since we found a normal secretion of Usp45 by MG1363[pNZ10α5], this explanation seems unlikely in lactococci. Alternatively, the combination of Usp45 signal peptide and mature α-amylase could result in premature folding of the protein into a form that cannot be recognized by molecular chaperones necessary for secretion (Lecker et al. 1990). This phenomenon

was found in *B. subtilis* when a penicillinase signal peptide was used to secrete α-amylase. In that case only 3% of the α-amylase was secreted (Himeno et al. 1986). The lack of activity of the intracellular fraction of MG1363[pNZ10α5] confirms an aberrant folding of the intracellular form found α-amylase. In addition, the ratio between the amount of active α-amylase secreted into the supernatant to the amount of inactive intracellular α-amylase remained the same using a less strong promoter sequence in front of the 27-residue Usp45 leader peptide fused to the α-amylase (data not shown).

We have shown that the 27-residue leader peptide of Usp45 could be used to secrete the α-amylase of *B. stearothermophilus*. However, the Usp45 leader contains two other sequences which could function as a site for signal peptidase I according to the rules proposed (Von Heijne 1986). The most probable sites are located after Ala^{19} and Ala^{20}. If one of these cleavage sites is used by lactococci, Usp45 would be produced as a preproprotein, such as the preproproteinase encoded by the *prtP* gene from *L. lactis* SK11 (Vos et al. 1989b). Such a pro-region could consist of only a few residues as is the case in the α-amylase of *B. subtilis* (Sasamoto et al. 1989). To test this hypothesis, pNZ10α6 and pNZ10α7 were constructed encoding the mature α-amylase preceded by a leader composed of the first 20 or 19 amino-terminal residues of Usp45, respectively. *E. coli* cells harbouring these plasmids were able to secrete the mature active α-amylase. In contrast, *L. lactis* cells harbouring pNZ10α6 and pNZ10α7 were unable to secrete the α-amylase into the culture supernatant. Hence, the 19 or 20 first residues of the Usp45 leader are not sufficient for the secretion of α-amylase in *L. lactis*. Furthermore, it suggests that the signal peptidase I cleavage site for Usp45 itself is located after Ala^{27} in *L. lactis*. Although cleavage sites for signal peptidase I of known signal peptides are well conserved, the length of signal peptides may differ significantly. It has been shown (Suominen et al. 1987) that besides the normal 35-residue signal peptide of *B. stearothermophilus*, also the first 31 residues are used in *E. coli*. However, the *S. aureus* protein A signal peptide (of 36 residues) is functional in *E. coli* and the staphylococcal signal peptidase cleavage site is used (Abrahmsén 1985). Our data with the 19-, 20- and 27- residue Usp45 leader peptide α-amylase fusions show that secretion in *E. coli* can also be accomplished with shorter leader peptides of Usp45. In *L. lactis*, however, the shorter signal peptides did not result in secretion of α-amylase, providing experimental evidence for the hypothesis (Von Heijne and Abrahmsén 1989) that Gram-positive bacteria require a longer signal

peptide for secretion than Gram-negative organisms.

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CHAPTER 4

ROLE OF SEQUENCES UPSTREAM OF THE -35 REGION OF THE *USP45* GENE IN EXPRESSION OF A HETEROLOGOUS α-AMYLASE GENE IN *LACTOCOCCUS LACTIS*.

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(Submitted for publication)

More Than This (Roxy Music)

ABSTRACT

The role of upstream sequences, gene dose and chromosomal location on heterologous gene expression in *Lactococcus lactis* was studied. For this purpose a series of gene fusions was constructed based on the expression and secretion signals of the *L. lactis usp45* gene, encoding the major lactococcal extracellular protein and part of the *Bacillus stearothermophilus amyS* gene encoding mature α-amylase. These fusions were introduced into *L. lactis* on plasmid vectors or integrated into the chromosome. The smallest amounts of α-amylase were produced by *L. lactis* containing only the -35 and -10 promoter sequences and DNA downstream of the *usp45* promoter. A sixfold increase in expression was obtained when the promoter region of the fusion was extended with an AT-rich region of 121 bp region upstream of the -35 sequence. Further extension of the region upstream of the promoter resulted in instable plasmids in *L. lactis*. Therefore, integration of several *usp45-amyS* fusions was accomplished in the *usp45* and the *lac* locus of *L. lactis*. Further extension of the *usp45* promoter upstream of the fusion resulted in an important additional increase of expression. These expression studies showed that the AT-rich region immediately upstream of the *usp45* -35 sequence contributes significantly to the level of α-amylase production. Moreover, DNA more than 120 bp upstream of the -35 sequence is of major importance for expression. Furthermore, the introduction of the *usp45-amyS* gene fusions in *L. lactis* resulted in amylolytic lactococci, which were able to use starch as a sole energy source.

INTRODUCTION

There is considerable interest in the development of microorganisms capable of secreting heterologous proteins to be used in the food industry. Lactic acid bacteria, in particularly strains of *Lactococcus lactis*, are known to secrete proteins into the extracellular environment and could be suitable for this purpose (18). To realize this goal, genetic tools have been developed for the expression and secretion of homologous and heterologous proteins by *L. lactis* (5, 25).

Recently, we have characterized the *usp45* gene of *L. lactis* MG1363 (23) encoding the major extracellular protein of lactococci. An expression and secretion

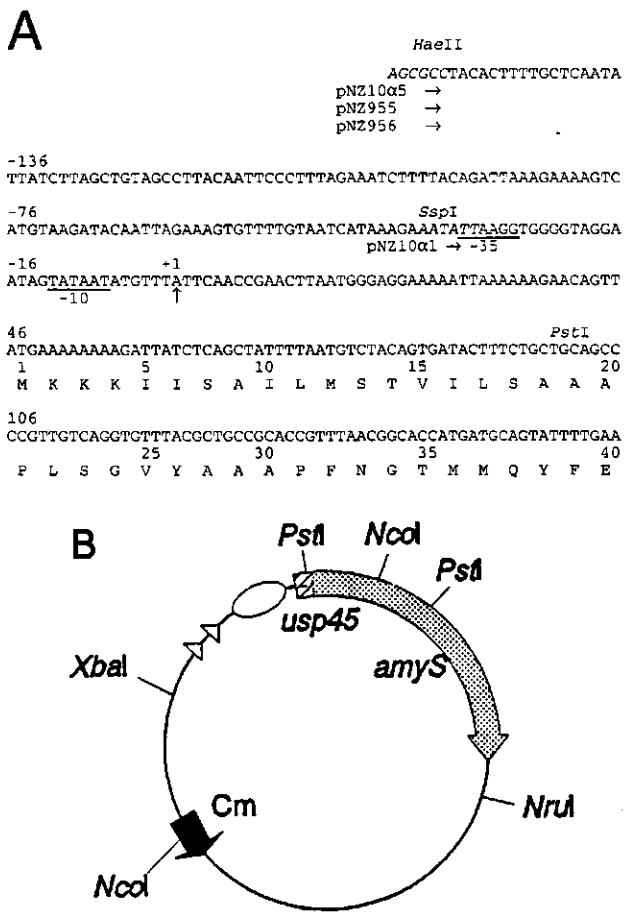


Fig. 1 (A) The nucleotide sequence encoding the N-terminal part of the Usp45-α-amylase fusion and upstream sequences. The start of transcription is indicated by a vertical arrow. The -35 and -10 promoter sequences are underlined. Relevant restriction sites are shown in italics. The α-amylase protein sequence is shaded. The startpoints of the gene fusion in the different plasmids are indicated. **(B) Schematic representation of pNZ10α5.** Relevant restriction sites and (parts of) genes are indicated. The *usp45* gene is shown by an hatched box and the *amyS* gene as a dotted box. The *usp45* -10 and -35 sequences are reperesented by arrowheads and the ribosome binding site by an ellipse.

system was developed using the promoter, ribosome binding site and signal- peptide-coding sequence of this gene (24). As a reporter gene we used the *Bacillus stearothermophilus amyS* gene devoid of sequences encoding the signal peptide. This system directed the production and secretion of α-amylase in *L. lactis*.

The transcription start of the *usp45* gene (23) is preceded by a consensus - 10 promoter sequence and an abberrant -35 sequence spaced by 14 nucleotides (Fig. 1). Immediately upstream of the *usp45* -35 sequence an AT-rich region is present, which appeared to be a common feature in lactococcal promoter regions (5, 25). In *E. coli*, regions upstream of the -35 sequence are known to contribute to promoter strength (12). The same phenomenon is observed in *Bacillus subtilis* (28), and in *Staphylococcus aureus* (15). In most of the investigated cases AT-rich sequences are

involved.

For an extended evaluation of the expression system, the role of the region upstream of the -35 sequence was investigated by studying the expression of *usp45-amyS* fusions. Various expression and secretion gene fusions were constructed and inserted in plasmid vectors or integrated in the chromosome to investigate the role of this region, gene dose and gene location in heterologous gene expression in *L. lactis*.

MATERIALS AND METHODS

Bacterial strains, plasmids and media. Strains, bacteriophages and plasmids used are listed in Table 1. *Escherichia coli* was grown at 37°C in TY broth (16) or on TY solidified with 1.5 % agar. *L. lactis* was grown at 30 °C in M17 medium (20) or in whey permeate medium (6), containing either 0.5 % glucose or 0.5 % starch. To screen for α-amylase-producing transformants, whey permeate agar plates containing 0.5 % starch were used as described previously (24). Chloramphenicol (10 μg/ml) or erythromycin (5 μg/ml) were used for selection. To increase the number of integrated plasmids, clindamycin (50 μg/ml) was used as described previously (4).

DNA manipulations. For transformation of *L. lactis*, cells were cultured, washed, electroporated and recovered according to the method of Holo and Ness (10) and plated on glucose M17 agar plates. Plasmid DNA and chromosomal DNA was isolated as described previously (19, 24). Enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, Md.) or New England Biolabs (Beverly, Mass.) and were used as recommended by the suppliers. General procedures for DNA manipulations were essentially as described by Sambrook et al. (17).

Secretion vector construction. Plasmid pNZ10α5 contains part of the *usp45* gene (pos -158 to 127, Fig. 1) fused to the *amyS* gene encoding the mature α-amylase (24). To construct pNZ10α1, plasmid pNZ10α5 was digested with *Xba*I, and the recessed ends were filled-in with the Klenow fragment of DNA polymerase I. Subsequently, it was partially digested with *Pst*I. The fragment in which only the *Pst*I site at position 102 of the *usp45* gene was cut was isolated. The 253-bp fragment containing the *usp45* promoter region, ribosome binding site and part of the signal peptide coding

Table 1. Strains, phages and plasmids used in this study.

Bacteria	Relevant properties[a]	Source/Reference
E. coli	plasmid free	
MC1061		(3)
L.lactis		
MG1363	plasmid free *lac*$^-$	(9)
MG5267	plasmid free *lac*$^+$	(7)
NZ9551	MG5267, harboring 1 copy of pNZ955 in the *lac* locus; Em^R	This study
NZ9564	MG1363, harboring 4 copies of pNZ956 in the *usp45* locus; Em^R	This study
NZ9571	MG1363, harboring 1 copy of pNZ957 in the *usp45* locus; Em^R	This study
NZ9572	MG5267, harboring 2 copies of pNZ957 in the *usp45* locus; Em^R	This study
NZ9573	MG5267, harboring 3 copies of pNZ957 in the *usp45* locus; Em^R	This study
Phages and Plasmids		
λ1001	λ 47.1 containing a 10 kb insert, containing the *usp45* gene.	(23)
pNZ1011	pUC19 containing a *KpnI-Eco*RI fragment carrying the *usp45* gene; Ap^R	(23)
pNZ10α1	pNZ123 carrying the *amyS* gene fused to nt -33 to 127 of the *usp45* gene; Cm^R	This study
pNZ10α5	pNZ123 carrying the *amyS* gene fused to nt -158 to 127 of the *usp45* gene; Cm^R	(24)
pNZ942	pNZ941, containing the 0.86 kb Cm^R marker from pNZ123; Cm^R, Em^R, Ap^R	(19)
pNZ950	pNZ956, in which the 1.1 kb *HindIII-KpnI usp45* fragment has been deleted.	This study
pNZ955	pNZ942, containing the 2.5 *NcoI-NruI* fragment of pNZ10α5 cloned into the *SalI-NcoI* sites; Cm^R, Em^R, Ap^R	This study
pNZ956	pNZ955, containing a 1.1 kb *HindIII-KpnI* fragment of λ1001, cloned into the *ClaI-HindIII* sites. Cm^R, Em^R, Ap^R	This study
pNZ957	pNZ950, containing a 1.5 kb *HindIII-PstI* fragment from λ1001 upstream of the *usp45-amyS* fusion. Cm^R, Em^R, Ap^R	This study

a. Abbreviations: Cm, Chloramphenicol; Em, Erythromycin; Ap, Ampicillin.

region was replaced by the 132-bp *SspI-PstI* fragment from pNZ1011, containing thesame elements except for the 121-bp -fragment located upstream of the -35 sequence.

Plasmid pNZ955, which was used for the integration of the *usp45-amyS* fusion into the *lacGX* locus of strain MG5267 (7) was constructed by digesting pNZ942 (19) with *SalI*, filling-in with Klenow fragment, and subsequently digesting with *NcoI*. The 6.0-kb fragment, consisting of the vector, the Ap and Em antibiotic resistance markers, part of the *cat*-194 gene and the *HindIII-ClaI lacGX* fragment, was ligated with a 2.0-kb *NcoI-NruI* fragment of pNZ10α5, containing the *usp45-amyS* fusion and the remainder part of the *cat*-194 gene. For the construction of pNZ956, the *lac* region of pNZ955 was replaced by an 1.1 kb *KpnI-HindIII* fragment containing *usp45* upstream sequences from λ1001, an EMBL3 derivative, containing the *usp45* gene (24). For this purpose the *KpnI* site of the λ1001 fragment had been made blunt with T4 polymerase

fragment. It was ligated to plasmid pNZ955 which had been digested with *Cla*I, filled-in with Klenow fragment and subsequently cut with *Hind*III. For the construction of pNZ957, the *Hind*III-*Kpn*I *usp45* fragment was deleted from pNZ956, generating pNZ950. Plasmid pNZ950 was partially cut with *Pst*I and subsequently with *Eco*RI. The 411-bp fragment generated by digestion of only the *Pst*I site at position 102 of the *usp45* gene and the *Eco*RI site upstream of the *usp45-amyS* fusion was isolated. The *Eco*RI site was filled-in with Klenow fragment. Phage λ1001 was digested with *Hind*III and *Pst*I. The *Hind*III-*Pst*I fragment containing 1.5-kb upstream of the *usp45* gene to the *Pst*I site at position 102 was isolated. The *Hind*III was filled-in with Klenow fragment and ligated to the *Pst*I-blunt fragment of pNZ1050, generating pNZ957.

Southern blot analysis. DNA was transferred from 0.8 % agarose gels to Genescreen Plus membranes (DuPont, Nen Research Products, Boston, Mass.). DNA transfer and hybridization was performed as described by the manufacturers. Radioactively (^{32}P) end-labelled oligonucleotides or nick-translated DNA fragments were used as probes. To determine the copy numbers of the plasmids, total DNA was analyzed by Southern blotting. An oligonucleotide probe complementary to nucleotide positions 34-61 of the *usp45* gene (Fig. 1), hybridizing with both the chromosome and the plasmid, was used. The hybridizing fragments were cut out from the filter and the radioactivity was determined. The number of integrated copies of the chromosomal gene fusions was determined by comparison of the radioactivity in the border fragments and the amplified band after Southern blotting.

Determination of the stability of transformants. *L. lactis* was grown in glucose M17 broth without antibiotics. After 100 generations, dilutions were spread on selective or non-selective agar plates, containing starch. The ratio between the number of halo-forming colonies obtained with or without selection determines the percentage of stable transformants.

α-Amylase activity assay. *L. lactis* cells were cultured in whey permeate medium for approximately 4 h until an OD_{600} of 1.0. Cells were removed by centrifugation. Aliquots of 5, 10, 25, and 50 µl of culture supernatant were incubated with 20 mg of amylose azure (Sigma Chemical Co; St. Louis, MO) for 60 min at 60 °C in 1 ml of α-amylase buffer (50 mM Tris-HCl [pH 7.5], 50 mM NaCl, 5 mM $CaCl_2$). After

centrifugation, the absorption at 595 nm of the supernatant was measured. α-Amylase of *B. stearothermophilus* (Cerestar, Vilvoorde, Belgium) (4600 u/ml) was used as a reference.

PAGE and immunoblotting A 100-fold concentrated supernatant fraction was obtained by centrifugating the culture supernatant in 30 K microsep tubes (Filtron; Northborough, MA). Sodium dodecyl sulphate-polyacrylamide gelelectrophoresis (SDS-PAGE) was performed according to Laemmli (13) and immunoblotting according to Towbin et al. (21) using antibodies against *B. stearothermophilus* α-amylase (24) and goat anti-rabbit peroxidase (BRL, Gaithersburg, Md.) as a second antibody.

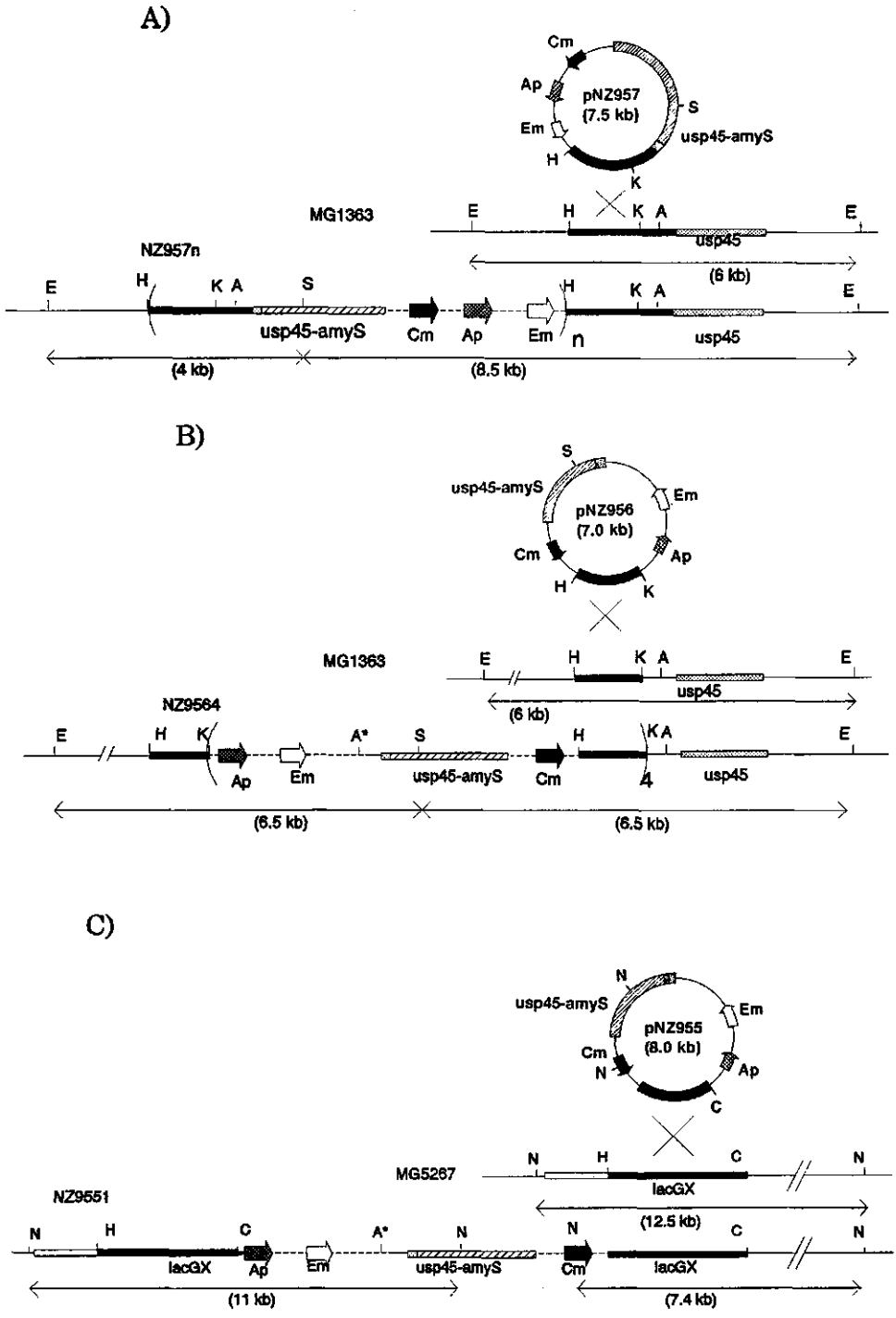
RESULTS

Deletion of the AT-rich region upstream of the *usp45* -35 sequence on plasmid pNZ10α5 results in a decreased expression of the *usp45-amyS* gene fusion. Plasmid pNZ10α5 encodes the first 27 amino acids of Usp45 followed by the mature α-amylase of *B. stearothermophilus* (Fig. 1, Table 1)(24). The expression of the fusion with the heterologous α-amylase gene is under control of the DNA region downstream of the *Hae*II site of the *usp45* gene, located at position -158. To analyze the role of the AT-rich region immediately upstream of the -35 sequence in the expression of the gene fusion, the 121 *Hae*II-*Ssp*I fragment of the *usp45* gene was deleted in pNZ10α5 to generate plasmid pNZ10α1. After transformation of pNZ10α1 to *L. lactis* MG1363, transformants were streaked on whey-agar plates containing starch. The obtained colonies displayed smaller halos than colonies of MG1363 harboring pNZ10α5. The copy numbers of pNZ10α1 and pNZ10α5 appeared to be identical (12 per chromosome).

Culture supernatant of the MG1363 cells harboring pNZ10α1 or pNZ10α5 was tested for α-amylase activity (Table 2). The supernatant fraction of MG1363 harboring pNZ10α5 displayed 600 mU/ml of α-amylase activity (Table 2)(24). Deletion of the 121 nucleotides upstream of the *usp45* -35 region in pNZ10α1, resulted in a 6-fold decrease in expression of the *usp45-amyS* fusion, indicating an important role in expression of this additional region in pNZ10α5.

Fig. 2 Schematic representation of the integration of pNZ957 (A), pNZ956 (B) and pNZ955 (C). The *usp45* structural gene is drawn as a grey box, the part of the *lacG* gene wich is not present on plasmid pNZ955 as a open box, and the *amyS* gene as a hatched box. Chromosomal DNA is shown as a continuous line, integrated plasmid DNA as a broken line. The black box in the recombinants represents the homology region used for recombination. The resistance markers are shown by arrows. Relevant restriction sites are marked as follows; C, *Cla*I; E, *Eco*RV; H, *Hind*III; S, *Sst*II; K, *Kpn*I; N, *Nco*I; A, *Hae*II. A* represents the position of the *usp45 Hae*II site which has been lost in to the construction of pNZ956 and pNZ955. Between brackets the amplificated unit is shown. n = 1, 2 or 3 for NZ9571, NZ9572 or NZ9573, respectively.

Chromosomal integration of the *usp45-amyS* fusion in the *usp45* locus results in an increased α-amylase production. If the *usp45* upstream region in the *usp45-amyS* gene fusion in plasmid pNZ10α5 was further extended, the transformation efficiency in *L. lactis* of the generated plasmid decreased more than tenfold. The few obtained transformants produced no halos on starch-containing plates and harbored plasmids in which the *usp45* region and parts of the *amyS* gene were deleted. To determine the influence of the regions upstream of the *Hae*II site on the α-amylase production, several *usp45-amyS* gene fusions were integrated into the lactococcal chromosome. In plasmid pNZ957 the *usp45-amyS* fusion was preceded by a 1.5 kb-region upstream of the *usp45* promoter. This region provided the homology region required for the integrative recombination event with the chromosome. As a result the *usp45-amyS* fusion will be located at the position of the *usp45* gene in the parent strain (Fig. 2A). Two isogenic plasmid-free *L. lactis* strains, were chosen for the integration of pNZ957 into the chromosome, i. e. MG1363 (lactose-deficient) and MG5267 carrying a chromosomal copy of the *lac* operon. Analysis of the DNA content of the transformants, revealed that no plasmid DNA was present. In Fig. 2A, the DNA arrangement of the wild-type *usp45* locus, and the situation after integration of one or more copies of pNZ957 is schematically depicted. Fig. 3A shows the Southern blot analysis of the chromosomal DNA of strains MG1363, and two transformants, NZ9571 and NZ9572 using an *usp45*-specific oligonucleotide probe. Digestion of chromosomal DNA of MG1363 with *Eco*RV and *Sst*II resulted in a 6-kb fragment hybridizing to the *usp45* probe (Fig. 3A, lane 2). Southern blot analysis of chromosomal DNA of NZ9571 digested with *Eco*RV and *Sst*II, showed two hybridizing fragments of 4 kb and 8.5 kb (Fig. 3A, lane 3). These sizes agree with those of the border fragments generated after integration of one copy of pNZ957 (Fig. 2A), and confirmed the site-specific integration of the vector in the *usp45* locus. In chromosomal DNA of NZ9572 an additional band with the size of the linear plasmid (7.5 kb) could be detected (Fig 3A,


A)
Cm
Ap
pNZ957
(7.5 kb)
S
Em
usp45-amyS
H
K
MG1363
E
H
K A
E
usp45
(6 kb)
NZ957n
E
H
K A
S
H
K A
E
usp45-amyS
Cm
Ap
Em
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usp45
(4 kb)
(8.5 kb)
B)
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usp45-amyS
pNZ956
(7.0 kb)
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Cm
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H
K
MG1363
E
H
K A
E
usp45
(6 kb)
NZ9564
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N
usp45-amyS
pNZ955
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Ap
C
MG5267
N
H
C
N
lacGX
(12.5 kb)
NZ9551
N
H
C
A*
N
N
C
N
lacGX
Ap
Em
usp45-amyS
Cm
lacGX
(11 kb)
(7.4 kb)

lane 4), indicating that more copies of the pNZ957 were present on the chromosome. The number of integrated copies of the gene fusions in the transformants was determined and showed that most strains, like NZ9571, carried one copy. Some transformants originating from MG5267, like NZ9572, carried two copies. To increase the number of copies, NZ9572 was cultured in the presence of clindamycin, an analogue of erythromycin. This resulted in NZ9573, carrying 3 copies of the integrated gene fusions (Fig. 3A, lane 5). Further amplification was not observed.

The α-amylase production of NZ9571, NZ9572 and NZ9573 was determined and showed that NZ9571, containing a single copy of the *usp45-amyS* gene fusion, produced an α-amylase activity of 800 mU/ml. This is an increase of 200 mU/ml, compared to MG1363 harboring 12 copies of pNZ10α5. Furthermore, an increase in the copy number of the *usp45-amyS* fusion, as in NZ9572 and NZ9573, resulted in a slight increase in α-amylase production (Table 2).

Integration of the *usp45-amyS* gene fusion in the *usp45* and *lac* locus of *L. lactis*. To investigate whether the increase in α-amylase production by NZ9571 was due to the additional upstream *usp45* sequences preceding the *usp45-amyS* gene fusion or was a consequence of the locus at which the *usp45-amyS* gene fusion was integrated, pNZ956 and pNZ955 were constructed. Plasmid pNZ956 was used for integration of the *usp45-amyS* fusion upstream of the *usp45* structural gene and plasmid pNZ955 for integration downstream of the *lac* operon of strain MG5267 (Fig. 3). As in pNZ10α5, the expression of these gene fusions was controlled by the DNA sequences at position -158 to 127 of the *usp45* gene and are preceded by vector sequences which are known to function as transcriptional terminators (19). Transformation of pNZ956 to MG1363 yielded strain NZ9564, which was difficult to obtain, probably due to the small size (1 kb) of the homology region (Fig. 2B). DNA from NZ9564 was digested with *Eco*RV and *Sst*II, and Southern blot analysis resulted in two overlapping hybridizing border fragments of 6.5 kb each (Fig. 3B). Furthermore, a fragment with the size of the plasmid was detected, showing a stronger hybridization signal than the two border fragments together, suggesting that more than one copy had been integrated in the chromosome. Determination of the number of integrated copies showed that NZ9564 contained four copies of the *usp45-amyS* gene fusion. The α-amylase activity secreted by NZ9564 was 300 mU/ml (Table 2).

Because the *lac* operon is chromosomally located in strain MG5267, this strain was used to transform pNZ955. Chromosomal DNA from MG5267 and transformants

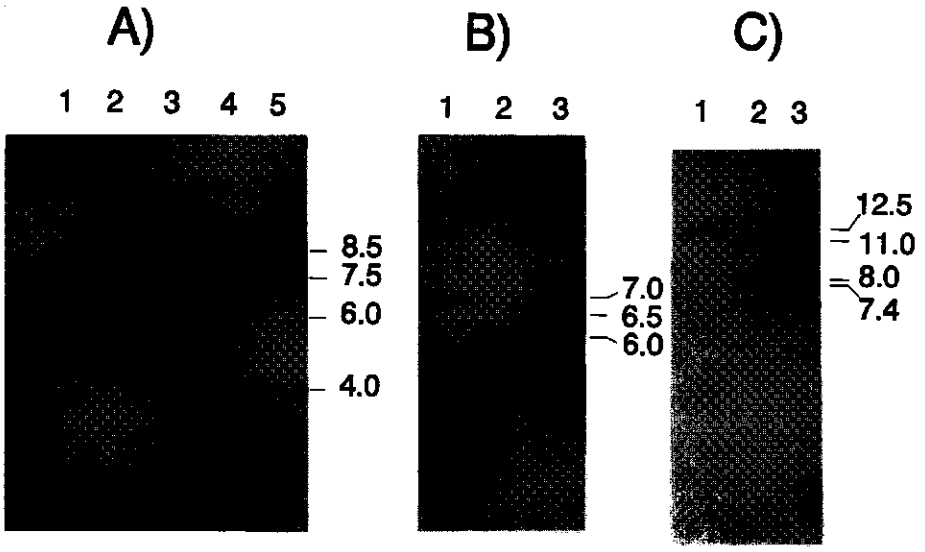

Fig 3. Autoradiogram of Southern analysis of chromosomal DNA. (A) MG1363, NZ9571 and NZ9572 and NZ9573, digested with *Eco*RV and *Sst*II, hybridized to an oligonucleotide complementary to position 34 to 61 of the *usp45*. Lane 1, pNZ957; lane 2, MG1363, lane 3; NZ9571, lane 4, NZ9572; lane 5 NZ9573. (B) MG1363 and NZ9564 digested with *Eco*RV and *Sst*II, hybridized to an oligonucleotide complementary to position 34 to 61 of the *usp45* gene. Lane 1, pNZ956; lane 2, MG1363, lane 3, NZ9564. (C) Chromosomal DNA of MG5267 and NZ9551, digested with *Nco*I, hybridized to a *lacX* probe. Sizes of the hybridizing fragments are indicated in kb.

was digested with *Nco*I. In MG5267 this resulted in a 11.5-kb fragment hybridizing to a *lacX*-specific probe (Fig 2C and 3C, lane 1). After integration of pNZ955 in MG5267, as in NZ9551, two bands, one sized 11 kb and the other 7.4 kb, could be visualized after hybridization with a *lacX*-specific probe, indicating that site-specific integration

Table 2. α-Amylase production of *L. lactis* strains.

Strain	*usp45-amyS* copies	α-amylase production (mU/ml)
MG1363 [pNZ10α1]	12	100 ± 10
MG1363 [pNZ10α5]	12	600 ± 25
NZ9551	1	70 ± 10
NZ9564	4	300 ± 20
NZ9571	1	800 ± 45
NZ9572	2	900 ± 40
NZ9573	3	1050 ± 55

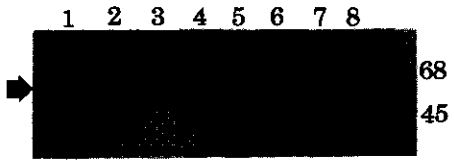

Fig 4. Immunoblot of 1 ml of supernatant of *L. lactis* strains incubated with α-amylase antibodies. Lane 1, MG1363 harboring pNZ123; lane 2, MG1363 harboring pNZ10α1; lane 3, MG1363 harboring pNZ10α5; lane 4, NZ9551; lane 5, NZ9564; lane 6, NZ9571; lane 7, NZ9572; lane 8, NZ9573; The size of the marker proteins are shown in kDa. The position of the mature α-amylase is indicated with an arrow.

had occurred (Fig 3C, lane 2). No transformants with multiple integrated copies were found. Strain NZ9551 secreted 70 mU/ml α-amylase into the culture supernatant (Table 2).

The segregational stability of the integrated DNA in NZ9571, NZ9572, NZ9573, NZ9564 and NZ9551 was tested. After 100 generations of growth without Em, cells were plated on plates with or without Em. All colonies showed Em resistance. Furthermore, α-amylase production remained unaffected, indicating that no loss of the integrated plasmids had occurred.

Immunoblot analysis. The supernatant fractions of the cultured strains were analyzed by SDS-PAGE. Wild-type amounts of Usp45 were found but no α-amylase band could be detected after staining with coomassie brilliant blue (results not shown). However, when the culture supernatant fractions were analyzed on immunoblots, a band with the same migration as mature *B. stearothermophilus* α-amylase could be detected in the supernatant of all strains (Fig. 4). The difference in α-amylase production by the strains is reflected by the intensity of the band. The ratio of intra- and extracellular α-amylase was estimated to be the same (4 : 1) in all strains (results not shown) and corresponds to that found in previous work (24).

Amylolytic properties of *L. lactis* harboring the *usp45-amyS* gene fusions. To test whether α-amylase production was high enough to obtain growth on starch, NZ9564, NZ9571 and MG1363 harboring pNZ10α1 or pNZ10α5 were cultured on whey permeate medium with starch as the sole usable energy source. Because whey permeate contains lactose, strains derived from MG5267 could not been used for this purpose. The growth curves of the various strains are depicted in Fig. 4. All strains reached an OD_{600} of 2.2 after growing on starch, when an *usp45-amyS* fusion was present in the cells. When no additional carbon source was available, cell division ceased at an OD_{600} of 0.25. Strains NZ9564, NZ9571 and MG1363 harboring

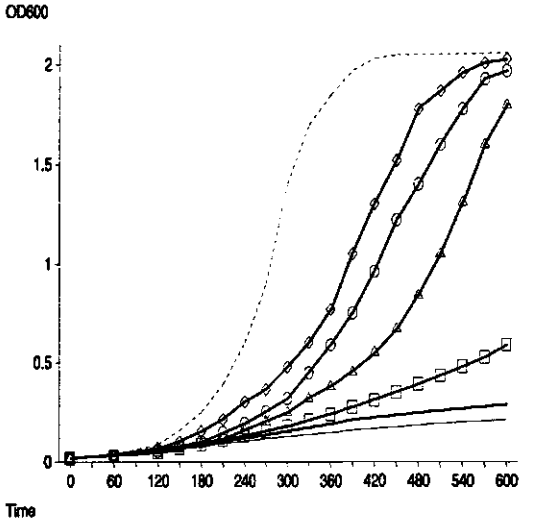

Fig 5. Growth curves on whey permeate with starch as carbon and energy source of MG1363 harboring pNZ123, - -, MG1363 harboring pNZ10α1 -□-, MG1363 harboring pNZ10α5 -○-, NZ9564 -△- and NZ9571 -◇-. Average growth characteristics on whey permeate with glucose are shown as a thin, broken line, and on whey permeate without any sugar as a thin continuous line.

pNZ10α5 displayed growth on starch, with maximal growth rates (μ_{max}) of 0.69, 0.71 and 0.76, respectively, indicating an efficient breakdown of the starch. These growth rates are still lower on starch than on glucose (μ_{max} = 1.07), but they are comparable with the growth rate of MG5267 on whey, using lactose as energy source (μ_{max} = 0.69) (19), indicating an efficient use of the starch. The differences in growth rate correspond with the differences in α-amylase production by the strains. Strain NZ9571, producing the largest amount of α-amylase exhibited the highest growth rate.

DISCUSSION

The functionality of the expression and secretion signals of the *usp45* gene, for the production of α-amylase has been reported previously (24). To investigate the role of the DNA region upstream of the -35 sequence of *usp45* on expression, various *usp45-amyS* gene fusions were constructed and transformed to *L. lactis*. Quantification

of the produced α-amylase showed significant differences in expression level of the strains. The results showed clearly that the AT-rich region immediately upstream of the -35 sequence affected the amount of α-amylase production. Moreover, an additional extension of the DNA region upstream of the *usp45* promoter also plays an important role in the efficiency of expression. Furthermore, the copy number of the gene fusion plays an additional role in the heterologous gene expression, but no effect of chromosomal locus on expression was observed.

Plasmid pNZ10α5 contains a region of 121 bp upstream of the -35 sequence. MG1363 harboring plasmid pNZ10α1, which is devoid of this extra region, show a six-fold decrease in α-amylase production, as compared to MG1363 harboring pNZ10α5. Both plasmids have the same copy number (12 per chromosome). The region upstream of the -35 sequence is AT-rich (73% for the first 100 nucleotides upstream of the -35 sequence) whereas the coding region of the *usp45* gene has an AT-content of 61% which is representative for *L. lactis* structural genes found so far (25). It has been demonstrated that curving of AT-rich sequences, contributes to an elevated expression of the downstream located DNA region (1, 8). Curving of the additional DNA region in pNZ10α5 may be involved in the enhanced expression of α-amylase compared to pNZ10α1.

Chromosomal integration of the *usp45-amyS* gene fusion under control of position -155 to 127, as in NZ9551 and NZ9564, resulted in a lower α-amylase production as compared to MG1363 harbouring pNZ10α5, carrying the same gene fusion on plasmid. Because readthrough of transcription of DNA upstream of the inserted *usp45-amyS* gene fusion could be excluded in all three cases, this is most probably the result of difference in copy number of the *usp45-amyS* fusions in these strains. Increase from one (NZ9551) to four copies (NZ9564) resulted in a 4 times increase in α-amylase secretion as was expected. This linear increase suggests that the locus used to integrate these gene fusions (*lac* operon or *usp45* gene) does not affect the expression of the *usp45-amyS* gene fusion. Further increase to 12 copies as in MG1363 harboring pNZ10α5 resulted in an additional 2 times higher expression. The importance of copy number in the (over)production of proteins in *L. lactis* has also been established for the production of the homologous proteinase PrtP and aminopeptidase N (2, 14, 22).

An additional effect on the expression of the *usp45-amyS* gene fusion has been observed in NZ9571, NZ9572 and NZ9573. Compared to pNZ10α5, pNZ955 and

pNZ956, the upstream region in pNZ957 is further extended. Comparison of the activities of NZ9551 (70 mU/ml), containing one copy of the *usp45-amyS* gene fusion with the additional 121 nucleotides located on the chromosome, and NZ9571 (800 mU/ml) permits the conclusion that extension of the promoter with a region more than 120 bp upstream of the -35 sequence results at least in a 12-fold increase of expression. The DNA region upstream of the promoter of the *L. lactis lacABCDFEGX* operon, has also been shown to contribute to its promoter strength (29). In this case extension of the region from position -75 to -204 resulted in a 1.5-fold higher expression. In addition, further extension to position -388 resulted in an additional 2-fold increase of transcription. Although no sequence conservation between the *lac* and *usp45* upstream region could be observed the results obtained with both promoters suggest a similar transcription activating role for DNA sequences more than 100 bp upstream of lactococcal promoters. The high expression of the gene fusion with an extended promoter region could explain in the instability in *L. lactis* of plasmids containing the region upstream of the *Hae*II site of the *usp45* gene.

The relatively small difference of α-amylase activity between NZ9571 (800 mU), harboring one copy of pNZ957 and NZ9572 (900 mU) or NZ9573 (1050 mU), in which two- or three-fold amplification of the integrated pNZ957 DNA has occurred, respectively, could be explained in several ways. First of all DNA sequences upstream of that cloned in pNZ957 could influence expression of the gene fusion. This could be caused by readthrough from genes upstream of the *usp45* gene. However, transcription analysis of the *usp45* gene showed only a single mRNA of 1500 nucleotides, which is initiated at position 1 (23). Furthermore, the amount of produced Usp45 should be significantly decreased, since the *usp45* structural gene is dislocated from its original position in NZ9564 and NZ9571. Wild-type amounts of Usp45 were produced by those strains which leaves the explanation very unlikely. Secondly, the amount of α-amylase produced by NZ9571 could be near the maximum of the secretion capacity of *L. lactis*. This explanation is also unfavourable, since there is no additional intracellular accumulation of precursor. Therefore, the most likely explanation for slight difference in expression between strains NZ9571, NZ9572 and NZ9573 is a non-linear gene dose effect. Allthough the gene dose correlates almost linearly with α-amylase production in NZ9551 and NZ9564, the level of expression is not always linearly related to the gene dose as has been demonstrated for the *prtP* gene in *L. lactis* (2, 14). After integration into the *L. lactis* chromosome an increase

from 2 to 5 copies resulted in an eight-fold increase of proteinase production. A further increase to 8 copies only resulted in a 1.5-fold higher proteinase level (14). When the gene was introduced on a plasmid with a ten-fold higher copy number than the plasmid present in the wild-type strain, a 3-fold increase of PrtP production was observed (2). The results obtained with the *prtP* gene and those obtained with NZ9571, NZ9572 and NZ9573, suggest that the role of gene dose diminishes at higher expression levels.

An important aspect of the production of α-amylase by *L. lactis* is the extension of its substrate range. *L. lactis* strains grow on several sugars, such as lactose, galactose, and in some cases sucrose or maltose. However, *L. lactis* is not able to utilize polysacharides, such as starch. By introducing a functional α-amylase gene in *L. lactis*, the amylolytic activity results in growth on starch (Fig.5). The results obtained with NZ9571 displaying growth rates on starch comparable to those on lactose indicate that the α-amylase production is sufficient for efficient growth.

In conclusion, the present study shows that the metabolic capacity of *L. lactis* can be increased by chromosomal integration of a single copy of the *usp45-amyS* gene fusion. The resulting *L. lactis* strains can ferment starch efficiently and have potential to be used as hosts for the production of homologous and heterologous proteins, that can grow on a cheap energy source, or as starter cultures for non-dairy fermentations.

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CHAPTER 5

MUTATIONAL ANALYSIS OF THE TRANSLATION INITIATION REGION OF THE *USP45* GENE OF *LACTOCOCCUS LACTIS* USING AN *USP45-AMYS* GENE FUSION.

Martien Van Asseldonk, Willem M. De Vos and Guus Simons.

What Difference Does It Make? (The Smiths)

ABSTRACT

The *usp45* gene encodes the major extracellular protein of *Lactococcus lactis* strain MG1363. The putative AUG start codon of this gene is preceded by two possible Shine and Dalgarno (SD) sequences; SD1 (GGAGG) and SD1 (AAAG), which are located 21 and 6 nucleotides upstream of the AUG codon, respectively. The vector pNZ10α5 contains an *usp45-amyS* gene fusion, encoding the Usp45 signal peptide fused to the *Bacillus stearothermophilus* α-amylase. Site-directed mutagenesis of the two *usp45* SD-sequences in pNZ10α5 has been performed to reduce their complementarity to the 3' end of 16S rRNA of *L. lactis*. In addition, the spacing between the putative translational start codon and SD1 was reduced to 7 nucleotides, to generate a consensus-like ribosome binding site. It appeared that none of the SD-mutations nor the optimization of the spacing between SD1 and the start codon resulted in a difference in α-amylase production in *L. lactis* as compared to the wild-type production found in *L. lactis* containing pNZ10α5.

INTRODUCTION

Usp45 is the major extracellular protein of *Lactococcus lactis* (21). To examine the capacity of *L. lactis* as a host for the production of heterologous proteins, the expression and secretion signals of the *usp45* gene were used in the construction of a secretion vector, designated pNZ10α5, employing the *Bacillus stearothermophilus amyS* as a reporter gene (22). Several *usp45-amyS* gene fusions have been constructed to investigate the influence of the upstream region of the *usp45* promoter on α-amylase production in *L. lactis*. Furthermore, the effect of gene dose and chromosomal location on the expression level was determined (23).

Besides the efficiency of transcription, also the translation efficiency of the mRNA has a significant effect on gene expression (16). The translational start codon of the *usp45* gene is preceded by 2 putative Shine-Dalgarno (SD) sequences (Fig. 1) (17). Neither of these SD sequences conform to the consensus SD sequences found in *L. lactis* that have a high complementarity to the 3' end of the lactococcal rRNA (11) (ΔG_0 values between -36 and -75 kJ/mol) and are located in between 5 and 16 bp upstream from the initiation codon (2, 4, 25). SD2 is located 6 bp upstream of the start codon, which is a normal spacing for lactococci (4, 25). However, the free energy of

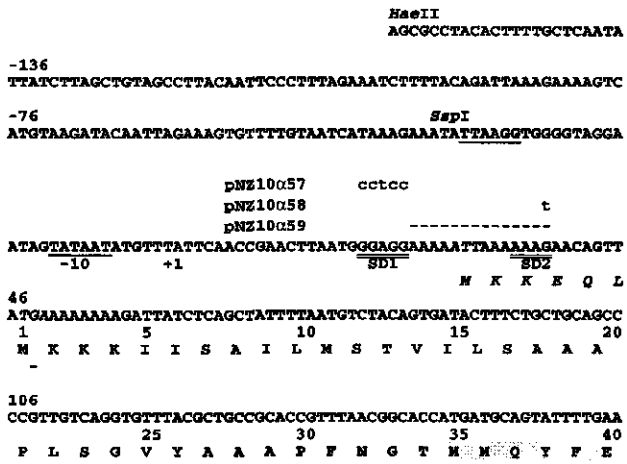

Fig. 1: Architecture of *usp45-amyS* gene fusions. Depicted is the nucleotide sequence of the *usp45* expression signals and that of the N-terminal part of the α-amylase preceded by the signal peptide of Usp45, present in pNZ10α5. The mutations in pNZ10α57, pNZ10α58, and pNZ10α59 are indicated. The deleted region in pNZ10α59 is shown by dashes. The start of transcription is indicated by a +1. The -35 and -10 promoter sequences are underlined. Relevant restriction sites are indicated. SD1 and SD2 are double underlined. The N-terminal residues of the *amyS* are shaded. The start and stop codon and the amino acids of the open reading frame possibly involved in the translational coupling are shown in italics.

the complementarity of this SD to the 3' end of the *L. lactis* 16S rRNA is only -19.3 kJ/mol. The complementarity of the second SD (SD1) sequence to the 3' end of the rRNA is high (ΔG_0 is -60.4 kJ/mol, and is more than average in lactococci (4, 25). However, the spacing between this SD sequence and the start codon is 21 bp, which is exceptional, both in Gram-positive and in Gram-negative bacteria (4, 6, 8, 25).

This report describes site-directed mutagenesis of these two SD sequences in pNZ10α5 to study their role in expression of the *usp45-amyS* gene fusion.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions. *E. coli* strain MC1061 (1) and *L. lactis* strain MG1363 (5) were used as hosts. *E. coli* was grown at 37°C in TY broth (14) or on TY solidified with 1.5 % agar. *L. lactis* was grown at 30 °C in M17 (19) or whey-permeate (3) medium containing 0.5 % glucose. For electroporation of *L. lactis*, cells were treated as described previously (22). To screen for α-amylase-producing transformants, whey-permeate agar plates containing 0.5 % starch were used. If appropriate, chloramphenicol was used at a concentration of 10 μg/ml.

DNA manipulations. Plasmid DNA was isolated as described previously (22). Enzymes were purchased from BRL (Gaithersburg, Md.) or Biolabs (Beverly, Mass.) and were used as recommended by the suppliers. General procedures for DNA manipulations were essentially as described by Sambrook et al. (15).

Site directed mutagenesis of the *usp45* SD regions in pNZ10α5. Plasmid pNZ10α5 contains the *usp45-amyS* gene fusion with the original *usp45* ribosome binding site (22). For the construction of mutations in the SD sequences (see Fig. 1) in pNZ10α5 the following oligonucleotides 5'CAACCGAACTTAATGCCTCCAAAAATTAAAAAAGAA-CAG [oligo 1], 5'GGGAGGAAAAATTAAAAAATAACAGTTATGAAAAAAAAG [oligo 2] and 5'CAACCGAACTTAATGGGAGGAACAGTTATGAAAAAAAAG [oligo 3] were synthesized on a Millipore/Biosearch Cyclone DNA synthesizer (Biosearch, San Rafael, Calif.). Following the PCR-mutagenesis protocol of Kuipers et al. (9), oligo 1 was used for the construction of pNZ10α57, oligo 2 for pNZ10α58 and oligo 3 for pNZ10α59. Oligonucleotides, complementary to *cat*86 and *amyS* (22) were used as border primers. The generated PCR products were cut with *Xba*I and *Sst*I and inserted into pNZ10α5, digested with the same enzymes. The mutations were verified by nucleotide sequencing (18).

α-Amylase activity assay. *L. lactis* and *E. coli* cells were grown to an OD_{600} of 1.0 and harvested by centrifugation. *E. coli* cell lysates were obtained by disrupting the cells 3 times for 3 min in a bead beater (Biospec Products; Bartlesville, Okla.) using zirconium beads (diameter 0.1 mm). Aliquots of *L. lactis* culture supernatant were incubated with 20 mg of amylose azure (Sigma Chemical Co; St. Louis, MO) for 60 min at 60 °C in 1 ml of α-amylase buffer (50 mM Tris-HCl [pH 7.5], 50 mM NaCl, 5 mM $CaCl_2$). After centrifugation, the absorption at 595 nm of the supernatant was measured. α-Amylase of *B. stearothermophilus* (Cerestar, Vilvoorde, Belgium) (4600 u/ml) was used as a reference.

PAGE and immunoblotting. Cellular extracts were obtained by disrupting the cells 3 times for 3 min in a bead beater (Biospec products) using zirconium beads (diameter 0.1 mm). A concentrated (100-fold) supernatant fraction was obtained by centrifugating the culture medium in 30 K microsep tubes (Filtron). SDS-PAGE was performed according to Laemmli (10) and immunoblotting according to Towbin et al. (20).

Antibodies against *B. stearothermophilus* α-amylase, purchased from Cerestar (Vilvoorde, Belgium), were raised in rabbits. Goat anti-rabbit peroxidase (Gibco-BRL) was used as a second antibody.

RESULTS AND DISCUSSION

Site-directed mutagenesis of the two *usp45* SD-regions. To locate the SD sequence of the *usp45* gene in the *usp45-amyS* gene fusion of pNZ10α5 (22), we constructed pNZ10α57 and pNZ10α58 (Fig. 1). In plasmid pNZ10α57, the SD1 sequence, GGAGG at position 19 to 23 was replaced by CCTCC. As a result, there is no complementarity of the mutated SD1 to the 3' end of 16S rRNA. In plasmid pNZ10α58 the G residue of SD2 at position 37 is mutated into a T residue. The complementarity of the mutated SD2, with the sequence AAA, to the 3' end of the 16S rRNA is reduced to AAA, which is almost negligible (Fig. 1). To generate a SD site with a high complementarity to the 3' end of 16S rRNA and an optimal spacing to the AUG initiation codon, the nucleotides at position 24 to 37 were deleted in pNZ10α59. In this way SD1 is positioned 7 nucleotides upstream from the *usp45* initiation codon. Plasmids pNZ10α57, pNZ10α58 and pNZ10α59 were transformed to *L. lactis* MG1363 and *E. coli* MC1061. The secreted α-amylase activities produced by the generated strains were determined (Table 1).

SD2 suffices for translational initiation in *L. lactis*. When SD1 was mutated as in pNZ10α57, expression of the *usp45-amyS* gene fusion in *L. lactis* and *E. coli* was hardly affected, suggesting that SD2 is the functional SD sequence of *usp45*. Recent studies performed on translation in *E. coli* and *B. subtilis* using the *lacZ* gene as marker gene (13, 27) have shown that a decrease in free energy of complementarity of a SD sequence and the 16S-rRNA resulted in a reduced translation efficiency. This effect was more drastic in *B. subtilis* than in *E. coli*. A decrease of ΔG_0 from -99.1 to -53.8 kJ/mol resulted in an approximately ten-fold decrease of translation efficiency in *E. coli*, but more than 100-fold in *B. subtilis* (27). *Staphylococcus aureus*, another Gram-positive organism, also requires SD sequences with a higher complementarity to the 3' end of 16S rRNA than *E. coli* (12). The degree of complementarity of SD-regions from *L. lactis* with the 3'end of 16S rRNA appears to be intermediate between

Table 1. α-Amylase production of *L. lactis* harboring the various plasmids.

Strain	Activity (mU/ml)	Strain	Activity (mU/ml)
L. lactis		*E. coli*	
MG1363[pNZ10α5]	600	MC1061[pNZ10α5]	5500
MG1363[pNZ10α57]	580	MC1061[pNZ10α57]	5500
MG1363[pNZ10α58]	600	MC1061[pNZ10α58]	4700
MG1363[pNZ10α59]	560	MC1061[pNZ10α59]	9900

those of SD-regions of other Gram-positive organisms and of *E. coli* (2, 4, 25). Free energy of complementarity of identified lactococcal SD-regions have a mean value of ΔG_0 -51.9 kJ/Mol (4). The results obtained with *L. lactis* harboring suggest that even a ΔG_0 of -19.3 kJ/Mol suffices for translational initiation.

Mutation of SD2 does not affect expression in *L. lactis*. Similar α-amylase production levels were observed in *L. lactis* harboring pNZ10α5 and pNZ10α58 in which SD2 was mutated (Table 1). This suggests that also SD1, that is separated by 21 nucleotides from the *usp45* start codon, can be functional in *L. lactis*. No secondary structure could be postulated between SD1 and the start codon, which might reduce their physical distance, as is the case in the T4 gene 38 translational initiation region (6). Three explanations could be envisaged for the wild-type level of α-amylase activity found by MG1363 harboring pNZ10α58.

First, translational coupling could be involved. The combination of start and stop codon, AUGA, has been shown to be a very efficient combination in the translational coupling of different open reading frames in *L. lactis* (24). The *usp45* start codon partially overlaps with an UGA stop codon. This stop codon is terminating an open reading frame of 6 amino acids using the AUU codon at position 28 as an initiation codon (Fig. 1). The spacing between SD1 and this initiation codon resembles the optimal spacing as observed in most ribosome binding sites, but so far this codon has not been found to initiate translation in bacteria. Nevertheless, it is has been observed that other codons, in which one substitution has taken place can serve as start codons, both in *B. subtilis* as in *E. coli* (6, 7, 8). In pNZ10α57, SD1 is mutated, which would eliminate the translation of the 6 aa leader peptide. In pNZ10α58 a stop codon (TAA) is introduced in the leader encoding reading frame. If translational coupling was involved, the expression of the *usp45-amyS* gene fusion in both plasmids would have

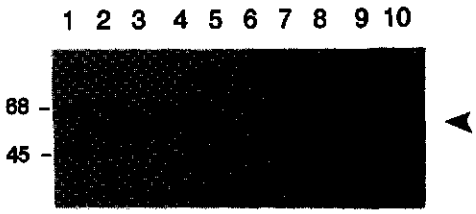

Fig. 2: Immunoblot of 1 ml of supernatant (sup) and 0.2 ml of cell pellet (cp) fractions of *L. lactis* strains incubated with α-amylase antibodies. Lane 1, molecular weight marker; lane 2, α-amylase; lane 3, MG1363[pNZ10α5] sup; lane 4, MG1363[pNZ10α57] sup; lane 5, MG1363[pNZ10α58] sup; lane 6, MG1363[pNZ10α59] sup; lane 7, MG1363[pNZ10α5] cp; lane 8, MG1363[pNZ10α57] cp; lane 9, MG1363[pNZ10α58] cp; lane 10, MG1363[pNZ10α59] cp. The position of the mature α-amylase is indicated with an arrowhead.

been decreased. Since this was not observed (Table 1) this explanation is very unlikely.

The second possible explanation is that a lower translation rate of the gene fusion in pNZ10α58 as compared to pNZ10α5 could result in a more efficient secretion of the α-amylase. Immunoblot analysis of intracellular proteins of the α-amylase producing *L. lactis* strains revealed that a substantial amount of inactive α-amylase remains intracellularly (22). This is probably due to premature folding of the protein into an export incompetent form. A presumed lower translation rate of the mRNA produced by *L. lactis* strain carrying pNZ10α58, could result in a more efficient secretion. However, analysis of the intracellular fractions of MG1363 harboring pNZ10α5, pNZ10α57, pNZ10α58 and pNZ10α59, showed no significant difference in the amounts of intracellular α-amylase (Fig. 2, lanes 7-10).

The third possible explanation could be that the allowed spacing between SD sequence and start codon in *L. lactis* is higher than in *E. coli* and *B. subtilis*. The spacing between SD-sequence and start codon has been the subject of investigations in *E. coli* and *B. subtilis*. Analysis of the effect on translation efficiency resulting from changes in the ribosome binding site have demonstrated that the longer spacing than observed for naturally observed ribosome binding sites are allowed, both in *E. coli* (13, 27), and in *B. subtilis* (27). Particularly *B. subtilis* is relatively more efficient than *E. coli* in recognizing ribosome binding sites with a greater than optimal spacing between SD and initiation site. Spacing of up to 12 nucleotides led to a 75% loss in translation efficiency in *E. coli*, but resulted in only 50% loss in *B. subtilis* (27) as compared with values obtained with an optimal spacing. Moreover, extension of the region between the SD sequence and the start codon to 21 nucleotides still caused, although drastically reduced (0.3% of optimal), *lacZ* expression in *E. coli* (13). Strain MC1061

harboring pNZ10α58 also showed a reduced expression of the *usp45-amyS* gene fusion, but the decrease in expression was by far not as dramatically as demonstrated with *lacZ* fusions (13). This could be due to the fact that expression of gene fusion in pNZ10α5 is directed by SD2, which is not optimized. The results of MG1363 harboring pNZ10α58 presented in this report suggest that *L. lactis* is able to use a ribosome binding site with a spacing of 21 nucleotides between the SD region and the start codon, without major loss of translation efficiency.

An optimization between SD sequence and start codon does not improve expression in *L. lactis*.

It seemed plausible that a combination of a strong SD sequence with a proper spacing in the *usp45-amyS* gene fusion would result in a higher production of the α-amylase. However, decrease of the spacing between SD1 and the AUG start codon, as in pNZ10α59, did not result in an increased expression of the *usp45-amyS* fusion in *L. lactis*. This could be due to the reduction of the non-translated region of the mRNA. Analysis of the *lac* promoter revealed that sequences downstream the start of transcription contribute significantly to promoter activity. A deletion of 54% of the non-translated 5' region resulted in a 12-fold decrease in expression level (26). In pNZ10α59, 32% of the non-translated 5' region is deleted. The possible negative effect of this deletion might conceal a conceivable higher rate of translation. This possibility should be investigated through RNA analysis. Furthermore, the results derived from pNZ10α57 and pNZ10α58 suggest that variations in the translational initiation region are not of major importance in the expression of the *usp45-amyS* gene fusion in *L. lactis*. The optimization of the ribosome binding site in pNZ10α59 results in an increase in α-amylase production in *E. coli*. However the difference in the observed activities is not as high as found with comparable ribosome binding sites used in the *lacZ* fusions.

Conclusions

Both SD sequences (SD1 and SD2) of the *usp45* gene are functional in translational initiation of the *usp45-amyS* fusion, both in *E. coli* as in *L. lactis*. Although differences in expression of the *usp45-amyS* gene fusion in *E. coli* have been observed no increase in production of the α-amylase in *L. lactis* was obtained using the optimized spacing between SD1 and the AUG initiation codon. These data suggest that *L. lactis* tolerates SD sequences with a low complementarity to 16S rRNA as well as a 21-

nucleotide spacing between the SD and the AUG start codon.

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CHAPTER 6

CLONING, NUCLEOTIDE SEQUENCE AND REGULATORY ANALYSIS OF THE *LACTOCOCCUS LACTIS DNAJ* GENE.

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The Heat (Dan Reed Network)

SUMMARY

The *dnaJ* gene of *Lactococcus lactis* was isolated from a genomic library of *L. lactis* strain NIZO R5 and cloned into pUC19. Nucleotide sequencing revealed an open reading frame of 1137 bp, encoding a protein of 379 amino acids. The deduced amino acid sequence showed homology to the DnaJ proteins of *Escherichia coli*, *Mycobacterium tuberculosis*, *Bacillus subtilis* and *Clostridium acetobutylicum*. The level of the *dnaJ* monocistronic messenger RNA increased approximately three-fold after heat shock. The transcription initiation site of the *dnaJ* gene was determined and appeared to be preceded by a typical Gram-positive vegetative promoter sequence (TTGCCA-17bp-TAAAAT). Upstream of the promoter region an inverted repeat is located, that is identical to those detected upstream of heat shock genes of other Gram-positive organisms. A transcriptional fusion between the *dnaJ* expression signals and an *usp45-amyS* secretion cassette caused a significant increase in α-amylase activity after heat shock induction. Deletion mutagenesis showed that the inverted repeat is involved in heat shock regulation of the *dnaJ* gene. The conservation of this palindromic sequence in Gram-positive heat shock genes suggests a common regulatory pathway, distinct from the system used in Gram-negative bacteria.

INTRODUCTION

An abrupt increase in growth temperature usually causes the induction of synthesis of a small group of proteins, called the heat shock proteins. This response is a common feature in eubacterial, archaebacterial and eukaryotic organisms. Not only the reaction to heat shock is similar, but also the structure and function of the induced proteins are highly conserved (for a recent review see 1).

The *dnaJ* gene of *Escherichia coli* was originally discovered as an essential gene for bacteriophage lambda replication (39). Recently, it has been demonstrated that DnaJ is also involved in the replication of phage P1 (47) and *oriC* plasmids (22). One of the major activities of DnaJ is to stimulate the ATPase activity of DnaK, the prokaryotic member of the HSP70 family. This enhanced ATPase activity may result in an efficient recycling of DnaK (20). Furthermore, DnaJ is also believed to "target" other proteins for action by DnaK (48). Due to its cooperation with DnaK, DnaJ also plays a role in protein folding (11) and the facilitation of export of homologous and

hybrid proteins (29, 49).

Analysis of the heat-shock response of *Lactococcus lactis* has revealed the induction of 13-16 proteins after temperature shift from 30 °C to 37 °C or 42 °C (2,50). Immunological screening of these induced proteins showed the presence of GroEL- and DnaK-like heat shock proteins in *L. lactis*. (2,50). In addition, the lactococcal counterparts of the heat shock proteins GrpE and DnaJ could also be detected (2). Recently, the *groELS* operon of *L. lactis* has been cloned and its nucleotide sequence has been determined (17).

In this report we describe the cloning and characterization of the *dnaJ* gene of *L. lactis*. We show that its expression is regulated at the transcriptional level and is critically dependent on the presence of a palindromic structure immediately preceding its promoter.

MATERIAL AND METHODS

Bacterial strains, plasmids, media and growth conditions. *E. coli* strain JM83 (45) and *L. lactis* strains MG1363 (12) and NIZO R5 (30) were used. The plasmids used are listed in Table 1. *E. coli* was grown in TY broth (34) or on TY solidified with 1.5% agar. *L. lactis* was grown in glucose M17 medium (40) or in whey-permeate broth (9). For the induction of heat shock response, *L. lactis* cells were grown at 30°C to an optical density at 600 nm of 0.6. Cells were pelleted by centrifugation and resuspended in whey-permeate broth of 30°C, 37°C or 42°C and incubated for 10, 20 or 30 min at those temperatures. For electroporation of *L. lactis*, cells were cultured, washed and recovered as described previously (15) and plated on glucose M17 agar plates. The antibiotics used for selection in media were chloramphenicol (10 µg/ml) and ampicillin (50 µg/ml).

DNA manipulations. Plasmid DNA was isolated as described previously (4). For *L. lactis* cells TMS buffer (44) containing 2% of lysozyme was used for 30 min at 37°C to protoplast the cells. Enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, Md.) and New England Biolabs Inc. (Beverly, Mass.) and were used as recommended by the suppliers. DNA manipulations were essentially as described previously (35).

Table 1. Plasmids used in this study

Plasmid	Relevant genetic characteristics[a]	Source/Reference
pUC19	Cloning vector; Ap^R	45
M13 mp 18/19	Cloning vector for sequencing purposes	23
pNZ2015	pUC19 carrying a *StyI-XhoI* fragment containing the *dnaJ* gene; Ap^R	This study.
pNZ2016	pUC19 carrying a *SphI-XhoI* fragment containing the *dnaJ* gene; Ap^R	This study.
pNZ10α5	pNZ123 carrying the *amyS* gene fused to position -158 to 127 of the *usp45* gene; Cm^R	44
pNZ20α1	pNZ123 carrying the *usp45-amyS* cassette fused to position -217 to 41 of the *dnaJ* gene; Cm^R	This study
pNZ20α3	pNZ123 carrying the *usp45-amyS* cassette fused to position -40 to 41 of the *dnaJ* gene; Cm^R	This study

a. Abbreviations: Cm, Chloramphenicol; Ap, Ampicilin

Cloning of the *dnaJ* gene and immunological methods. A genomic library of *L. lactis* NIZO R5 partial *Sau3A* fragments was prepared in *E. coli* MB406 (Promega, Madison, Wisc.) using the EMBL arms cloning system (Packagene Lambda Packaging System; Promega, Madison, Wisc.) as described previously (33). Cellular extracts of *L. lactis* strain NIZO R5 were separated on SDS/PAGE (18). A mixture of proteins with a molecular weight of approximately 40 kDa, including glyceraldehyde-3-phosphate dehydrogenase, was excised from the gel and recovered by isotachophoresis (27). Antibodies were raised against this partially purified protein fraction and were used to screen the genomic library. Immunoblotting was performed as described previously (42). Screening of the library, preparation of liquid lysates and DNA isolation of positive recombinant phages was performed as described previously (43).

DNA sequence analysis and data evaluation. Restriction fragments of pNZ2015 (Table 1) were inserted into the appropriate sites of M13 mp18 or mp19 (23). DNA sequencing by the dideoxy chain method (36) was performed using Sequenase (U. S. Biochemical Corp., Cleveland, Ohio) and the universal M13 primer or oligonucleotides synthesized on a Cyclone DNA synthesizer (Biosearch, San Rafael, Calif.). The DNA sequence was analyzed with the PC/GENE software system (IntelliGenetics Inc., Geneva, Switzerland). The database search was performed with the CaosCamm facilities in Nijmegen, The Netherlands (10).

RNA analysis. Following 10 min of induction at 30°C, 37°C or 42°C, 25 ml of cells were pelleted by centrifugation and immediately frozen in liquid nitrogen. After

resuspension in 0.5 ml of TE buffer (10 mM Tris-HCl [pH 8.0], 1mM EDTA) total RNA was isolated using macaloid clay (32). After addition of 0.6 g Zirconium beads (0.1-mm, Biospec Products, Bartlesville, Okla.), 0.17 ml 4% macaloid clay suspension, 0.5 ml phenol and 50 µl 10% sodium dodecyl sulphate, cells were disrupted in a bead beater (Biospec Products, Bartlesville, Okla.). After centrifugation, a phenol/chloroform extraction was performed. The RNA was precipitated and stored at -80 °C. Northern blot analysis was performed as described previously (31).

Primer extension analysis. A synthetic oligonucleotide complementary to position 3 to -27 of the *dnaJ* gene was used in a primer extension experiment. 1 picomol primer was annealed to 30 µg of RNA, followed by cDNA synthesis as previously described (8). The product was analyzed on a 6% polyacrylamide-urea sequencing gel together with a dideoxy sequencing reaction using the same primer.

Construction of a *dnaJ-amyS* transcriptional fusion. A transcriptional fusion between the *dnaJ* expression signals and a *usp45-amyS* gene fusion (44) was constructed. For this fusion a recombinant PCR protocol (16) was adjusted (Fig. 1). Three primers were used: (i) A fusion primer with the sequence 5'-GGAAGTGAGTAATTTAGAAATGAAAAAAAAGATTATCTCAGC-3', of which the 5' end was complementary to position 23 to 44 of *dnaJ* and the 3' end to the first 23 nucleotides of the *usp45* signal sequence, (ii) an oligonucleotide with the sequence 5'-CGACTTCGGGATGATCC-3', complementary to the *amyS* gene, and (iii) the reverse sequencing primer (New England Biolabs Inc. Beverly, Mass.). Plasmids pNZ10α5 (Table 1)(44), containing the *usp45-amyS* fusion encoding the Usp45 signal peptide fused to the mature α-amylase of *Bacillus stearothermophilus*, and plasmid pNZ2016 (Table 1) were used as templates. The 3 primers and 2 templates were used simultaneously in one PCR reaction with annealing at 46 °C. In the first 2 cycles a product will be generated from the fusion primer, the α-amylase primer and pNZ10α5 (Fig. 1 A and B). The 3' end of this product will be complementary to the fusion primer, and serves as a primer on pNZ2016 (Fig. 1 C). The generated product can be further amplified with the reverse primer (Fig. 1 D) and the α-amylase primer. After 30 cycles of PCR the two expected products were found. One is the product of the fusion primer and the α-amylase primer using pNZ10α5 as template, the other is a fragment containing the *dnaJ* expression signals fused to the *usp45-amyS* fusion (Fig. 1 E).

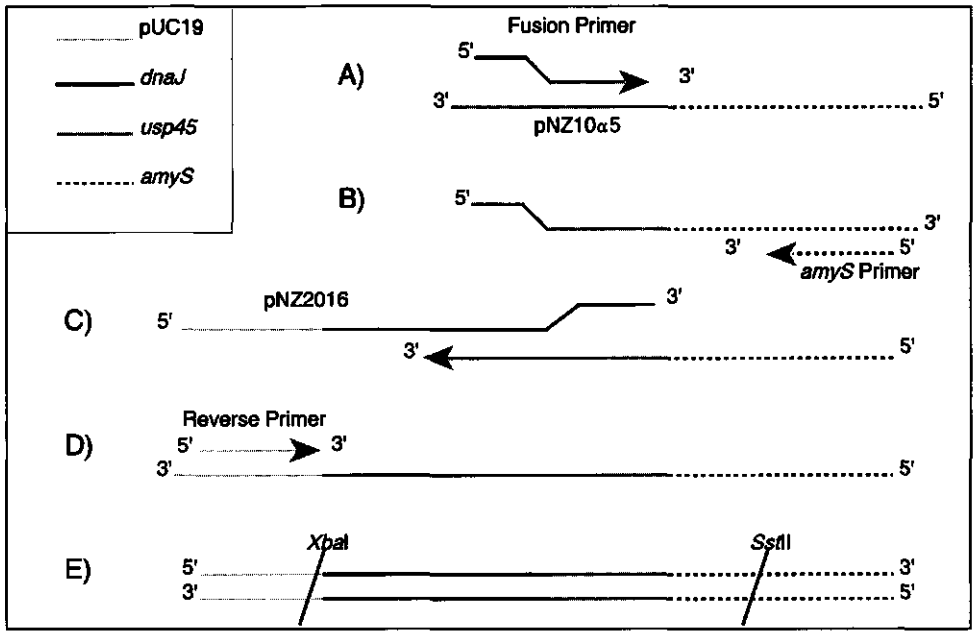

FIG. 1. Schematic drawing of the recombinant PCR method for the construction of a transcriptional fusion *of the dnaJ* expression signals with the *usp45-amyS* fusion as described in the Materials and Methods section. Only steps involved in the production of the fusion and their products are displayed. A) annealing first cycle. B) annealing second cycle. C) annealing third cycle. D) annealing fourth cycle. E) end product.

Subsequently, the fusion product was cut with *Xba*I and *Sst*II and ligated into pNZ10α5, digested with the same enzymes, resulting in pNZ20α1. For the construction of pNZ20α3, DNA of pNZ20α1 served as a template in a PCR reaction with the α-amylase primer and a primer complementary to position -40 to -14 of the *dnaJ* gene, preceded by a *XbaI site* (5'-GGGTCTAGATTTTTTTGCCAAAAAT-GAAAAAACGTG-3'). The product was cut with *Xba*I and *Sst*II and ligated into pNZ10α5, digested with the same enzymes.

α-Amylase activity assay. Culture supernatant was incubated with 20 mg of amylose azure (Sigma Chemical Co; St. Louis, MO) for 60 min at 60 °C in 1 ml of α-amylase buffer (50 mM Tris-HCl [pH 7.5], 50 mM NaCl, 5 mM $CaCl_2$). After centrifugation, absorption at 595 nm of the supernatant was measured.

Nucleotide sequence accession number. The DNA sequence shown in Fig. 2 has been assigned GenBank data base accession no. M99413

RESULTS

Isolation of the *dnaJ* gene from *L. lactis* NIZO R5. A genomic library of strain R5 was screened with antibodies raised against intracellular proteins of *L. lactis* with a size of approximately 40 kDa. This screening resulted in the isolation of four recombinant phages. As expected, analysis of phage lysates of these recombinant phages by immunoblotting revealed that they all directed the synthesis of proteins with a size of approximately 40 kDa. Analysis of the DNA of the phages showed that they contained inserts of 12-19 kb. Restriction endonuclease mapping revealed a 8-kb *Xho*I-*Kpn*I fragment present in all inserts. This fragment was used for further analysis. Different overlapping fragments were inserted into pUC19. Immunoblot analysis of *E. coli* JM83 harboring these plasmids showed that a 2-kb *Sty*I-*Xho*I DNA fragment directed the synthesis of the 40 kDa protein. The plasmid containing this fragment was designated pNZ2015.

Nucleotide and encoded aa sequences. The nucleotide sequence of the *Sty*I-*Xho*I fragment of pNZ2015 was determined (Fig. 2). The largest open reading frame present on this fragment was ORF1 with a size of 1173 bp. ORF1 could encode a protein of 379 residues, with a calculated molecular mass of 40786 Da. This molecular mass is in agreement with the size of the protein reacting with the antibodies used. The deduced amino acid sequence of the protein shows a high glycine content (16%), and contains four repeats of a motif consisting of CxxCxGxG (residues 154-161; 171-178; 197-204; 211-218). Furthermore, the sequence GGFGG is repeated 3 times in the N-terminal part (residues 75-79; 80-84; 96-100). ORF1 is preceded by a Shine-Dalgarno sequence (38) at position 20 for which a free energy of -11.8 kcal/mol could be calculated (41). A second ORF (ORF2) of at least 200 bp terminates at position -257; only the 3' terminal part of ORF2 is present on the *Sty*I-*Xho*I fragment. Downstream of ORF1, no ORFs larger than 133 bp are present.

An inverted repeat (IR), extending from positon -69 to position -39 is located between ORF1 and ORF2. Downstream ORF1, no sequences with significant secondary structures were found.

Relationship of the determined ORFs to other amino acid sequences. The deduced amino acid sequence of ORF1 was compared to sequences in the NBRF-Pir

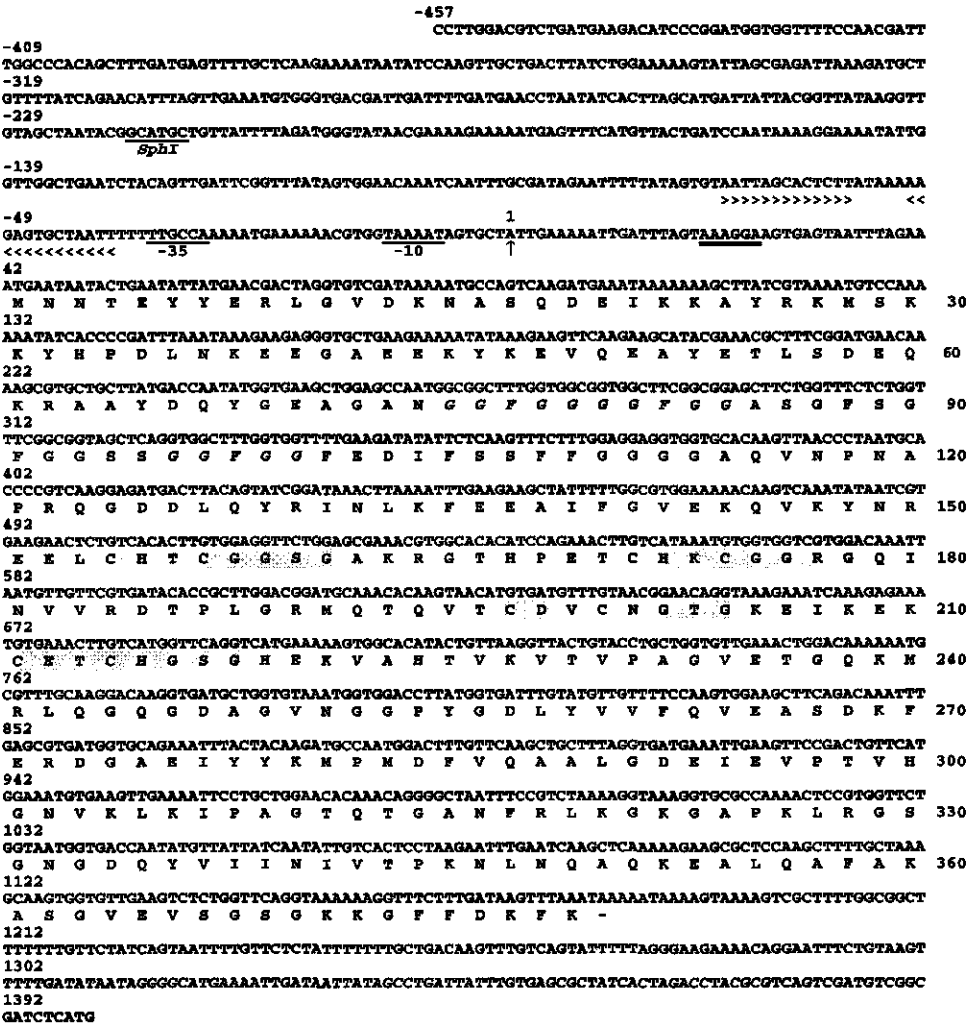

FIG. 2. Nucleotide and deduced amino acid sequence of the *dnaJ* gene of *L. lactis*. Indicated are the putative Shine-Dalgarno sequence (double underlined) , the -10 and -35 sequences (underlined) and the IR (arrowheads below the sequence). The 5' end of the mRNA as identified by primer extension is marked (arrow). The repeated CXXCXGXG motif in the protein sequence is shaded and the repeated GGFGG sequences are shown in italics.

(Version 32.0) and SWISS-PROT (Version 21.0) data bases and published protein sequences. This analysis revealed a high similarity of the encoded protein with several bacterial DnaJ heat shock proteins; DnaJ from *E. coli* (3, 28) (45.2% identity), from *Mycobacterium tuberculosis* (19) (37.5% identity), and from *Bacillus subtilis* (46) (57% identity) (Fig. 3). In addition, similarity with the published N-terminal sequence of DnaJ of *Clostridium acetobutylicum* (51% identity)(25) was found. The amino acid sequence

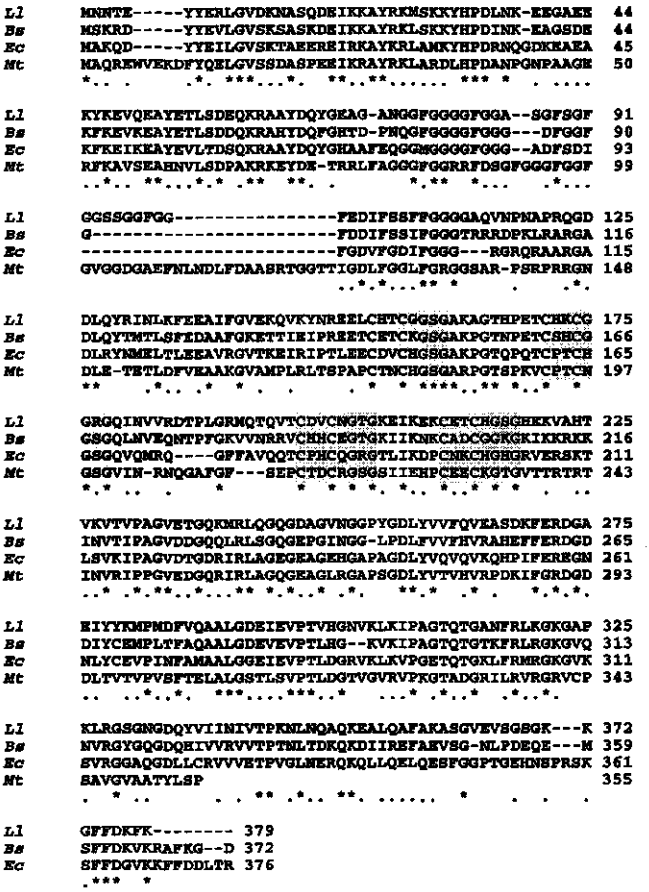

FIG. 3. Alignment of the deduced amino acid sequences of the DnaJ proteins of *L. lactis* (*Ll*), *B. subtilis* (*Bs*) (46), *E. coli* (*Ec*) (3, 28) and *M. tuberculosis* (*Mt*)(19). Identical amino acids are indicated by asterisks, conserved residues are indicated by points (14). Gaps to obtain maximum fit are indicated by dashes. The conserved CXXCXGXG repeats are shaded.

also shared significant homology with SIS1 (21), YD1 (6) and SCJ1 (5), three eukaryotic counterparts of this heat shock protein from yeast. Alignment of the proteins revealed the conservation of the CxxCxGxG repeats. From these data, together with the heat shock regulation data described below, we conclude that ORF1 encodes the lactococcal *dnaJ* gene. Homology analysis of ORF2 and the small ORFs downstream ORF1 revealed no significant homology with known proteins.

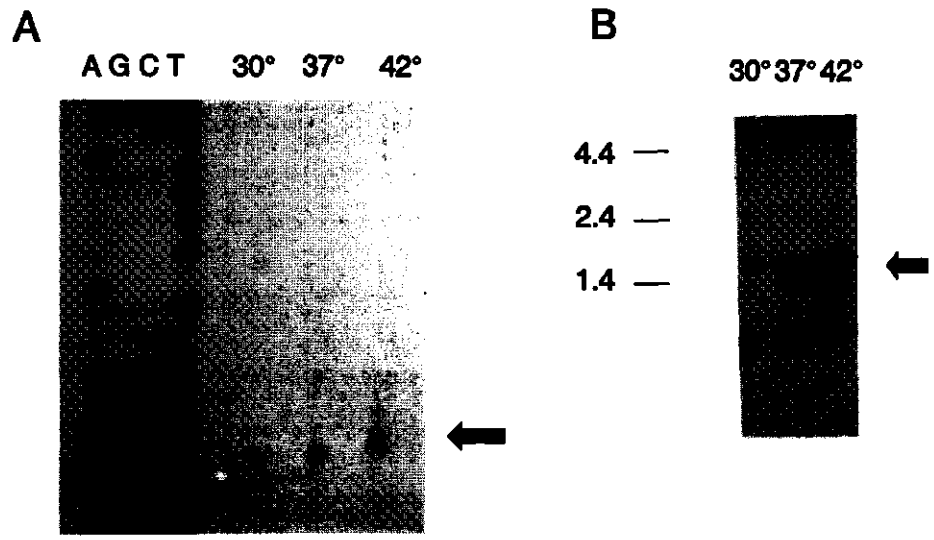

FIG. 4. Transcriptional analysis of the *dnaJ* gene. A) Autoradiogram of the sequence gel used to analyze the primer extension products of RNA of NIZO R5, isolated after 10 min of heat shock induction at the indicated temperatures. The primer extension product is indicated (arrow). The sequence ladder obtained with the same primer is also shown. B) Autoradiogram of a Northern blot of total RNA, isolated at 30°C, or after heat shock, hybridized with a radioactively labeled *Hpa*I-*Xho*I fragment of the *dnaJ* gene. The 1.8 kb messenger is indicated (arrow). To the left, the size of RNA markers (Bethesda Research Laboratories, Gaithersburg, Md.) are shown in kb.

Transcriptional analysis of the *L. lactis dnaJ* gene. The start of transcription of the *dnaJ* gene was determined by primer extension analysis of RNA isolated at 30°C or after heat shock (Fig. 4A). These experiments revealed that the transcription initiation starts at an adenine at position 1. This start of transcription is preceded by the sequence TTGCCA-17bp-TAAAAT (position -35 to -7), resembling the consensus for vegetative Gram-positive promoters (13). The putative -10 sequence is preceded by TGN, which is also present in more than 50% of the lactococcal promoters determined so far (37). In RNA isolated from a 30°C culture, a small amount of cDNA could be observed. In equal amounts of RNA isolates from cultures after heat shock, the quantity of primer extension product increased approximately 2-3 fold with respect to 30°C, indicating an elevated amount of transcripts after heat shock. Furthermore, no additional transcription initiation sites could be detected under these conditions.

Northern blot analysis was performed by use of a radioactively labeled *Hpa*I-*Xho*I fragment containing the part of the *dnaJ* gene downstream position 390. Analysis of RNA isolated from *L. lactis* NIZO R5 grown at 30°C, showed that the *dnaJ* gene was transcribed as a 1.8-kb mRNA (Fig. 4B). The amount of mRNA increased approximately two-fold after heat shock of 37°C and 3-4-fold after heat shock of 42°C.

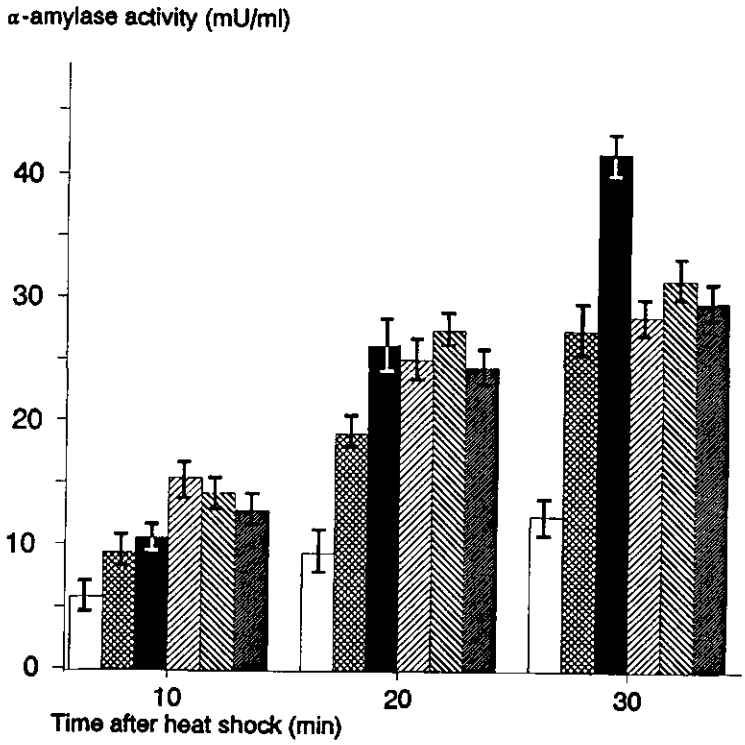

FIG. 5. Relative amounts of α-amylase activity produced by *L. lactis* MG1363 carrying a transcriptional *dnaJ-amyS* fusion. pNZ20α1 contains the *dnaJ* promoter region including the IR, while in pNZ20α3 the IR was deleted. Heat shock was induced as described in the Materials and Methods section. Samples were taken after indicated times, and newly secreted α-amylase activity was assayed.
MG1363[pNZ20α1] at: 30°C □ , 37°C ▩ , 42°C ■ . MG1363[pNZ20α3] at: 30°C ▨ , 37°C ▧ , 42°C ▨ .

A 1.0-kb product also hybridized with the probe. When a probe complementary to the 5' end of the *dnaJ* gene was used this product could not be detected, indicating that it probably represents a 3' terminal breakdown product of the *dnaJ* mRNA (results not shown). No large products were detected after heat shock.

A palindromic DNA structure is involved in heat shock regulation of *dnaJ*.
The IR found at position -69 shows similarity to palindromic structures that are located at corresponding positions upstream of heat shock genes of *B. subtilis*, *C. acetobutylicum*, *Synechocystis* sp., *Synechococcus* sp., *Mycobacterium* sp. and *Chlamydia psittaci* (46). It has been postulated that this IR could be involved in the temperature sensitive regulation of transcription of these heat shock genes. To address this hypothesis, a construction was made in which the DnaJ-encoding region was exchanged with a *usp45-amyS* gene fusion encoding the *B. stearothermophilus* mature α-amylase, preceded by the *usp45* signal peptide (44). In plasmid pNZ20α1, the *usp45-amyS* fusion is preceded by a region of *dnaJ* including position -217 to 44

that contains the IR (Fig. 2). In plasmid pNZ20α3, only position -40 to 44 of the *dnaJ* gene is present and hence the IR is deleted. After introduction of pNZ20α1 or pNZ20α3 in *L. lactis* MG1363, α-amylase activities were measured 10, 20, or 30 min after heat shock at 37 or 42 °C (Fig. 5). The final optical density of the cells after heat shock induction at 37 °C or 42 °C was not higher compared to the optical density of cells grown at 30 °C at the indicated times. Strain MG1363 harboring pNZ20α1 resulted in 2-4 times higher α-amylase production after heat shock induction. However, strain MG1363 harboring pNZ20α3 showed a constitutive α-amylase production at 30°C and at elevated temperatures. The level of α-amylase production of this strain was comparable to that of MG1363 harboring pNZ20α1 after heat shock induction.

DISCUSSION

In this report the cloning, sequencing and characterization of the *dnaJ* gene of *L. lactis* is described. Besides homology of the encoded protein with the DnaJ protein of *E. coli* (3, 28) and *M. tuberculosis* (19), identity was found with the DnaJ proteins from *B. subtilis* (46) and from *C. acetobutylicum* (25). The Met residue at position 1 fits perfectly in the alignment of the different DnaJ proteins. Alignment of the reported proteins revealed a significant overall homology and the conservation of a motif consisting of CxxCxGxG, which is repeated four times (Fig. 3). This motif was also found in the eukaryotic homologues of the DnaJ proteins; SCJ1 (5), YD1 (6) and SIS1 (21). The biological meaning of this motif is not yet clear. The organisation into two larger repeats CxxCxGxG$(x)_8$CxxCxGxG as in *YDJI* (6), was not found in the other reported proteins and is unlikely to be characteristic for DnaJ proteins. The conservation of the GGFGG sequence is less significant. Only one of the three lactococcal copies of this sequence is present in the bacterial DnaJ species of *E. coli* and *B. subtilis*.

In prokaryotes, most *dnaJ* genes characterized so far are preceded by *dnaK*, encoding another heat shock protein that is conserved among prokaryotes and eukaryotes (3, 25, 28, 46). Upstream of the *L. lactis dnaJ* gene another ORF, designated ORF2, was found, but its deduced amino acid sequence shared no homology with known DnaK proteins, suggesting another genomic organization of these heat shock genes in *L. lactis*. The *dnaJ* gene of *M. tuberculosis* is located 788 bp downstream of the *dnaK* gene (19). This intergenic distance exceeds the DNA

region sequenced from *dnaJ* from *L. lactis*, hence a conservation in genetic organization of the *dnaK* and *dnaJ* genes between *M. tuberculosis* and *L. lactis* cannot be totally excluded. The possibility that the *dnaK* is situated downstream of the *dnaJ* gene or elsewhere on the chromosome is also still conceivable.

In *B. subtilis* (46), *C. acetobutylicum* (25), and *E. coli* (3, 28) *dnaJ* is located in an operon that also includes *dnaK*. In *L. lactis* however, the start of transcription of *dnaJ* is located immediately upstream of the *dnaJ* gene. In addition, the size of the RNA messenger is 1.8 kb. This is too small to contain both genes. From these data, it can be concluded that the lactococcal *dnaJ* and *dnaK* are not organized in a single operon. The same holds for the *dnaK/ dnaJ* gene organisation of *Synechocystis* (7). Transcriptional analysis of the *dnaK* gene of this organism revealed that it is transcribed as a monocistronic messenger. Hence, a putative *dnaJ* gene will also be on a separate transcriptional unit.

The induction of expression of the *dnaJ* gene by heat shock was determined by three methods. First, the primer extension carried out with RNA isolated at 30 °C, or after heat shock at 37 °C and 42 °C, demonstrated a significant increase of *dnaJ* mRNA. Second, Northern blot analysis showed a 2-fold increase in the amount of *dnaJ* RNA after heat shock at 37 °C. The amount of messenger was even higher after heat shock at 42 °C. These results confirm that the heat shock response is controlled at the transcriptional level. The same has been found for the heat shock genes of other Gram-positive bacteria such as *B. subtilis* (46) and *C. acetobutylicum* (24, 25). Third, the fusion between the *dnaJ* promoter region and an *usp45-amyS* cassette, caused a significant increase of α-amylase production after heat shock. Similar results were obtained in a comparable experiment in *B. subtilis* (46) using a transcriptional fusion between the *dnaK* promoter and the *amyL* gene.

Analysis of the transcription initiation site of the *dnaJ* gene revealed that it was preceded by Gram-positive vegetative -10 and -35 sequences (13). The IR, located upstream of the -35 sequence was also found upstream of the heat shock genes characterized thus far in Gram-positive organisms, like the *groELS* operons from *L. lactis (17)* and *C. acetobutylicum* (24) and the *dnaK* operons from *B. subtilis* (46) and *C. acetobutylicum* (25). Furthermore, an IR with the same sequence is located upstream heat shock genes of *Synechocystis, Synechococcus* and *C. psittacci* (46). However, the IR is, at least partial as for *Synechococcus*, or entirely as in *B. subtilis* or *C. acetobutylicum* located on the 5' end of the mRNA in these operons, whereas

in the *L. lactis dnaJ* gene it is located upstream of the start of transcription. The IR at position -69 of the lactococcal *dnaJ* gene shares complete identity with the consensus as proposed by Wetzstein et al. (46). To examine the function of this IR in heat shock regulation, pNZ20α1 and pNZ20α3 were constructed. These plasmids contain a *usp45-amyS* fusion, preceded by the promoter region of the *dnaJ* gene. In MG1363 harboring pNZ20α1, containing the IR, the level of α-amylase activity is 2-4 times higher after heat shock. MG1363 harboring pNZ20α3, in which the IR has been deleted, showed no heat shock induction of α-amylase production. These results indicate a major role for the IR in the heat shock regulation of the *dnaJ* gene of *L. lactis*.

In transcription of *E. coli* heat shock genes, a specific sigma factor (σ^{32}) is involved that recognizes a promoter sequence deviant from the vegetative -35 and -10 sequences (26). In the heat shock genes from *B. subtilis* (46) and *C. acetobutylicum* (24, 25), the transcription start sites are preceded by vegetative promoter sequences. The function of the IR in the heat shock regulation of the *dnaJ* gene of *L. lactis*, and its conservation in sequence and location in heat shock genes of Gram-positive bacteria, strongly suggests a significant difference in heat shock regulation between *E. coli* and Gram-positive organisms. Moreover, in *Synechocystis, Synechococcus* and *C. psittacci* both an *E. coli* heat-shock consensus promoter sequence and the IR are present in the promoter region suggesting that the IR is not specific for Gram-positive heat-shock genes. However, in the *L. lactis dnaJ* gene the IR is unlikely to protect against RNA degradation as suggested for *B. subtilis* by Wetzstein et al. (46), because it is located upstream of the start of transcription. For the same reason it is unlikely to cause a pausing of the RNA polymerase as proposed by Narberhaus and Bahl (24). The amount of α-amylase produced by MG1363 harboring pNZ20α3 at all tested conditions was comparable with the amount produced by MG1363 harboring pNZ20α1 after heat shock. This constitutive high level of α-amylase production by MG1363 harboring pNZ20α3, suggests that the repeat is a target for a repressor, the activity of which is disturbed after heat shock. However, further analysis of the system is required to be conclusive about this hypothesis.

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CHAPTER 7

SUMMARY AND CONCLUDING REMARKS

Don't You Forget About Me. (Simple Minds)

SUMMARY AND CONCLUDING REMARKS.

Lactococcus lactis strains have been used for centuries in food fermentation, now appreciated as traditional biotechnology. They have been applied in the cheesemaking process and for the manufacturing of other dairy products. Years of experience with these lactic acid bacteria have led to a profound understanding of the microbiological and technological aspects of *L. lactis*. Recent progress in the genetics of *L. lactis* made this organism a suitable candidate for the use in modern biotechnology as a host for the production of homologous, heterologous, or engineered proteins. The purpose of the research, described in this thesis, was to investigate the capacity of *L. lactis* to produce and, in particular, to secrete heterologous proteins.

Chapter 1 presents a brief overview of the knowledge about gene expression and secretion systems in procaryotes, with specific attention to heterologous gene expression in *L. lactis*.

In **Chapter 2** the characterization of Usp45, the major extracellular protein of *L. lactis* is described. The aim of isolating the *usp45* gene was to use its promoter, ribosome binding site and export signal for the development of an expression and secretion system for *L. lactis*. Determination of the nucleotide sequence of the gene revealed several specific characteristics of the Usp45 protein and its gene. i) The primary amino acid sequence of the protein revealed an unusual amino acid composition and did not show homology with any protein of known function. ii) The mature protein starts at amino acid residue 28. It is preceded by a leader peptide which contains 3 possible signal peptidase I cleavage sites, after ala^{19}, ala^{20} and ala^{27}. iii) The open reading frame encoding Usp45 is preceded by two Shine and Dalgarno (SD) sequences, both with uncommon properties. iv) The A/T content of the region 100 nucleotides upstream of the *usp45* promoter is significantly (10%) higher than that found in *L. lactis* coding sequences. All these characteristics have been investigated.

No function could be postulated for Usp45. Its deduced amino acid sequence showed 30% homology with that of P54 of *Enterococcus faecium*, but the function of this protein is also unknown. However, a weak cross reaction has been observed of antibodies raised against surface layer (SL) proteins from *Lactobacillus helveticus* with the Usp proteins from several *L. lactis* strains (15), and of the Usp45 antibodies with the surface proteins of *Lactobacillus plantarum* NCFB 1988 (8). This could indicate that *L. lactis* Usp proteins are related to SL-proteins.

Several attempts to inactivate the chromosomal *usp45* gene have been performed. (15). Various plasmids have been constructed in which the *usp45* homologous region carried deletions or several mutations in the 3' and 5' region of the structural gene. Campbell-like integration of these plasmids would result in truncation of the *usp45* gene. Furthermore, plasmids have been constructed for the inactivation of the *usp45* gene by replacement recombination. Although various studies have shown that homologous recombination can be used for gene inactivation in *L. lactis* (7, 13) none of the tested strategies resulted in the inactivation of the *usp45* gene, suggesting that *usp45* is an essential gene in *L. lactis*.

Chapter 3 describes the construction of an expression and secretion system, based on the *usp45* gene, which was evaluated with the *prtP* gene of *L. lactis* SK11 and the *amyS* gene of *B. stearothermophilus* as reporter genes. Fusions in which the 27 amino acid leader peptide directed the secretion of the homologous proteinase, were used to show that the *usp45* leader is sufficient for the efficient secretion of PrtP. In addition, to examine the functionality of two shorter leader peptides (aa 1-19 and aa 1-20) as signal peptides, plasmids encoding the first 19, 20 or 27 residues of Usp45 fused to the mature α-amylase were introduced in *E. coli* and *L. lactis*. In *E. coli* these plasmids resulted in secretion of active α-amylase into the periplasm and even into the external growth medium. However, in *L. lactis* only the 27-residue leader peptide functioned as an export signal. These results provide experimental evidence for the postulated difference in length between signal peptides from Gram-positive and Gram-negative organisms. When the Usp45 signal peptide was used for the secretion of the α-amylase, less than 50% of this reporter protein was located in the extracellular medium. The remaining fraction was present as an unprocessed, inactive precursor in the intracellular fraction.

Analysis of the *usp45* promoter region is described in **Chapter 4.** Several *usp45-amyS* gene fusions were constructed and introduced on plasmids or in the chromosome of *L. lactis*. These gene fusions were used to demonstrate the role in transcription of the A/T-rich region immediately upstream of the *usp45* -35 region. The highest levels of α-amylase production were obtained when the *usp45-amyS* fusion was located at the position of the *usp45* gene in MG1363. These data showed that a large DNA region of more than 200 bp upstream of the -35 region was of major importance for expression. Similar results have been reported by Van Rooijen et al. (20) for the *lac* promoter. This could indicate a general role in transcription activation

for the region upstream of lactococcal promoters. The *L. lactis* strains harboring the various *usp45-amyS* fusions produced sufficient α-amylase activity to allow growth on media containing starch as a sole energy source.

Chapter 5 describes the effect of the translational initiation region on expression of the *usp45 -amyS* gene fusion. Both postulated SD regions SD1, an extremely weak ribosome binding site 6 bp upstream of the ATG start codon, and SD2, with a higher complementarity to the 3' end of the 16S rRNA, but 21 bp upstream of the ATG codon, were altered in the *usp45-amyS* secretion vector. These studies revealed that translation of the *usp45-amyS* fusion is possible from both SD regions. Deletion of either one of the SD regions resulted in normal α-amylase expression, suggesting that *L. lactis* tolerates SD-sequences with low complementarity to the 3' end of 23S rRNA, and ribosome binding sites with a 21 bp spacing. A reduction of the spacing between SD2 and the AUG start codon, resulted in a ribosome binding site which corresponds to the consensus in *L. lactis*, with respect to both spacing and free energy. However, this ribosome binding site did not lead to an significant increase in α-amylase production.

To enhance the level of heterologous gene expression, a search for stronger promoters was initiated. Several studies have been performed to isolate strong lactococcal promoter sequences, using promoter probe vectors (4, 6, 19). One of the isolated promoters resulting from these studies, has already been used in a heterologous expression system for the production of hen egg lysozyme and *B. subtilis* neutral protease (16, 17). We have used different approaches to isolate strong promoters.

Based on the N-terminal sequence of an abundant intracellular 30 kDa protein, oligonucleotide probes were designed and used to screen a genomic library from *L. lactis*. This resulted in the isolation of the 1-kb *Hind*III-*Pst*I fragment containing an open reading frame of 612 nucleotides that could encode a polypeptide of 204 aa (Fig. 1). The determined N-terminus was found at at position 2-11 of the deduced amino acid sequence, indicating that the N-terminal methionine had been removed in *L. lactis*. Homology analysis revealed that the amino acid sequence showed considerable homology with the aminoterminal part of glyceraldehyde 3-phosphate dehydrogenase from *B. subtilis, E. coli*, yeast, mouse and humans. Based on this homology and on the molecular weight of the *L. lactis* protein it could be calculated that the cloned fragment lacked approximately 350 nucleotides of the 3' part of the glyceraldehyde 3-

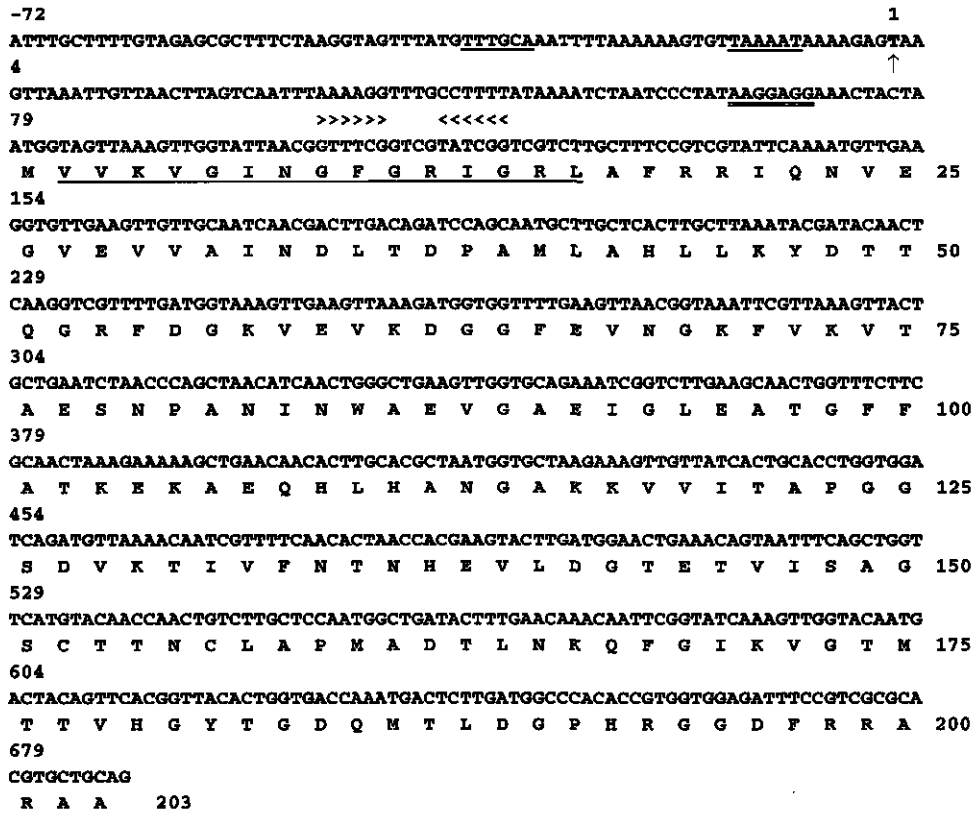

Fig. 1. Nucleotide and deduced amino acid sequence of the *L. lactis* glyceraldehyde 3-phosphate dehydrogenase gene. The 5' end of the mRNA as identified by primer extension is marked with an arrow. The -10 and -35 promoter sequences are underlined. The putative Shine and Dalgarno sequence is double underlined. The short inverted repeat is marked (arrowheads below the sequence. The deduced n-terminal sequence is also underlined.

phosphate dehydrogenase gene. Upstream of the ATG start codon a putative SD sequence was located. The complementarity of this SD sequence to the 3' end of the *L. lactis* 16S rRNA was 74.76 kJ/mol, which is the highest value found untill now in *L. lactis* (2, 18). The start of transcription was determined and revealed that the promoter (TTTGCA-16bp-TAAAAT-7bp-T) differed at 3 positions with the consensus for lactococcal promoters (Fig. 1)(2). A small inverted repeat is present in the non-translated 5' part of the mRNA. It remains unclear whether this repeat plays a role in the high expression of the gene.

Another approach to isolate strong promoters was based on the screening of

a *L. lactis* genomic library with antibodies raised against abundant intracellular proteins. Interestingly this resulted in the cloning of the *dnaJ* gene as described in **Chapter 6**. The *dnaJ* gene is one of the first heat shock genes characterized in *L. lactis*. Investigation of the promoter region showed that heat shock regulation in *L. lactis*, and very likely in other Gram-positive organisms, is not achieved by an alternative sigma factor as is the case in *E. coli* (9). An inverted repeat which is highly conserved in the promoter region of heat shock genes from Gram-positive organisms, is responsible for the repression of transcription of the *dnaJ* gene at non-stress conditions. The *dnaJ* promoter was used in the *usp45-amyS* fusion, and a 2-4 fold induction of α-amylase was accomplished after heat shock. However, the *dnaJ* promoter did not result in higher expression of the α-amylase, as compared to the *usp45* promoter.

In the last decennium a lot of research has been performed on the exploration of several microorganisms, such as yeast, fungi, *E. coli* and *B. subtilis* as a potential host for heterologous protein production and secretion. Initially, *B. subtilis* appeared to be a good organism for this purpose (3). It is a Gram-positive organism, capable of secreting large amounts of proteins into the extracellular medium. In addition, it can be cultivated in large amounts, at low costs. One of the main obstacles that limits the application of *B. subtilis* as a production host is its high extracellular proteolytic activity, resulting in degradation of the heterologous proteins of interest (11, 21). *L. lactis* could be a suitable alternative. It is also a Gram-positive organism. However, it has a low extracellular proteolytic activity and *Prt* strains are available. Furthermore, it possesses the additional desired features which make this organism a suitable candidate for heterologous gene expression. It is a safe, non-pathogenic organism and has a widespread use in the food industry. These properties have stimulated investigation on the use of *L. lactis* as a production organism (10, 12, 13, 16, 17). The overproduction of several homologous proteins has now been accomplished in *L. lactis* (1, 14) and the production of several heterologous proteins has been established (Chapter I). However, the secretion of heterologous proteins in *L. lactis* is inefficient (Chapter III). The investigation on the secretion of proteins in *L. lactis* has been initiated (5, 10, 12, Chapter II and Chapter III) and the results invite for continuing these investigations.

Besides a view on the several approaches which can be used in these investigations, the work presented in this thesis has yielded a set of expression and

secretion vectors, which could be employed to express heterologous genes in *L. lactis*. Furthermore they can be used in the further unravelling of the expression and secretion mechanism of *L. lactis*.

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CHAPTER 8

SAMENVATTING

Later is alllang begonnen (Klein Orkest)

SAMENVATTING

Melkzuurbacteriën worden al eeuwen gebruikt in de traditionele biotechnologie bij de bereiding van verschillende gefermenteerde voedingsmiddelen. Nog steeds vormt de melkzuurbacterie *Lactococcus lactis* de belangrijkste component van starter culturen bij de bereiding van Goudse kaas, boter en karnemelk. Jaren ervaring met *L. lactis* hebben geleid tot een uitgebreid inzicht in de microbiologische en procestechnologische aspecten van deze micro-organismen. De toenemende kennis van de genetica van *L. lactis* maakt dit organisme een geschikte kandidaat voor gebruik in de moderne biotechnologie als gastheer voor de productie van homologe (soorteigen) en heterologe (soortvreemde) eiwitten. Dit proefschrift beschrijft het onderzoek naar de mogelijkheid om *L. lactis* te gebruiken voor de productie en in het bijzonder de secretie van heterologe eiwitten.

Expressie signalen.

Voor de productie en secretie van eiwitten door organismen zijn verschillende onderdelen nodig. Als basis dient natuurlijk het structurele gen: het DNA dat uiteindelijk codeert voor het eiwit. Om dit gen te vertalen in eiwit is eerst een tussenstap nodig. Van het DNA wordt een kortlevende kopie, een boodschapper RNA molecuul gemaakt. Om dit proces, transcriptie genaamd, op gang te brengen is een promoter nodig. Dit element ligt op het DNA voorafgaand aan het gen, en bestaat uit twee redelijk geconserveerde domeinen van 6 nucleotiden (DNA bouwstenen). Het boodschapper RNA moet daarna vertaald worden in eiwit. Het juiste startpunt van deze vertaling wordt aangegeven door de ribosoom bindingsplaats. Het ribosoom, een belangrijk onderdeel van het RNA vertalend apparaat van de bacteriële cel, bindt aan een domein op het RNA, dat 6 tot 12 nucleotiden voorafgaand aan de start van het structurele gen ligt. De start van de vertaling wordt aangegeven door een startcodon, vaak AUG, dat vertaald wordt in het eerste aminozuur (eiwit bouwsteen) van het eiwit. Hoewel er tussen de beschreven expressiesignalen van de verschillende bacteriesoorten een zekere mate van conservering waar te nemen is, zijn er toch soortspecifieke kenmerken.

Het RNA molecuul eindigt vaak met sequenties die een secundaire structuur (een "hairpin") aan kan nemen. Dit gedeelte, terminator van transcriptie genoemd, speelt een rol in de stabiliteit van het boodschappermolecuul en dus ook in de

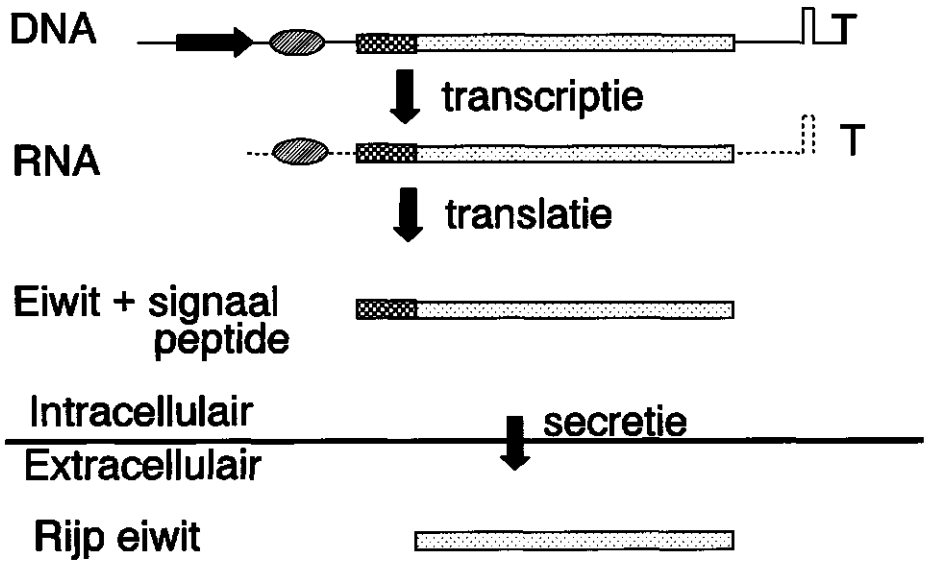

Fig. 1. Schematische weergave van de bacteriële expressie- en secretie route. DNA is weergegeven als doorlopende lijn, RNA als onderbroken lijn. De promoter regio is weergegeven als zwarte pijl, de ribosoom bindingsplaats als gearceerde ellips en de terminator van transcriptie is met een T aangeduid. De geblokte en gestippelde balk stellen respectievelijk het signaalpeptide en het rijpe eiwit voor of het DNA of RNA dat daarvoor codeert. Voorts is de celmembraan als dikke stippellijn aangegeven.

expressie van het gen.

Secretiesignaal

Indien eiwitten bestemd zijn om te worden uitgescheiden door bacteriën, worden ze aangemaakt als een "precursor". Het eiwit bevat voor het "rijpe" eiwit een extra stukje, het signaalpeptide. Van dit signaalpeptide is de opbouw geconserveerd in alle levende organismen. Het start met enkele positief geladen aminozuren, daarna komt een hydrophoob ("waterafstotend") gedeelte gevolgd door enkele ongeladen aminozuren, die de knipplaats voor een enzym, het signaalpeptidase, bevatten. Op deze knipplaats wordt het eiwit gesplitst tijdens transport naar buiten de cel, zodat het rijpe eiwit vrijkomt. Ook voor signaalpeptiden gelden soortgebonden kenmerken. Signaalpeptiden van bacteriën zijn in de regel langer dan die van eukaryoten (zoals planten en dieren).

De expressie van een gen en de secretie van het eiwit, met de beschreven signalen wordt schematisch weergegeven in Fig. 1.

Usp45

Om een efficiente productie en secretie van heterologe eiwitten in *L. lactis* te bewerkstelligen, werd onderzoek verricht naar geschikte expressie- en secretiesignalen. Daarvoor is het eiwit dat in de grootste hoeveelheid wordt gesecreteerd door *L. lactis* als basis genomen. De isolering en karakterisering van het gen dat voor dit eiwit, Usp45 genaamd, codeert, wordt beschreven in hoofdstuk 2 van dit proefschrift. De afgeleide aminozuurvolgorde van dit eiwit is vergeleken met de aminozuurvolgorden die bekend waren van andere eiwitten. Hierbij werd echter alleen overeenkomst gevonden met P54 uit *Enterococcus faecium*, een eiwit met een onbekende functie, zodat over de functie van Usp45 in het duister wordt getast. In de nucleotiden volgorde van het gen en de daarvan afgeleide aminozuurvolgorde werden de verschillende expressie en secretiesignalen aangetroffen. De karakterisering van deze elementen wordt beschreven in hoofdstuk 3, 4 en 5.

De Usp45 expressie- en secretiesignalen

In hoofdstuk 3 wordt het onderzoek naar het Usp45 signaalpeptide beschreven. Het rijpe Usp45 eiwit wordt voorafgegaan door een stukje van 27 aminozuren. Dit stukje voldoet aan de regels die voor signaalpeptiden gelden, zoals hierboven beschreven. Als het DNA, coderend voor het rijpe eiwit werd vervangen door DNA, coderend voor een *L. lactis* proteinase, bleek dat deze 27 aminozuren voldoende waren om dit proteinase efficient te secreteren. Naast de knipplaats tussen aminozuur 27 en 28, vertonen ook twee andere gebieden de structuur van een knipplaats. Deze knipplaatsen worden dan voorafgegaan door een signaalpeptide van de eerste 19 of van de eerste 20 aminozuren van Usp45. Om uit te zoeken of die peptiden voldoende waren voor secretie is in plaats van het proteinasegen, een α-amylase gen (*amyS*) van *Bacillus stearothermophilus* achter de expressie en secretiesignalen gecloneerd. Indien dit α-amylase gesecreteerd wordt is dit gemakkelijk aantoonbaar op groeibodems, waarin zetmeel aanwezig is. Het α-amylase breekt het zetmeel af en na kleuring met een jodide oplossing, zijn er ophelderingszones rond de α-amylase secreterende bacteriekolonies waarneembaar (Dit wordt onder andere geïllustreerd op de omslag van dit proefschrift).

In de *usp45-amyS* gen fusies werden of de eerste 19, de eerste 20 of de eerste 27 aminozuren als secretiesignaal gebruikt. Uit deze proeven bleek het verschil in lengte tussen signaalpeptiden van de verschillende bacteriesoorten. In *Escherichia coli*, een gram-negatieve bacterie was met gebruik van de eerste 19 en de eerste 20 aminozuren α-amylase activiteit in het medium aantoonbaar. Echter, in *L. lactis*, een gram-positieve bacterie, waren alleen de eerste 27 aminozuren in staat tot de secretie van het α-amylase. Een gedeelte van het gesynthetiseerde α-amylase bleef ook binnen de cel achter. Het signaalpeptide was niet van het α-amylase geknipt en er kon ook geen activiteit worden aangetoond. Het proteinase werd wel volledig uitgescheiden. Hieruit blijkt dat naast het signaalpeptide ook de combinatie tussen signaalpeptide en het te secreteren eiwit zelf invloed heeft op de secretie.

Hoofdstuk 4 beschrijft de analyse van het promotergebied, door verschillende gebieden stroomopwaarts van het *usp45* gen als promotergebied te gebruiken voor de *usp45-amyS* gen fusie. Hieruit bleek dat niet alleen de twee hiervoor beschreven domeinen van belang zijn voor de transcriptie, maar ook een vrij groot gebied dat voor deze promotersequentie is gelegen. Verder werd aangetoond dat als er meerdere kopieën van een *usp45-amyS* fusie in de bacteriecel werden ingebracht, er ook meer productie van α-amylase plaatsvond. Als laatste werd aangetoond dat *L. lactis* ook gebruik kan maken van het ingebrachte soortvreemde *amyS* gen. Er ontstond een *L. lactis* stam die kon groeien op een medium met zetmeel als enige energiebron.

De ribosoom bindingsplaats voor het *usp45* gen werd onderzocht in hoofdstuk 5. Deze bevat namelijk twee sequenties die betrokken kunnen zijn bij de binding van het ribosoom. Omdat beide sequenties afwijkend waren in vergelijking met de tot nu toe gevonden ribosoom bindingsplaatsen werd in verschillende *usp45-amyS* telkens één van die twee afzonderlijk verwijderd, zodat kon worden uitgezocht wat de rol van deze sequenties was. Uit deze experimenten bleek dat beide sequenties afzonderlijk voldoende waren voor de translatie van het door de *usp45-amyS* fusie geproduceerde boodschapper RNA. Verder werd een optimale ribosoom bindingsplaats geconstrueerd die overeenkomt met de consensus voor *L. lactis*. Dit leverde echter nauwelijks verhoging van de expressie op, en er kan dus worden gesteld dat de rol van de verandering van ribosoombindingsplaats in dit expressiesysteem te verwaarlozen is.

DnaJ

Hoofdstuk 6 beschrijft de klonering en karakterisering van *dnaJ*, een gen uit *L.*

lactis dat codeert voor een intracellulair eiwit dat betrokken is bij de hitte-schok reactie van dit organisme. Dit gen heeft een regelbare promoter, waardoor er tijdens normale groei een lage hoeveelheid boodschapper RNA wordt geproduceerd. Worden de cellen echter aan een plotselinge temperatuurverhoging blootgesteld (hitte-schok), dan vindt er een verhoogde transcriptie van dit gen plaats. Door het promoter gebied van dit gen in de *usp45-amyS* fusie te gebruiken en in dit promoter gebied enkele veranderingen (mutaties) aan te brengen kon een stuk DNA in het promotergebied worden aangewezen dat een belangrijke functie vervult in de regulatie van deze verhoogde expressie na hitte-schok. Er werd echter geen hogere α-amylase productie bewerkstelligd dan met de *usp45* expressie signalen werd bereikt.

Conclusies

Samenvattend kan worden gesteld dat het mogelijk is om met behulp van expressie en secretie signalen van een *L. lactis* gen, de productie van een vreemd eiwit (als model is α-amylase gekozen) in *L. lactis* te bewerkstelligen. Het door *L. lactis* gesynthetiseerde α-amylase werd maar gedeeltelijk door de cellen gesecreteerd. Dit hoeft echter bij andere commercieel interessante heterologe eiwitten niet het geval te zijn. Het productieniveau is echter nog niet zo hoog dat dit organisme zou kunnen concurreren met de organismen die al worden gebruikt als gastheer, zoals *Bacillus subtilis* of *E. coli*. Dit zou mischien kunnen worden verbeterd door sterkere expressie signalen te gebruiken, de secretie te verbeteren of te zoeken naar stammen met een hogere secretiepotentie.

Curriculum vitae

Martien van Asseldonk werd geboren op 10 maart 1964 te Sas van Gent. Na het behalen van het diploma VWO-B aan het Titus Brandsma Lyceum te Oss begon hij in 1982 aan de studie biologie aan de Landbouwuniversiteit te Wageningen. De doctoraalfase bevatte als hoofdvakken Plantencytologie en -Morfologie, en Moleculaire biologie. Voordat in maart 1988 het doctoraalsexamen werd behaald, werd nog een stage vervuld bij de de werkgroep Moleculaire Genetica aan het Nederlands Zuivelinstituut te Ede, waar hij in april van dat jaar in tijdelijke dienst trad. Tijdens dit dienstverband werd het in dit proefschrift beschreven onderzoek verricht. Sinds Oktober 1992 is hij in tijdelijke dienst van Keygene te Wageningen, waar hij werkzaam is in het kader van het E.E.G. Arabidopsis Genome Sequencing project.
Martien is getrouwd met Karin, en heeft twee zoons, Wouter en Jelle.