Nisin Immunity and Food-Grade Transformation in Lactic Acid Bacteria

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by their Roman numerals. In addition, some unpublished data are presented.

I Koponen, O., **Takala, T. M.**, Saarela, U., Qiao, M., and Saris, P. E. J. (2004) Distribution of the NisI immunity protein and enhancement of nisin activity by the lipid-free NisI. FEMS Microbiol. Lett. 231:85-90.

II **Takala, T. M.,** Koponen, O., Qiao, M., and Saris, P. E. J. (2004) Lipid-free NisI: Interaction with nisin and contribution to nisin immunity via secretion. FEMS Microbiol. Lett. 237:171-177.

III **Takala, T. M.**, and Saris, P. E. J. (2002) A food-grade cloning vector for lactic acid bacteria based on the nisin immunity gene *nisI*. Appl. Microbiol. Biotechnol. 59:467-471.

IV **Takala, T. M.**, Saris, P. E. J., and Tynkkynen, S. S. H. (2003) Food-grade host/vector expression system for *Lactobacillus casei* based on complementation of plasmid-associated phospho-β-galactosidase gene *lacG*. Appl. Microbiol. Biotechnol. 60:564-570.

The author's contribution in articles:

I
- Experimental work: purification of NisI protein, nisin and NisI ratios in cell and supernatant fractions, binding of NisI to cells, influence of externally added lipid-free NisI to nisin activity in NisF/E/G strain.
- Methods: nisin sensitivity agar diffusion bioassay, SDS-PAGE and Western blotting, glutathionine-linked affinity chromatography, basic methods of microbiology and molecular biology
- Partial writing and interpretation of the results.

II
- Experimental work: purification of NisI protein, kinetic analyses of the interaction between nisin and NisI, construction of lipid-free NisI secretion plasmid, role of the secreted NisI in nisin immunity.
- Methods: glutathionine-linked affinity chromatography, surface plasmon resonance analysis by BIACORE 2000, SDS-PAGE and Western blotting, basic methods of microbiology and molecular biology
- Main responsibility of writing the paper and interpretation of the results.

III
- All experimental works.
- Writing the paper and interpretation of the results.

IV
- Experimental work: localization of lactose operon, construction of integration plasmid, construction of food-grade complementation plasmid, expression of *pepI* in *Lactobacillus casei*, growth characterization of *lacG* complementation strains
- Writing the paper and interpretation of the results.

ABBREVIATIONS

aa	amino acid
ABC	ATP-binding cassette
Abu	2-aminobutyric acid
amp	ampicillin
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BGSC	Bacillus Genetic Stock Center
bp	base pair(s)
cam	chloramphenicol
C-terminal	carboxyterminal
DCO	double cross-over
Dha	2,3-didehydroalanine
Dhb	2,3-didehydrobutyrine
DSM	Deutsche Sammlung von Mikroorganismen
e.g.	*exempli gratia*, for example
erm	erythromycin
et al.	*et alii*, and others
GMO	genetically modified organism
GST	glutathionine-S-transferase
i.e.	*id est*, in other words
IS	insertion sequence
IU	international unit
kb	kilobase pair(s)
LAB	lactic acid bacteria
LF-NisI	lipid-free NisI
M17G	M17 medium + 0.5% glucose
M17GS	M17 medium + 0.5% glucose + 0.5 M sucrose
MDa	megadalton
NCIMB	National Collection of Industrial and Marine Bacteria
NSLAB	non-starter lactic acid bacteria
N-terminal	aminoterminal
P-β-gal	phospho-β-galactosidase
PCR	polymerase chain reaction
PTS	phosphotransferase system
RCR	rolling circle replication
SCO	single cross-over
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
ts	thermosensitive
VTT	Finnish state technological research center

ABSTRACT

Lactic acid bacteria (LAB) are used as starters by the dairy industry. Several strains of LAB produce substances called bacteriocins, which inhibit the growth of competitive bacteria. Certain strains of *Lactococcus lactis* produce bacteriocin nisin, which kills Gram-positive bacteria by forming pores in the cell membrane. Since nisin inhibit the growth of pathogenic bacteria, it is used as a food preservative in the food industry.

As a Gram-positive bacteria, *L. lactis* is sensitive to nisin. To protect their own cell membrane against the pore-forming activity of nisin, the producer strains express several self-protection proteins, which constitute the nisin immunity system. The system includes the transporter complex NisF/E/G, and the membrane-bound lipoprotein NisI.

In this study, the nisin immunity protein NisI, in addition to the membrane-bound form, was shown to be secreted to the external environment in the nisin-producing *L. lactis* strain N8. The excretion of the lipid-free (LF) NisI was demonstrated to increase nisin tolerance of sensitive cells, suggesting that the secreted form of the protein may have a supportive role in the nisin immunity mechanism. In a strain carrying the NisFEG complex, the LF-NisI-mediated nisin resistance was more evident. This indicates that LF-NisI assisted the NisFEG transporter in export of nisin from cell surface. Purified LF-NisI, added exogenously to the growth medium, did not protect the cells against nisin, but, on the contrary, it enhanced the activity of nisin. This suggests a dual role for NisI: as a cell-associated immunity protein, and as a soluble factor stimulating nisin activity in the environment.

The interaction of LF-NisI and nisin was shown by biomolecular interaction analysis. The affinity between LF-NisI and nisin was determined to be in the micromolar range, indicating that the LF-NisI:nisin complex is rather unstable and easily dissociated. The observed weak affinity supports the idea of NisI as a nisin intercepting/delivering protein. Membrane-bound lipoprotein NisI may hinder nisin from inserting to the membrane, or assist the NisFEG complex to export inserted nisin molecules from the membrane. LF-NisI assists the NisFEG complex as well, either by delivering nisin to the transporter, or, by functioning as a carrier transferring nisin from the transporter to the external environment.

In addition to nisin immunity studies, two food-grade cloning systems were developed. The safe use of genetically modified bacteria, aimed for food processes, requires cloning systems that are solely composed of DNA from food organisms, and which do not rely on antibiotic resistance for their selection.

First, the gene *nisI*, encoding NisI, was exploited as a dominant selection marker with nisin selection. The constructed *nisI* expression plasmid pLEB590 consisting of merely lactococcal DNA was introduced into an industrial *L. lactis* starter, and into a *Lactobacillus plantarum* strain using nisin selection. Thus, the plasmid pLEB590 can be regarded as a food-grade vector for LAB.

Secondly, for a non-starter *Lb. casei* strain, a complementary food-grade expression system based on lactose selection was constructed. The plasmid-associated gene *lacG*, encoding lactose-degrading enzyme phospho-β-galactosidase in *Lb. casei*, was inactivated by gene replacement. The deletion in the *lacG* gene resulted in a lactose-negative host strain. The *lacG* complementation plasmid pLEB600 was then constructed out of DNA from LAB. The plasmid was introduced into the lactose-negative host strain using lactose as the selective agent. The plasmid pLEB600 is thus regarded as a food-grade vector for *Lb. casei*.

The applicabilities of the constructed food-grade vectors were shown by cloning the proline-iminopeptidase gene *pepI* from *Lb. helveticus* into pLEB590 and pLEB600. The results demonstrated that the food-grade vectors constructed in this study show potential as tools for overexpression of useful genes in dairy starter bacteria.

TIIVISTELMÄ (abstract in Finnish)

Maitohappobakteereita käytetään meijeriteollisuudessa hapatteina. Monet maitohappobakteerit tuottavat yhdisteitä, jotka estävät kilpailevien bakteerien kasvua. Tietyt *Lactococcus lactis* –maitohappobakteerikannat tuottavat nisiini-nimistä proteiinia, joka tappaa bakteereita tekemällä reikiä niiden solukalvoon. Koska nisiini tappaa myös elintarvikkeiden pilaajabakteereita, käytetään sitä elintarviketeollisuudessa ruoan säilöntäaineena.

Nisiiniä tuottavan *L. lactis* –bakteerin solukalvo on herkkä nisiinille. Välttääkseen itsemurhan, nisiinintuottajakannoilla on erikoistuneita proteiineja, jotka suojaavat niiden solukalvoa tuottamaltaan nisiiniltä. Tätä itsesuojelumekanismia kutsutaan nisiini-immuniteetiksi. Immuniteettijärjestelmään kuuluvat solukalvolla toimiva nisiininkuljetus-kompleksi NisF/E/G, sekä solukalvoon rasvahappoankkuroitu lipoproteiini NisI.

Tässä työssä osoitettiin, että *L. lactis* N8 -nisiinintuottajakannassa nisiini-immuniteettiproteiini NisI esiintyy solukalvositoisen muotonsa lisäksi liukoisena erittyvänä proteiinina. Tämän erittyvän LF-NisI:n (lipid-free, rasvavapaa) tuotto nisiiniherkässä *L. lactis* kannassa nosti sen nisiininsietotasoa, mikä viittaa LF-NisI:n erityksen olevan osa nisiini-immuniteettijärjestelmää. NisFEG-kompleksin sisältävässä *L. lactis* –kannassa LF-NisI:n eritys nosti vastustuskykyä nisiinille huomattavasti enemmän, mikä kertoo LF-NisI:n kyvystä avustaa NisFEG:n nisiininkuljetusta solukalvolta ulos solusta. Puhdistettu LF-NisI –proteiini lisättynä kasvatusalustaan ei kuitenkaan suojannut soluja nisiiniltä, vaan päinvastoin, se tehosti nisiinin tappokykyä. Tulosten perusteella NisI:llä on solussa kaksi erilaista tehtävää: solusitoinen suojaproteiini, ja solun ulkopuolinen liukoinen nisiinitehostaja.

Työssä osoitettiin LF-NisI:n ja nisiinin sitoutuvan toisiinsa. Proteiinien muodostaman kompleksin osoitettiin olevan melko heikko, ja osien irtautuvan helposti toisistaan. Proteiinien heikko yhdistelmä vahvistaa esitettyä mallia NisI:n toiminnasta nisiinin siirtäjänä. Solukalvoon ankkuroitu NisI estää nisiiniä tunkeutumasta solukalvoon, tai auttaa NisFEG-kompleksia kuljettamaan nisiinimolekyylejä pois solukalvolta. Erittyvä LF-NisI avustaa myös NisFEG:tä, joko toimittamalla nisiiniä kompleksille, tai toisinpäin, vastaanottamalla nisiiniä kompleksilta ja kuljettamalla sen ulos solusta.

Nisiini-immuniteettitutkimuksen lisäksi työssä kehitettiin kaksi elintarviketasoista geenien kloonausmenetelmää. Geneettisesti muunneltujen bakteerien käyttö ruuassa edellyttää elintarviketasoisia geeninsiirtovektoreita, joissa kaikki siirrettävä DNA on peräisin syötävistä bakteereista, ja jotka eivät sisällä antibiooteille vastustuskykyä antavia geenejä.

Ensimmäisessä sovelluksessa käytettiin hyväksi nisiini-immuniteettigeeni *nisI*:n tuottamaa vastustuskykyä nisiinille. Geenin *nisI* sisältämä plasmidi pLEB590 tehtiin *L. lactis* bakteerista peräisin olevasta DNA:sta. Plasmidi siirrettiin teolliseen *L. lactis* hapatekantaan, sekä *Lactobacillus plantarum* –kantaan. Plasmidin sisältämät solut seulottiin plasmidittomista soluista kasvattamalla niitä nisiiniä sisältävällä alustalla. Plasmidi pLEB590:ää voidaan täten pitää elintarviketasoisena kloonausvektorina maitohappobakteereille.

Toinen elintarviketasoinen kloonausvektori tehtiin juustosta eristetylle *Lb. casei* –kannalle. Laktoosia hajottavaa entsyymiä koodaavan *lacG*-geenin keskeltä poistettiin pala. Tämän seurauksena syntynyt mutaatiokanta ei kyennyt käyttämään laktoosia maitohapoksi. Ehjä, toimiva *lacG*-geeni liitettiin maitohappobakteerien DNA:sta tehtyyn plasmidivektoriin, joka siirrettiin laktoosinegatiiviseen mutanttiin. Plasmidin, nimeltä pLEB600, sisältämät solut seulottiin kasvattamalla niitä alustalla, jonka ainoa energianlähde oli laktoosi. Plasmidi pLEB600:aa voidaan täten pitää elintarviketasoisena kloonausvektorina *Lb. casei* –bakteerille.

Kehitettyjen kloonausvektorien pLEB590 ja pLEB600 käyttökelpoisuus osoitettiin kloonaamalla niihin proliini-iminopeptidaasigeeni *Lb. helveticus* –bakteerista. Tulosten perusteella työssä kehitetyt plasmidivektorit ovat toimivia työkaluja uusien geenien ja ominaisuuksien siirtämiseen ruuassa käytettäville bakteereille.

INTRODUCTION

1. Nisin

In 1928, “lactic streptococci” in milk fermentation were found to produce a substance, which inhibited the growth of other bacteria (Rogers 1928; Rogers and Whittier 1928). The inhibitory substance was shown not to be lactic acid, but some other heat-stable, soluble, and diffusible factor excreted by lactic bacteria. A few years later the unidentified substance was found to be proteinaceous (Whitehead 1933), and in 1947 (Mattick and Hirsch 1947), it was given a name “nisin” (group **N** *Streptococcus* **I**nhibitory **S**ubstance, -**in** ending indicating an antibiotic). Due to its ability to kill bacteria, nisin could be exploited in the food industry in order to prevent bacterial growth in food. Nisin was accepted in 1969 by the Food and Agriculture Organization (FAO)/World Health Organization (WHO) as a food preservative (WHO 1969). Its use is approved by many countries including the EU (food additive E234) and the USA. The previous name of the nisin producing bacterium, *Streptococcus lactis*, was renamed in 1985 to *Lactococcus lactis* (Schleifer et al. 1985). Today, nisin is the only bacteriocin available commercially. It is manufactured industrially from *L. lactis* culture supernatant by a low-cost and simple foaming process (Waites et al. 2001).

The structure of nisin was solved in 1971 (Gross and Morell 1971) (Fig. 1). The 34-aa long ribosomally synthesized and post-translationally modified peptide contains five lanthionine rings, which can be divided into two parts. The N-terminal half including three rings (A, B, and C in Fig. 1) is more hydrophobic than the C-terminus containing two rings (D and E in Fig. 1). The rigid ring structures are separated by a flexible hinge region. The three-dimensional structure of nisin has been determined by NMR (van de Ven et al. 1991; van den Hooven et al. 1993). The ring structures give nisin a screw-like conformation that possesses amphipathic characteristics. In water its solubility and stability increases with decreasing pH, showing maximum solubility 57 mg/ml at pH 2 (Liu and Hansen 1990), and maximum stability at pH 3 (Davies et al. 1998). Nisin is insoluble in non-polar solvents (Thomas et al. 2000). Three nisin variants, nisin A, Z, and Q, show similar antimicrobial properties and differ only in a few amino acids (Twomey et al. 2002; Zendo et al. 2003). According to the lanthionine rings and antibiotic properties, nisin belongs to a group of bacteriocins called lantibiotics (Schnell et al. 1988).

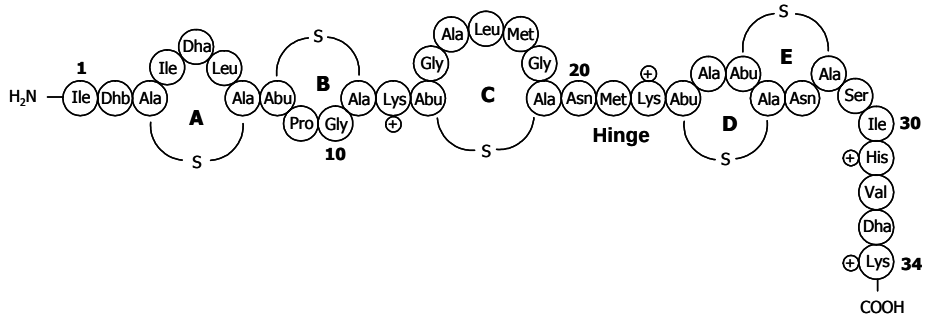

Fig. 1. Structure of nisin Z. Lanthionine rings are labeled A-E. Dha = dehydroalanine; Dhb = dehydrobutyrine; Ala-S-Ala = lanthionine; Abu-S-Ala = β-methyllanthionine. Adapted from Gross and Morell 1971. The flexible hinge region comprises residues 20-22.

1.1. Mode of action

Nisin kills bacteria by (i) forming voltage-dependent pores in the cell membrane (Héchard and Sahl 2002; Wiedemann et al. 2004), (ii) preventing murein synthesis (Reisinger et al. 1980), and (iii) inducing autolysis of susceptible cells (Bierbaum and Sahl 1985). Autolysis of cells is a consequence of the release of two cell wall hydrolysing enzymes, *N*-acetylmuramoyl-L-alanine amidase and *N*-acetylglucosaminidase (Bierbaum and Sahl 1987). Nisin has also been shown to reduce the thermal resistance of *Bacillus* spores (Beard et al. 1999), and to prevent the germination of *Bacillus* (Nissen et al. 2001) and *Clostridium* (Thomas et al. 2002) spores. In normal circumstances, Gram-negative bacteria are usually resistant to nisin mainly due to their impermeable outer membranes (Gänzle et al. 1999).

For pore formation and inhibition of cell wall synthesis, nisin binds at nanomolar concentrations to the peptidoglycan precursor molecule undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)-GlcNAc, shortly lipid II. The N-terminus of nisin binds in 1:1 stoichiometry to lipid II, followed by the interaction of C-terminus with the membrane and insertion of nisin across the membrane (Wiedemann et al. 2001; Hsu et al. 2002). Lipid II stays associated to nisin in pore formation, and finally it is an intrinsic component of the nisin pore (Breukink et al. 2003). Nisin at micromolar concentrations can form membrane pores in the absence of lipid II, as shown in studies with black lipid membranes (Ruhr and Sahl 1985; Sahl et al. 1987). A “wedge-model” for lipid II -independent pore formation has been proposed (Driessen et al. 1995; van den Hooven et al. 1996). The formation of the nisin pore results in leakage of the cell and loss of the proton motive force, which leads to cell death. Models for nisin pore formation are presented in Fig. 2.

Lipid II is also the recognition site for some antibiotics, e.g., vancomycin, which interacts with the D-Ala-D-Ala –residues in lipid II (Perkins 1968). Lipid II can be considered as a “docking molecule” for nisin, whereas, it is a “target” for vancomycin, since vancomycin acts by inhibiting peptidoglycan synthesis. Vancomycin blocks the membrane leakage activity of nisin (Breukink et al. 1999). However, even though nisin and vancomycin share the same receptor, they bind to different locations in lipid II, since vancomycin resistant enterococci, in which lipid II has been altered, are still sensitive to nisin (Breukink et al. 1999). The precise location in lipid II for nisin interaction is not known.

Resistance to nisin is not dependent on the level of lipid II content in the cell membrane, as shown in a study where protoplasts containing different amounts of lipid II in their membranes were equally sensitive to nisin (Kramer et al. 2004). In that study, it was suggested that the cell wall is an important factor in acquired nisin resistance. This same conclusion has also been drawn in several previous studies. Excluding lactococcal nisin resistance/immunity proteins (Froseth and McKay 1991b; Kuipers et al. 1993) and bacillar proteases degrading nisin (Jarvis 1967), the resistance to nisin among Gram-positives has been connected to cell wall structure (Davies et al. 1996; Maisnier-Patin and Richard 1996; Peschel et al. 1999). An increased production of certain cell wall elements, such as anionic polysaccharides (Breuer and Radler 1996), lipoteichoic acids (Mantovani and Russell 2001), or penicillin-binding proteins (Gravesen et al. 2001) have been observed in nisin resistant cells. Thus, changes in cell wall architecture are plausible explanations for acquired nisin resistance.

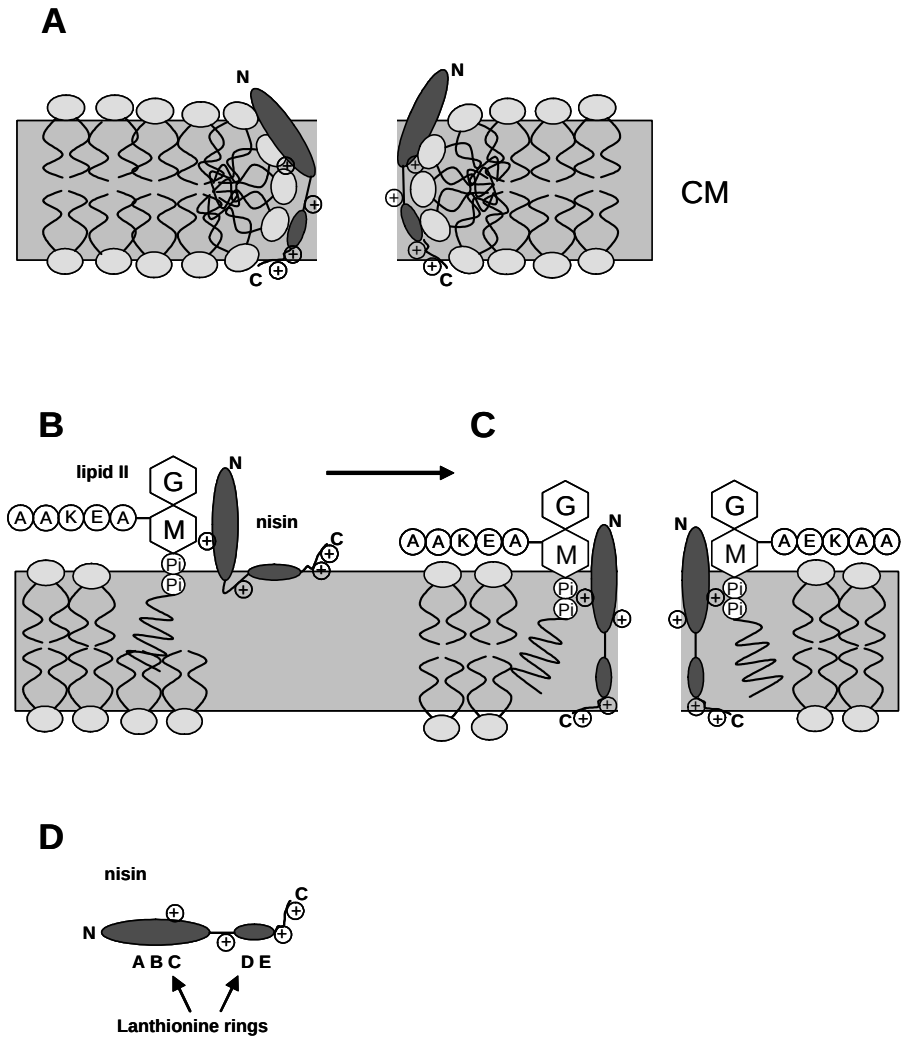

Fig. 2. Models for formation of membrane pores by nisin. **A**, Wedge-like, target independent pore. At micromolar concentrations, nisin interacts with the hydrophilic heads of phospholipids causing a locally disturbed bilayer structure. **B**, Nisin binds to lipid II by its N-terminus. **C**, The C-terminus of nisin translocates across the membrane. The pore consisting of approximately 5 to 8 nisin:lipid II complexes is formed. **D**, Functionally important segments of nisin; the N-terminal rings ABC for lipid II binding, the C-terminal rings which insert through the membrane, and the flexible hinge region between them. For the more detailed structure of nisin, see Fig. 1. CM, cytoplasmic membrane. G, *N*-acetylglucosamine. M, *N*-acetylmuramic acid. The amino acids attached to *N*-acetylmuramic acid in lipid II are: L-Ala, D-Glu, L-Lys, D-Ala, D-Ala. Adapted from Héchard and Sahl 2002 (A), and Wiedemann et al. 2001 (B-D).

1.2. Biosynthesis and transcriptional regulation

Nisin is synthesized ribosomally as an inactive prepeptide. After post-translational modifications including dehydration, lanthionine ring formation, secretion, and leader cleavage, mature nisin becomes biologically active. The production of active nisin requires a

total of 14 kb of DNA in conjugative nisin-sucrose transposon carrying 11 *nis* genes, *nisA*/*Z*/*QBTCIPRKFEG* (Fig. 3; McAuliffe et al. 2001; Zendo et al. 2003).

The structural gene *nisA*/*Z*/*Q* encodes the 57-aa prepeptide containing an N-terminal leader peptide (23 aa) (Kaletta and Entian 1989; Dodd et al. 1990; Steen et al. 1991; Graeffe et al. 1991; Mulders et al. 1991; Zendo et al. 2003). Nisin operon includes the genes responsible for intracellular post-translational modifications (*nisBC*) in which amino acids are dehydrated and lanthionine rings are formed (Engelke et al. 1992; Kuipers et al. 1993; Koponen et al. 2002; Okeley et al. 2003). The peptide is secreted by the translocator protein NisT, and the leader peptide is cleaved by the protease NisP (van der Meer et al. 1993; Qiao and Saris 1996). Production of nisin is autoregulated by a two-component regulation system; *nisR* encodes a response regulator, and *nisK* a histidine kinase (Engelke et al. 1994; Kuipers et al. 1995; Qiao et al. 1996). The *nisA* promoter in a certain nisin producer strain has been found to be induced not only by nisin but also by galactose (Chandrapati and O`Sullivan 1999; Chandrapati and O`Sullivan 2002).

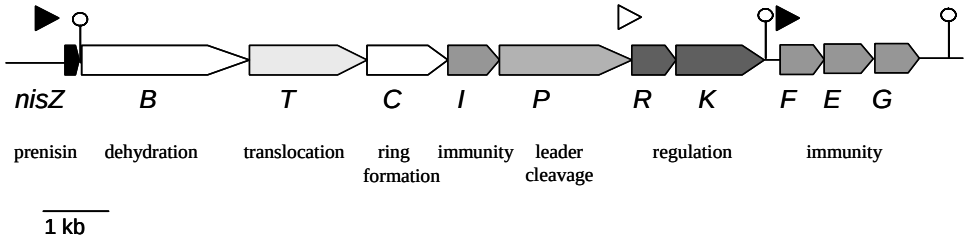

Fig. 3. Genes involved in nisin Z production, regulation, and immunity. The entire nisin gene block, from the *nisZ* promoter through the 11 *nis* genes to the transcription terminator 350 bp downstream from *nisG* is approximately 14 kb. Filled and open triangles indicate nisin-inducible and constitutive promoters, respectively. Hairpins indicate transcription terminator loops.

1.3. Immunity

The membrane of nisin-producing lactococci is sensitive to nisin. In order to protect their membrane, nisin producers express a complex resistance mechanism. This self-protection mechanism is called nisin immunity, and encoded by four genes in the nisin operons. Three of the immunity proteins, NisF, NisE, and NisG, form an ABC transport complex (Siegers and Entian 1995; Immonen and Saris 1998). NisF is a cytoplasmic ATP-binding protein. NisE and NisG are hydrophobic integral membrane proteins. The function of NisFEG complex is to export cell-associated nisin to the external environment (Stein et al. 2003).

The fourth nisin immunity protein, NisI, encoded by the gene *nisI*, is a lipoprotein attached to the extracellular side of the cell membrane (Kuipers et al. 1993; Qiao et al. 1995). The protein seems to be unique; the protein or gene sequence has no significant homology to any other sequence known. Even though the structure of nisin is very similar to the lantibiotic subtilin, the corresponding immunity proteins NisI and SpaI show no homologies (Klein and Entian 1994). Pre-NisI contains a typical 19 aa long N-terminal lipoprotein signal peptide and the site for lipid-modification (Cys in position 1 in mature protein) (Sutcliffe and Russell 1995). Lipid moieties are first added to the protein, the leader peptide is cleaved by signal peptidase II, the mature protein is secreted through the membrane via *sec*-dependent pathway, and finally attached to the membrane by its lipid groups. The mature NisI is a negatively charged hydrophilic protein (net charge -7). If the leader peptide is cleaved before lipid groups are added, the resulting protein becomes merely secretable and will not be attached to

the membrane. NisI has been shown to exist, not only as a lipoprotein, but also in a lipid-free form in a growth medium of a heterologous host *Bacillus subtilis* (Stein et al. 2003).

NisI protects the cell against nisin. The actual mechanism of NisI-mediated immunity is not fully clear. It has been shown that nisin:NisI interaction results in an unstable complex (Stein et al. 2003). Based on the interaction results it was proposed that NisI could function as an interceptive protein lowering the quantity of nisin molecules on the membrane. Previously, it has also been suggested that NisI may prevent nisin from reaching the membrane, hinder nisin insertion into the membrane (Saris et al. 1996), or it may act as a binding protein delivering nisin to NisFEG transporter, thus functioning similarly as extra-cytoplasmic binding lipoproteins of import systems in *Streptococcus pneumoniae* and *Mycoplasma hyorhinis* (Immonen and Saris 1998; Gilson et al. 1988).

The immunity protein LciA for the bacteriocin lactococcin A interacts with the receptor of the bacteriocin thus blocking the binding of lactococcin A to the receptor (Venema et al. 1994). A similar mechanism, shielding the target of the bacteriocin, has been proposed for the immunity protein PepI providing resistance to the lantibiotic Pep5 (Hoffmann et al. 2004). The nisin receptor lipid II is the binding site for some other lantibiotics as well (Brötz et al. 1998a; Brötz et al. 1998b). As there are no cross-immunity between nisin and other lantibiotics (McAuliffe et al. 2001), it is unlikely that NisI would interact with lipid II.

Disruption of the *nisI* gene in a nisin-producing strain resulted in at least an 80% loss in nisin resistance (Siegers & Entian 1995; Ra et al. 1999). This fits well with the results by Duan et al. (1996), who prensented that plasmid-associated *nisFEG* provided approximately 20% of the wild-type immunity level. Based on these studies, the NisI-mediated immunity level should be approximately 80% of the wild-type immunity. However, expression of *nisI* gene in a nisin sensitive background resulted in only 1 to 4% of the wild-type immunity level (Kuipers et al. 1993; Engelke et al. 1994; Qiao et al. 1995). This suggests that there may be some synergistic function between NisI and NisFEG. In spite of this, there are two distinct views about that: (i) a co-operative function between NisI and NisFEG, based on several observations that NisI and NisFEG produced together result in a higher level of immunity than expressed separately (Ra et al. 1999), and (ii) NisI and NisFEG are two independent systems, based on immunity studies in *B. subtilis* (Stein et al. 2003).

2. Food-grade cloning and expression systems for lactic acid bacteria

Genetic modification of lactic acid bacteria (LAB) started more than twenty years ago when the first cloning vectors based on endogenous plasmids were established. The first-generation cloning vectors were equipped with antibiotic resistance genes. For scientific purposes, and for use in a laboratory, the antibiotic resistance genes offer useful markers for the selection of transformants. However, in development of genetically modified bacteria aimed to be used in the food processes, the genes coding for resistance to antibiotics used in medical therapy are not acceptable, both for legal and ethical reasons. It was realized already in the 1980´s that other types of selection markers are needed for constructing improved starter bacteria (de Vos 1986). The development of genetically modified starters for food applications requires gene cloning systems that (i) are devoid of DNA from non-food origin, and (ii) do not rely on antibiotic resistance genes for selection. These alternative selection markers and cloning techniques, food-grade systems, have been developed extensively during the last two decades, and a number of new ones are still published every year.

Possibilities for genetic improvement of starter bacteria include many different DNA transformation procedures, such as transduction, conjugation, protoplast transformation, and electroporation. Transduction and conjugation have been used for transferring foreign DNA into LAB since the 1970´s (McKay and Baldwin 1974; McKay et al. 1976; Kempler and McKay 1979; McKay et al. 1980). Protoplast transformation for LAB was described in 1982 by Kondo and McKay, and electroporation was established a few years later (Chassy and Flickinger 1987; Powell et al. 1988; Somkuti and Steinberg 1988; Luchansky et al. 1989). Despite the enormous progress in the understanding and the technology of LAB genetics, conjugation is still the only accepted way in the EU to alter DNA content of LAB for use in food processes. This backwardness is due to the fact that in addition to the final construct, EU legislation considers relevant "the way" the modification has been performed (Anonymous 2001; Renault 2002). Non-recombinant DNA modifications, such as conjugation, can occur naturally between bacteria. Bacterial strains constructed by non-natural but specific genetic engineering are considered as genetically modified organisms (GMO) in the EU, and are thus not yet allowed for use in food production. GMO products for use in food are accepted according to the regulation (EC) No 1829/2003 (Anonymous 2003a). To date, the only bacterial GMO product accepted for deliberate release in the EU is a non-food test kit for the detection of antibiotic residues in milk in the dairy industry (Anonymous 2003b, 2004). The kit T102 contains a GMO-bioluminescent *Streptococcus thermophilus* strain sensitive to several dairy-relevant antibiotics (Jacobs et al. 1995).

2.1. Conjugation

Wild-type LAB strains often contain several indigenous plasmids harbouring relevant and useful properties (McKay 1983). For transfer of these desired properties to strains suitable for fermentation processes, conjugation is an acceptable way for this, since according to EU legislation, transconjugants are not considered as GMOs (Anonymous 2001). The acceptability is limited to the natural conjugative plasmids; no alterations by genetic engineering are allowed to be performed in the plasmids.

Endogenous plasmids have been transferred by conjugation in a food-grade manner between strains of *L. lactis* for almost two decades. Conjugal food-grade transfer was first demonstrated by Sanders et al. (1986) with the plasmid pTR2030 conferring resistance to bacteriophages. Transconjugants were selected based on the resistance to the phages Φ12 and Φ13. The same selection method, resistance to a phage, was also more recently used by O´Sullivan et al. (1998). They constructed a phage-insensitive Cheddar cheese starter strain from the plasmid-free *L. lactis* host MG1614 (Gasson 1983), by sequential conjugal transfer of three different phage resistance plasmids and the fourth plasmid, pLP712 carrying genes for lactose utilization and protease activity. The quality of the resulting Cheddar manufactured with this single strain was shown to be as high as the cheese produced with a commercial starter.

Native plasmids have been transferred by food-grade conjugation into *L. lactis* also by selection based on resistance to bacteriocins, including dricin (Powell et al. 1990; Powell et al. 1994), nisin (Harrington and Hill 1991), lacticin 3147 (Ryan et al. 1996; Coakley et al. 1997; O´Sullivan et al. 2003b), and lacticin 481 (Mills et al. 2002; O´Sullivan et al. 2003b). These selections were based on endogenous lactococcal plasmids carrying genes for immunity to the particular bacteriocin.

2.2. Cotransformation

Indigenous plasmids in LAB do not necessarily contain any factors that could be exploited for their selection in transformation. Still, wild-type plasmids might carry some properties, which could be useful in starter strains. Transfer of these cryptic plasmids may be desirable for constructing improved starters. Screening of plasmid transformants without any selection from the non-transformed background is impossible. This problem has been overcome by cotransformation of competent cells with two separate plasmids, one selectable and the other unselectable. Cotransformation of *L. lactis* protoplasts in a food-grade manner was demonstrated in 1985 (Simon et al. 1985). In fact, this paper seems to be the first publication of constructing food-grade LAB strains with altered genetic content. By using a plasmid vector equipped with an erythromycin resistance (Erm^r) gene, in combination with a mixture of four different cryptic plasmids, 70% of the obtained transformants contained one or more cryptic plasmids in addition to the Erm^r-vector. The final food-grade transformants were obtained in an erm-free medium enabling curing of the Erm^r-vector. Resulting derivatives contained one or more cryptic plasmids of sizes varying from 6 to 42 kb. A similar strategy was used in constructing nisin resistant *L. lactis* with a plasmid mixture of pFM011 (Nis^r) and pSA3 (Erm^r), followed by curing of the Erm^r helper vector (Froseth et al. 1988). In addition, cotransformation by electroporation has been established for *L. lactis* (Corneau et al. 2001).

An advanced food-grade cotransformation system for *L. lactis* was presented a few years ago (Émond et al. 2001). The helper plasmid pCOM1 contained Erm^r gene, origin of replication, but no gene for replicative function. The actual food-grade vector pVEC1 contained no selection marker, but the gene for autonomous replication, thus providing the replication function for Erm^r plasmid *in trans*. When selecting with erm, both plasmids were needed for the Erm^r phenotype. After successful transformation, the helper plasmid could be cured by cultivating cells without selective pressure resulting in a strain with food-grade status. The benefit of using this elegant system is that the erm selection is very powerful and functional in most LAB, and that background transformants containing only the Erm^r helper plasmid cannot be obtained. A disadvantage, which concerns other cotransformation systems as well, is that because of the lack of a selection marker in the food-grade plasmid, no selection pressure can be maintained, and the food-grade vector may be cured. Another weakness in this particular system is that, as mentioned previously, wild-type LAB often contain indigenous plasmids, and further, most lactococcal plasmids replicate via theta mechanism (Seegers et al. 1994). If the replication-deficient Erm^r companion plasmid gets replication protein *in trans* from some indigenous plasmid, the food-grade plasmid is not needed anymore, and background problems will occur.

Taken together, cotransformation is a suitable method for the construction of food-grade lactococcal strains with new combinations of wild-type cryptic plasmids carrying no selection markers. However, according to the EU regulations, strains achieved by cotransformation are considered as GMOs, since “the way” the final food-grade strain is obtained does not occur in nature.

2.3. Food-grade plasmid vectors and markers

Food-grade transformation systems are based on circular plasmid DNA. Plasmids replicate via two different types, namely theta and rolling circle replication (RCR). Theta replicative plasmids have been proposed to be less sensitive to segregational and especially structural rearrangements (Kiewiet et al. 1993). During the replication process of the RCR plasmids, single-stranded DNA is formed, which is the source for the lower stability (Bron et al. 1991). In addition, multimerization of the RCR plasmids has been shown to cause instability

(Leonhardt and Alonso 1991). Thus, it seems that theta type plasmids would be the better choice for vectors. In spite of this, both plasmid types have been widely used for food-grade vectors. Apparently, the choice of the vector is not a simple decision; RCR plasmids are not necessarily unstable, especially when smaller than 13 kb (Kiewiet et al. 1993), and among the theta type plasmids, less stable constructions are found as well (Simon and Chopin 1988). The instability of the latter was, however, due to the lack of a stability determinant *orfH*, which was later cloned into the unstable theta-vector pIL252, resulting in a stable plasmid pSR11 (Kiewiet et al. 1993).

Endogenous plasmids from LAB, as such, may in principle be regarded as food-grade vectors, but in practice, a replicative food-grade plasmid vector requires a selection system. For example, the studies by Biet et al. (1999; 2002) describing -according to the titles- the development or use of food-grade vectors for *Leuconostoc mesenteroides,* do not actually contain any kind of development of food-grade constructs. Their so-called "food-grade vectors" were indigenous cryptic plasmids from *L. mesenteroides* without selection markers. The applicability of the plasmids as cloning vectors was demonstrated by equipping the plasmids with antibiotic resistance genes.

Food-grade cloning markers can be divided in two classes, based on their selection: dominant and complementary markers (de Vos 1999). Dominant markers are similar as antibiotic selection markers; they can be transferred and selected virtually in any host sensitive to the selective agent (e.g., bacteriocin), or lacking the ability to metabolize the agent used in selection (e.g., carbohydrate utilization). Complementary markers have more limited host range; they need host strains specifically engineered to lack a certain essential property, which is then complemented by the food-grade marker (e.g., carbohydrate utilization). In addition to these two selection types, there are other systems, which do not meet with these criteria. For instance, integrative transformation systems do not necessarily need food-grade markers for a final construct showing food-grade status.

2.4. Dominant selection markers

2.4.1. Nisin resistance

The first expression for the need of genetic engineering methods suitable for food bacteria was published in 1984 (McKay and Baldwin 1984). A 60 kb endogenous plasmid, pNP40, conferring resistance to nisin was found in *L. lactis*. It was realized that this property, nisin resistance, could potentially be used as a selection marker for cloning purposes. Furthermore, the resistance was a plasmid-associated trait, which would allow the use of pNP40 plasmid as a cloning vector. Since nisin had been accepted as a food additive, pNP40 could be the first food-grade plasmid cloning vector. However, transformation experiments using nisin selection were only proposed, not performed.

A year later, Klaenhammer and Sanozky (1985) attempted to use nisin resistance as a primary selection marker in conjugation. The attempt failed, due to spontaneous nisin resistant mutants. They suggested that although nisin resistance cannot be used as a primary selection marker, it could still be useful as a secondary marker after the primary selection had been carried out by some other selective agent. Froseth et al. (1988) used a cotransformation strategy to transfer nisin resistance phenotype into *L. lactis*. A 7.6 kb fragment from the nisin resistance plasmid pNP40 was self-ligated and successfully cotransformed with a helper plasmid pSA3 into *L. lactis* with erm selection. Later, the constructed food-grade vector, pFM011 was transferred into *L. lactis* with primary nisin selection, and a gene providing resistance to phage was cloned (Froseth and McKay 1991a). The resulting plasmid was finally transferred into *L. lactis* starter strains (Hughes and McKay 1992). The gene *nsr* responsible

for nisin resistance phenotype was shown to code for an integral membrane protein, based on a hydrophobic transmembrane domain in the N-terminus (Froseth and McKay 1991b). The mode of action of *nsr*-mediated nisin resistance has not yet been elucidated.

Another food-grade vector using nisin resistance for selection was published approximately at the same time as pFM011. Nisin resistance in primary selection was first demonstrated by von Wright et al. (1990). Theta-replicating plasmid pVS40 (7.8 kb) conferring nisin resistance was constructed and transferred into *L. lactis* (von Wright et al. 1990). Even though the origin of this nisin resistance DNA region was different from pFM011, the gene responsible for the nisin resistance was identical. Lactose fermentation genes in a 15-kb fragment were cloned and expressed in pVS40, showing the applicability of this vector for cloning large DNA fragments (Wessels 1993).

Nisin resistance combined with phage resistance has been used in the transfer of conjugative plasmid pNP40 (Harrington and Hill 1991). An interesting observation in their study was that primary nisin selection was successful in a liquid, but not on a solid media. This phenomenon might be a partial reason why the previous studies had no success in using nisin resistance in primary selection (Klaenhammer and Sanozky 1985; Froseth and McKay 1988).

2.4.2. Resistance to other bacteriocins

Immunity genes for bacteriocins for food-grade selection markers have been exploited rather sparingly, in consideration with the huge number of different characterized bacteriocins. Only two examples have yet been published, and in both of them, no final food-grade vector was constructed; just the functionality of the selection was shown. The first such selection published was the immunity gene *lafI*, providing immunity to lactacin F (Allison and Klaenhammer 1996). Lactacin F is a class II bacteriocin produced by *Lactobacillus johnsonii* (Muriana and Klaenhammer 1991). The use of *lafI* as a selection marker is limited to *Lactobacillus* strains that are sensitive to lactacin F. The second food-grade bacteriocin immunity selection published was the immunity gene *ltnI*, conferring immunity to the lantibiotic lacticin 3147 produced by *L. lactis* (McAuliffe et al. 2000). Lacticin 3147 selection was shown to be functional also in a heterologous host *Enterococcus faecalis*.

2.4.3. Resistance to metal ions

Metal ions, such as cadmium and copper are toxic to LAB. For resistance to metal ions, LAB have genes which confer them tolerant to metals. Since these genes occur naturally in LAB, and they are not antibiotic resistance genes, they are generally considered as food-grade markers. It was proposed already in the 1980´s that the gene encoding resistance to cadmium can be used as a selection marker (de Vos 1987). However, the first food-grade vector based on cadmium resistance was published not until nine years later (Liu et al. 1996). The 8.8-kb indigenous plasmid pND302 which could be selected with cadmium was found. A 1.6 kb fragment containing the nisin resistance gene was cloned into pND302, resulting in plasmid pND625, which could be transferred into *L. lactis* with cadmium and nisin selection. Use of the cadmium resistance plasmid pAH90 (Harrington and Hill 1992) as a food-grade vector was proposed by O´Sullivan et al. (2001). The cadmium resistance gene from pAH90 was cloned into the nisin- and phage resistance plasmid pNP40 (McKay and Baldwin 1984), simplifying its selection in industrial *L. lactis* starter strains (Trotter et al. 2001). Cadmium resistance has also been exploited as a food-grade cloning marker in *S. thermophilus* (Wong et al. 2003).

The second metal ion that has the potential for food-grade selection is copper (de Vos, 1986). A plasmid vector pND968 containing copper and nisin resistance genes was constructed in 1997, and it could be selected with copper or nisin (Leelawatcharamas et al. 1997). However, a DNA fragment originating from *Escherichia coli* was left in the plasmid, and thus, the final construction does not possess full food-grade status.

The genes encoding resistance to metal ions meet the requirements for food-grade cloning; they are not antibiotic resistance genes, and their origins are food organisms. The drawback of these selective agents is that heavy metals are toxic and cannot be added into food, thus eliminating the possibility of selection pressure for plasmid maintenance.

2.4.4. Carbohydrate utilization

The ability to ferment sugars can be used for selection on media containing the particular sugar as a sole source of energy. The potential for food-grade selection markers for *Lb. plantarum* and *Lb. casei* has been shown with xylose genes from *Lb. pentosu*s (Posno et al. 1991a), and inulin fermentation by a levanase gene from *B. subtilis* (Wanker et al. 1995).

Two true food-grade plasmids containing the genes needed for the sugar utilization as sole selection markers have been constructed. These include sucrose genes from *Pediococcus pentosaceus* (Leenhouts et al. 1998b), and melibiose genes from *L. raffinolactis* (Boucher et al. 2002). In the first study (Leenhouts et al. 1998b), sucrose genes were cloned into plasmid vectors containing a lactococcal chromosomal fragment for homologous recombination, and the origin of replication, but lacking the *repA* gene for replicative function (Leenhouts et al. 1991a). Introduction of these pORI plasmids into plasmid-free *L. lactis* strain resulted in integration into the chromosome followed by amplification of the integrated plasmid. The amplification was required for increasing the marker gene dosage for a sufficient expression level of the sucrose genes allowing selection. The system was used for cloning end expressing the proline iminopeptidase gene from *Propionibacterium shermanii* in *L. lactis* (Leenhouts et al. 1998a). Possible drawbacks of the system are poor applicability in dairy fermentations, due to the lack of selection pressure, and limitation to plasmid-free strains. Sucrose is a cheap and common sugar, but does not exist in milk. Strains carrying indigenous RCR plasmids will provide RepA protein for plasmid replication *in trans*, thus disturbing the integration of the plasmid into the chromosome.

Melibiose is the second sugar which has been used for selection as a dominant marker (Boucher et al. 2002). The α-galactosidase gene *aga* from *L. raffinolactis* was cloned into a theta-type plasmid vector, and the resulting plasmid pRAF800 was used as a food-grade vector for *L. lactis* and *Pediococcus acidilactici* using melibiose as a selective energy source. The applicability of pRAF800 was shown by cloning the phage defense mechanism AbiQ. In addition to the α-galactosidase, the melibiose positive phenotype requires a transporter for melibiose. Galactose permease GalA was shown to transport melibiose as well. Thus, the system is limited to GalA-positive strains of LAB.

2.4.5. Resistance to heat shock

A food-grade cloning system, based on the *shsp* gene encoding a small heat shock protein, was constructed for *S. thermophilus* and *L. lactis* (El Demerdash et al. 2003). Heat shock proteins in bacteria provide the ability to survive in harsh circumstances, for example, at low pH or high temperature. The *shsp*-vector pSt04 could be selected by incubating the transformed cells at a restricted temperature 52°C or 42°C for *S. thermophilus* and *L. lactis*, respectively. The applicability of the vector was demonstrated by cloning and expressing restriction-modification genes for phage resistance from a *S. thermophilus* strain.

2.5. Integrative expression systems

Recombination between an introduced plasmid and genomic DNA is a technique which can be used for inserting, deleting, or altering DNA content in the genome (Fig. 4; Mills 2001). By integration of a foreign gene into the chromosome, strains devoid of extra DNA, other than the target gene, can be built. Integrative food-grade systems may involve DNA from non-food-grade origin, since such DNA is not present in the final construction. Therefore, integrative systems can be selected with antibiotic resistance genes, and vectors originating from e.g., *E. coli* can be used for clonings and transformations. In addition to these benefits, integrative systems are generally more stable than those based on plasmids (Leenhouts et al. 1990). Foreign plasmids are likely to suffer more from segregational or structural instability than genes integrated into the chromosome. On the other hand, especially a double-cross-over (DCO) recombination, resulting in one gene inserted into the chromosome, may suffer from a low expression level due to the low gene dosage (Leenhouts et al. 1998a). In some cases, a single cross-over (SCO; Campbell-like integration) leads to the amplification of the integrated gene, thus enhancing gene expression (Leenhouts et al. 1989; Chopin et al. 1989; Leenhouts et al. 1998a). However, without selection pressure, Campbell-like integration may recombine for a second time resulting in a loss of the target gene.

2.5.1. Homologous recombination

The most common way to integrate foreign DNA into the chromosome is via homologous recombination. A chromosomal fragment is cloned into a plasmid not replicating in LAB, and the construction is transferred into LAB strain. The homologous fragments in the plasmid and in the chromosome recombine, resulting in integration of the plasmid into the chromosome (Fig. 4), and the integrants can be found with selection. Depending on the arrangement of the homologous fragments, SCO or DCO resulting in insertions or deletions in the chromosome will occur. Fig. 4 displays the principle of homologous recombination leading to gene replacement (Biswas et al. 1993; Mills 2001).

Sources for non-replicative plasmids for LAB are: (i) replicons from other bacteria, such as *E. coli* (Leenhouts et al. 1989; Scheirlinck et al. 1989; Mollet et al. 1993) or *Staphylococcus aureus* (Chopin et al. 1989), (ii) thermosensitive (ts) replicons which replicate in lower, but not in higher temperatures (Thompson and Collins 1989; Maguin et al. 1992), and (iii) plasmids lacking the *repA* gene required for replicative function (Leenhouts et al. 1996). RepA-deficient plasmids require a helper plasmid providing RepA *in trans* for clonings. These vector types, and their combinations (*repA*$^-$ plasmid combined with ts helper plasmid) have since been widely used in food-grade gene expression applications and modifications of cell metabolism.

Campbell-like integration requires a marker gene for selection. The only published food-grade marker used for Campbell-like integration was the sucrose utilization gene block from *P. pentosaceus* integrated into *L. lactis* chromosome (Leenhouts et al. 1998a). This approach was described in more detail in paragraph 2.4.4.

Replacement recombination resulting in insertion or deletion in the chromosome was first established in *L. lactis* by Leenhouts et al. (1991b). Since the gene inserted into the chromosome was the erythromycin resistance gene, this first report cannot be regarded as a food-grade system, but an establishment of a new technique for food-grade constructions in LAB. In fact, excluding non-food gene insertions, gene replacement practically always results in a food-grade construct. A number of deletions and insertions have been created into LAB genomes by gene replacement. Examples of them are listed in Table 1.

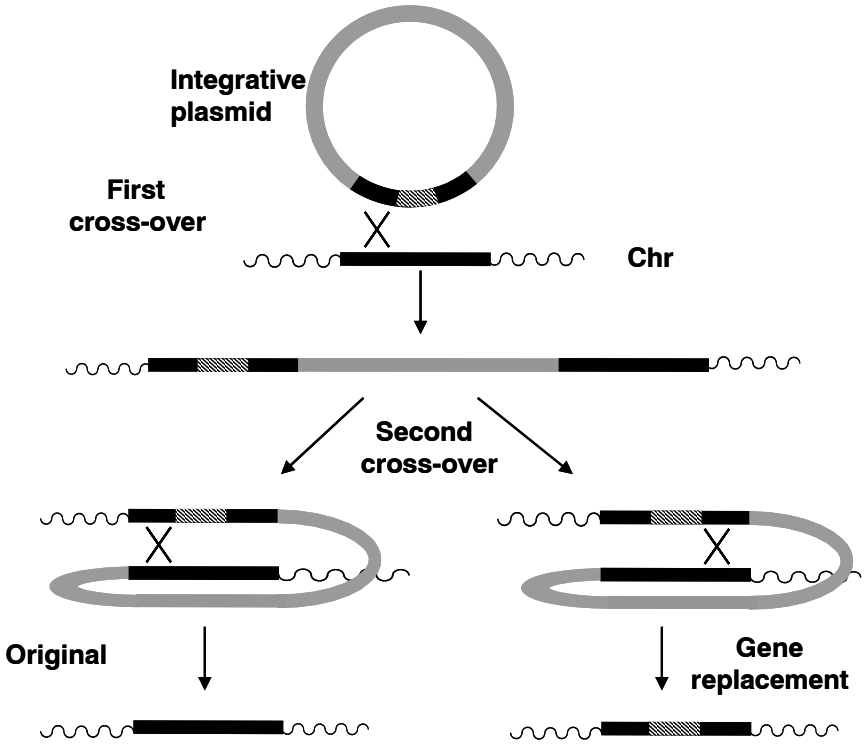

Fig. 4. Gene replacement via two homologous recombination events. Chromosomal target gene (black bar) has been cloned into a plasmid, and a fragment in the middle of the gene has been mutated (hatched area). First cross-over between a plasmid and the chromosomal (Chr) DNA results in Campbell-type integration of the plasmid into the chromosome. Campbell-type recombinants can be selected with a marker gene in the plasmid. Second recombination may occur either before or after the mutated area, resulting in the original intact gene, or the mutated gene left into the chromosome. Double recombination colonies are sensitive to the selective agent used in the first recombination and can be found by this property.

2.5.2. Insertion sequences

Transposons are mobile DNA elements which integrate randomly into bacterial chromosomes with the help of insertion sequences (IS). IS from LAB transposons have been used for insertional mutagenesis (Polzin and McKay 1992; Romero and Klaenhammer 1992; Dinsmore et al. 1993; Baccigalupi et al. 2000), and as tools for inserting rare restriction sites for chromosomal mapping (Le Bourgeois et al. 1992). Food-grade insertional mutagenesis was developed by using IS*S1* from *L. lactis*, associated with a ts replicon (Maguin et al. 1996). In addition to lactococci, random transposition of this vector was obtained in *Enterococcus faecalis* and *S. thermophilus*. This system could be used for generating food-grade insertional mutant strains.

Table 1. Examples of food-grade deletions and insertions constructed in LAB.

Deletion (Δ) / insertion (::)	LAB species	Replicon / integration	Objective	Ref.
::*nsr*	*L. lactis*	colE1(*E. coli*)	Nisin resistance	1
Δ*nisA*	*L. lactis*	colE1(*E. coli*)	Inactivation of nisin production	2
::*amyL*	*Lb. plantarum*	pE194(*S. aureus*)	α-amylase production	3
::*βgal*	*Lb. acidophilus*	colE1(*E. coli*)	Lactose utilization	4
Δ*ldhD*, ::*ldhL*	*Lb. helveticus*	pSA3(ts)	L-lactic acid production	5
::*c*I	*Lb. casei*	colE1(*E. coli*), site-specific int.	Phage resistance	6
::*ilvBN*	*Lb. casei*	colE1(*E. coli*)	Diacetyl production	7
::*pepC*, *pepG*, *pepI*, *pepL*, *pepQ*, *pepW*	*L. lactis*	pG^+host9(ts)	Nisin-inducible expression of peptidases	8
slpA::*c-myc*	*Lb. brevis*	*repA*$^-$	c-Myc epitope in S-layer	9
Δ*ltnA1*, Δ*ltnA2*	*L. lactis*	*repA*$^-$	Inactivation of lacticin 3147 production	10
::*abiD*	*L. lactis*	group II intron	Phage resistance	11
Δ*ldh* Δ*mtlA*, Δ*ldh* Δ*mtlF*	*L. lactis*	*repA*$^-$	Mannitol production	12

References within this table: 1) McIntyre and Harlander 1993; 2) Kuipers et al. 1993; 3) Hols et al. 1994; 4) Lin et al. 1996; 5) Kylä-Nikkilä et al. 2000; 6) Martín et al. 2000; 7) Gosalbes et al. 2000; 8) Henrich et al. 2002; 9) Åvall-Jääskeläinen et al. 2002; 10) Cotter et al. 2003; 11) Frazier et al. 2003; 12) Gaspar et al. 2004.

2.5.3. Site-specific recombination

In contrast to random integration by insertion sequences, targeted gene integration can be done by site-specific recombination. Chromosomes of LAB contain short "bacterial attachment sites" *attB*. Phages integrate to these *attB* sites by the phage attachment site *attP*. These two recombination sites have been exploited for site-specific insertion of genes to the chromosomes of LAB including species *L. lactis* (van de Guchte et al. 1994; Christiansen et al. 1994; Lillehaug et al. 1997), *Lb. gasseri* (Raya et al. 1992), *Lb. plantarum* (Dupont et al. 1995), *Lb. delbrueckii* (Auvray et al. 1997), and *Lb. casei* (Alvarez et al. 1998). A food-grade application of a site-specific recombination for *Lb. casei* was constructed by Martín et al. (2000). An integrase-dependent integration, followed by transformation with a second plasmid expressing β-recombinase, facilitating DCO, resulted in a strain with a single copy of the target gene, in this case, the phage A2 repressor gene *c*hi, integrated into the chromosome. Benefits of this system are generally the same as with other integrative systems: primary cloning of the target gene to be expressed can be done in *E. coli*, and transformants can be selected with antibiotics.

2.5.4. Group II introns

Group II introns are catalytic RNA elements present in many bacterial species (Donny and McKay 1999). They can mobilize autonomously to allelic sites in the chromosomes. This process called homing is efficient enough to eliminate the use of a selection pressure for driving integration. A food-grade gene insertion system was developed by cloning the gene *abiD* for phage resistance into the group II introne M1083-opt in a plasmid (Frazier et al. 2003). The integration of the intron::*abiD* into the malate decarboxylase gene *mleS* in the *L. lactis* chromosome was achieved without selection pressure. The intron donor plasmid was then cured, resulting in a food-grade insertional *mleS* mutant showing increased phage resistance.

2.6. Complementary selection markers

Complementary markers require host strains, which lack some essential property. The lack of the property is then complemented by a plasmid expressing the particular gene. Since mutants with an inactive gene are difficult to find in nature, and because artificial mutagenesis may cause several unspecific mutations, the strains aimed for complementation hosts are more reasonable to construct by genetic engineering. A common way to construct complementable LAB strains is to delete a gene or a part of a gene by gene replacement (Fig. 4).

2.6.1. Lactose genes

The ability to utilize lactose is essential for starter bacteria in milk fermentations. Lactose is the best source of energy present in milk. LAB can ferment lactose by several different mechanisms (de Vos and Vaughan 1994). Food-grade complementary cloning systems constructed, thus far, are based on genes encoding enzymes involved in the lactose phosphotransferase system (PTS) (de Vos et al. 1989; MacCormick et al. 1995; Platteeuw et al. 1996). One “nearly-food-grade” lactose selection system has been constructed based on complementation of the β-galactosidase from the lactose permease system (Hashiba et al. 1992).

The smallest gene *lacF* coding for lactose-specific phosphocarrier protein EIIA in the lactose-PTS-operon of *L. lactis* strain YP2-5 was found to be inactive due to a point mutation. The lactose utilization was restored by introducing a plasmid vector expressing a functional *lacF* gene (de Vos et al. 1989). These experiments showed that the direct selection with lactose was possible. The first real food-grade system based on lactose selection was published in 1995 (MacCormick et al. 1995). In this study, the *lacF* gene was deleted from the chromosome of *L. lactis* by gene replacement. The 2.5-kb food-grade complementation plasmid pFI846 was constructed of lactococcal pSH71 replicon, lactococcal promoter P32, and the *lacF* gene. A similar strategy was used in the studies by Platteeuw et al. (1996) and Xiang et al. (2003). In the former, they constructed several different *lacF*-based vectors, which applicability were shown by expressing *E. coli gusA* gene in *L. lactis*. In the latter study, a *lacF*-vector was used to overexpress human glutathione *S*-transferase and Cu/Zn superoxide dismutase in *L. lactis*.

The lactose selection plasmid with *lacF* gene as a marker was mentioned in a study investigating the influence of cell lysis to cheese ripening (de Ruyter et al. 1997). However, the results in this study, including the cheese trials, were presented only with plasmids carrying chloramphenicol resistance gene as their markers.

2.6.2. Nonsense suppression

A complementary food-grade approach based on nonsense suppression was first presented in 1995 (Dickely et al. 1995). This method can be considered as a complementary selection, even though it differs from the others in a way that the mutated gene in the host strain is not the same as the complementing gene. First, a nonsense ochre mutation was created in the target gene of the *L. lactis* host strain. The target was chosen to be an essential gene needed for growth in purine-free medium, e.g., milk. As a selection marker, the constructed complementation plasmid pFG1 contained a nonsense suppressor gene *supB*, whose product was an altered transfer RNA, transferring serine for ochre codon. Thus, ochre codon did not any more serve as a stop codon, and the functional protein was translated. The ochre suppressor vector pFG1 has been used in studies examining the influence of overproduction of several lactococcal (Guldfeldt et al. 2001) or lactobacillar (Joutsjoki et al. 2002) peptidases in *L. lactis*. A drawback of the vector is that most lactococcal genes (approximately 72%) have an ochre codon for termination, resulting in disturbance in protein synthesis (Cancilla et al. 1995). Therefore, the scheme was improved by applying the nonsense mutation as an amber codon, and using the amber suppressor *supD* gene as a selection marker. (Sørensen et al. 2000). Amber codon terminates only approximately 10% of *L. lactis* genes (Cancilla et al. 1995). The resulting plasmid was named as pFG200, and its applicability was shown by expressing the *pepN* gene in several industrial *L. lactis* starters.

2.6.3. Thymidylate synthase

The use of thymidylate synthase as a food-grade selection marker originates from the studies by Ross et al. (1990a; 1990b). They characterized the *thyA* gene of *L. lactis*, and constructed a "nearly-food-grade" vector with *thyA* as a selection marker. A weakness of these studies was that there were no *thyA*-deficient LAB host strains available, and the selection could not be applied for LAB. Ten years later, *thyA* mutant host strains of *Lb. acidophilus* were obtained by trimethoprim treatment (Fu and Xu 2000). A plasmid expressing *thyA* was constructed, and selected for in a thymidine-free medium. The pUC19 replicon was left to the plasmid facilitating the cloning in *E. coli*. This feature, however, gives the plasmid a non-food-grade status.

Finally, a true food-grade cloning vector, with *thyA* as a selection marker for *S. thermophilus*, was constructed (Sasaki et al. 2004). Again, the *thyA* mutants were obtained by trimethoprim treatment. The applicability of the vector was shown by expressing the amylase gene *amyA* from *S. bovis* in *S. thermophilus*.

2.6.4. Alanine racemase

The D-form of alanine is an essential component of the bacterial cell wall. However, it is not a regular constituent in industrial fermentation media. Thus, starter bacteria need an enzyme, alanine racemase, for converting common L-Ala to D-Ala. A selection system was constructed for *L. lactis* and *Lb. plantarum* based on complementation of alanine racemase deficiency (Bron et al. 2002). By homologous recombination, the gene *alr*, encoding alanine racemase, was first disrupted in the chromosome resulting in strains depending D-Ala supplementation for growth (Hols et al. 1999). The plasmid expressing the *alr* gene was then constructed and selected for in a medium lacking D-Ala (Bron et al. 2002). An interesting feature with this plasmid was that, in addition to the *alr* mutants, it could also be transferred into wild-type LAB using D-cycloserine, a competitive inhibitor of alanine racemase, as a selective agent. Thus, the *alr* expression represents both complementary and dominant selection.

AIMS OF THE STUDY

The main objectives of this study were to investigate the nisin immunity mechanism in nisin producing bacteria (I, II), and to develop food-grade cloning methods for lactic acid bacteria (III, IV). The detailed objectives of the research were to:

1. Characterize the interaction between nisin and the immunity protein NisI, determine the distribution of the NisI protein in nisin producing cell culture, and evaluate the role of lipid-free NisI in nisin immunity system (I, II).

2. Exploit the nisin immunity gene *nisI* as a selection marker, using nisin as a food-grade selective agent, and establish the nisin immunity in lactobacilli (III).

3. Adapt the use of gene replacement in construction of deletion mutation in a gene located in a multi-copy plasmid, and to complement the mutation by a food-grade expression plasmid (IV).

MATERIALS AND METHODS

The plasmids used in this study are presented in Table 2, bacterial strains in Table 3, and PCR primer sequences in Table 4. The analysis methods used in this study are substantially presented in Table 5. Descriptions of the methods in more detail are presented in the materials and methods sections in the publications I – IV.

Table 2. Plasmids used in this study.

Plasmid	Relevant properties	Reference/source	Used in
pHELIX2	3.0 kb, *E. coli* cloning vector, Amp^r	Boehringer Mannheim	IV
pLEB124	4.5 kb, Erm^r, *L. lactis* expression vector	Qiao et al. 1995	IV
pLEB415	5.2 kb, Erm^r, pLEB124 + *nisI*	Qiao et al. 1995	III
pLEB579	2.9 kb, Erm^r, *L. lactis* cloning vector	Beasley et al. 2004	II
pLEB580	4.2 kb, Erm^r, *nisI* expression	This study	II, III
pLEB588	3.1 kb, pHELIX2 + P_{pepR}	This study	IV
pLEB590	3.1 kb, *nisI* as a selection marker	This study	III, IV
pLEB591	5.8 kb, pVS89(ΔP45) + P_{pepR}	This study	IV
pLEB600	3.6 kb, *lacG* as a selection marker	This study	IV
pLEB604	4.6 kb, pLEB600 expressing *pepI*	This study	IV
pLEB605	4.1 kb, pLEB590 expressing *pepI*	This study	III
pLEB628	pCR4-Topo + P45ss_{usp45}, Amp^r	Laboratory collection	II
pLEB643	4.2 kb, Erm^r, lipid-free NisI	This study	II
pLS19	pUC19 + Erm^r	Kees Leenhouts, The Netherlands	IV
pVS2	5.0 kb, Cam^r, Erm^r	von Wright et al. 1987	III
pVS87	pLS19 + *lacG* from *Lb. casei* E	This study	IV
pVS88	pVS87, 141 bp *Sph*I-*Cla*I deletion in *lacG*	This study	IV
pVS89	pLEB124 + *lacG* from *Lb. casei* E	This study	IV

Table 3. Bacterial strains used in this study.

Bacterial strain	Relevant properties	Reference/source	Used in
E. coli DH5α	Transformation host	Hanahan 1983	IV
E. coli TG1	Transformation host	Sambrook et al. 1989	I, II, III
E. coli ECO395	Produces LF-NisI as a GST-fusion	Qiao et al. 1995	I, II
L. lactis MG1614	Plasmid-cured NCDO712, Nis^s	Gasson 1983	I- IV
L. lactis N8	Wild-type nisin producer	Graeffe et al. 1991	I, II, III
L. lactis NZ9840	Nisin producer mutant, Δ*nisA*, Δ*nisIP*, no nisin production	Oscar Kuipers, The Netherlands	I, II
L. lactis SSL110	Edam-type cheese starter, Nis^s	Valio Ltd.	III
L. lactis LAC230	MG1614 carrying plasmid pLEB579	This study	II
L. lactis LAC231	MG1614 carrying plasmid pLEB580	This study	II, III
L. lactis LAC233	MG1614 carrying plasmid pLEB590	This study	III, IV
L. lactis LAC241	MG1614 carrying plasmid pLEB605	This study	III
L. lactis LAC273	MG1614 carrying plasmid pLEB643	This study	II
L. lactis LAC276	NZ9840 carrying plasmid pLEB579	This study	II
L. lactis LAC277	NZ9840 carrying plasmid pLEB580	This study	II
L. lactis LAC278	NZ9840 carrying plasmid pLEB643	This study	II
Lb. casei E	Wild-type non-starter cheese strain	Valio Ltd.	IV
Lb. casei EΔ*lacG*	Lac^- derivative of strain E, Δ*lacG*	This study	IV
Lb. helveticus 53/7	Swiss-type cheese starter	Valio Ltd.	III, IV
Lb. plantarum 755	Plasmid-free, Nis^s	Valio Ltd.	III
Lb. plantarum NRRLB-192	Nis^s	NRRLB	I
Lb. rhamnosus 1/6	Wild-type non-starter cheese strain	Valio Ltd.	IV
M. luteus Al NCIMB 8166	Nis^s indicator strain	NCIMB	I
S. faecalis N	Nis^s indicator strain	Pazur et al. 1971	I
B. subtilis BRB1	Nis^s indicator strain	Sibakov et al. 1983	I
B. megaterium ATCC12980	Nis^s indicator strain	ATCC	I
B. stearothermophilus ATCC12980	Nis^s indicator strain	ATCC	I
B. amyloliquefaciens VTTE18	Nis^s indicator strain	VTT	I
B. coagulans DSM459	Nis^s indicator strain	DSM	I
B. natto BGSC27A1	Nis^s indicator strain	BGSC	I

Table 4. Sequences of PCR-primers used in this study. Relevant restriction sites added to the primers are shown underlined.

Primer name, use, restriction sites	**Sequence 5´→ 3´**	**Used in**
NIS234, LF-*nisI* forward *Xho*I	AGATCTCGAGTATCAAACAAGTCAAAAAAAGGTG	II
NIS235, LF-*nisI* reverse *Xho*I *Bam*HI	AGATCTCGAGGGATCCCTAGTTTCCTACCTTCGTTGCAAG	II
NIS173, *repA* reverse *Xho*I *Xba*I *Eco*RV *Bss*HII	CTCGAGTCTAGAGATATCGCGCGCTATTAATCGCAACATCAAACC	III
NIS174, *ermC* forward *Cla*I	CTGGAGATCGATGTTAAACCGTGTGCTCTACGACC	III
NIS213, *pepI* forward *Bam*HI	GTTCGGATCCAAGTAGGCAAAAAGATGGAAATTATTG	III, IV
NIS214, *pepI* reverse *Xho*I	AAATCTCGAGACAAACGCAGTGAAAGAATGAAG	III, IV
51446, *lacG* forward *Mlu*I	TGAACTACGCGTGTCGACTGGTCACAACAACTGGTC	IV
51445, *lacG* reverse *Bam*HI	CTGCCAGGATCCTTAATCCGGAATGATGTG	IV
71963, P_{pepR} forward *Eco*RI	ATGTGGAATTCTGCTTTGATACTCACCA	IV
71962, P_{pepR} reverse	AGCCGGATCCTTAGGTCAGGATCGTTGTTC	IV

Table 5. Methods used in this study.

Method	Used in	Reference
Basic DNA techniques, including PCR, enzyme modifications, electrophoresis, plasmid isolation from *E. coli*	II-IV	Ausubel et al. 1987; Sambrook et al. 1989; Catalogues of enzyme suppliers
DNA isolation from LAB		
- *Lb. casei*	IV	Anderson and McKay 1983
- others	II-IV	O´Sullivan and Klaenhammer 1993
DNA transformation		
- *E. coli*	II-IV	Zabarovsky and Winberg 1990
- *L. lactis*	II-IV	Holo and Nes 1989
- *Lb. plantarum*	III	Luchansky et al. 1989
- *Lb. casei*	IV	Varmanen et al. 1998
Southern blotting	IV	Ausubel et al. 1987
Nisin sensitivity assay, agar diffusion	I	Qiao et al. 1996
Bioscreen C growth analysis	II-IV	Labsystems Ltd.
Nisin quantification by nisin-induced GFP fluorescence	I	Reunanen and Saris 2003
NisI purification by Glutathionine-linked affinity chromatography	I, II	Qiao et al. 1995
SDS-PAGE + Western blotting	I, II, III	Sambrook et al. 1989
P-β-gal activity assay	IV	Okamoto and Morichi 1979; Varmanen et al. 1996
PepI activity assay	III, IV	El Soda and Desmazeaud 1982; Varmanen et al. 1996
Surface plasmon resonance	II	Myszka 1997

RESULTS AND DISCUSSION

1. Nisin immunity (I, II)

1.1. Lipid-free NisI (I)

The nisin immunity protein NisI has previously been shown to be a lipoprotein attached to the extracellular side of the cytoplasmic membrane by a lipid-modified N-terminal cysteine (Qiao et al. 1995). The leader peptide of NisI is a strongly conserved lipoprotein signal peptide, with a functional site for lipid-modifications (von Heijne 1989; Sutcliffe and Russell 1995). If the signal peptide of a protein aimed for lipid modification is cleaved before the lipid groups are added, the polypeptide is secreted to the external environment without lipid modifications. To examine if NisI is secreted by a wild-type nisin producer strain, the NisI contents in cell and supernatant fractions of the nisin producer *L. lactis* N8 liquid culture were analysed. Samples were taken at different growth stages, from early log-phase to late stationary phase, and run in SDS-PAGE, followed by Western analysis with polyclonal NisI antibody. The results showed that, during the first hours of growth, nearly all of the produced NisI was located in the cell fraction. In the later phases of growth, an increasing amount of NisI was released and found in the growth medium. In a stationary-phase culture, there were almost equal amounts of cell-bound and soluble NisI. Thus, NisI is secreted similarly in the wild-type nisin producer *L. lactis* strain N8, as in the heterologous overexpression host *B. subtilis* (Stein et al. 2003), and exists *in vivo* in two different forms, as a lipoprotein, and in a lipid-free form (LF-NisI). The high level of the LF-NisI excretion suggests that the secretion and/or the lipid-free form of the protein may have a function in the nisin immunity system.

1.1.1. Effect of externally added LF-NisI on nisin immunity (I)

To study the possible biological role of LF-NisI, the ability of LF-NisI to inactivate nisin or to protect cells against nisin in the growth medium was determined. LF-NisI was purified according to Qiao et al. (1995) from *E. coli* ECO395 as a GST fusion followed by cleavage of the GST by thrombin. The purified LF-NisI was mixed with nisin in a 1:1 ratio, and the mixture was added to liquid and solid media. LF-NisI did not inactivate nisin, nor did it protect cells against nisin. Contrary to expectations, on solid media LF-NisI enhanced the activity of nisin. In fact, it is reasonable that LF-NisI had no protective capacities, but instead, was capable of enhancing the activity of nisin. It would not make sense that a bacterium producing a bacteriocin, would provide a protective protein to the target cells at the same time. No significant effect, either protective or stimulative, could be seen in liquid media. This could be explained as LF-NisI may hinder nisin adsorption to surfaces on agar media, thus increasing the concentration of soluble active nisin. LF-NisI did not affect the nisin immunity level of the NisFEG expressing strain NZ9840. It seems that NisFEG is somehow able to protect cells against nisin as effectively as against the LF-NisI:nisin mixture.

NisI has been shown to form complex with nisin (Qiao 1996; Stein et al. 2003). If LF-NisI had affinity to cells, it could adsorb to cell surfaces and bind nisin from the environment thus bringing nisin to cells. To determine, if this was the reason for the nisin-stimulatory effect, the affinity of LF-NisI to cells was examined. Purified LF-NisI and *L. lactis* cells were mixed, the cells were collected by centrifugation, and NisI content in cells and remaining supernatant fraction was analysed. LF-NisI was not found in cell fraction, indicating that it had no affinity to cells. Thus, the nisin-stimulatory function of LF-NisI is not to enhance the binding of nisin to cells.

The nisin-enhancing effect of LF-NisI was not seen in the protein ratios below 1:5 (LF-NisI:nisin). The protein ratios in nisin producer's culture supernatant were shown to be 1:30 to 1:90, i.e., much lower than 1:5. From that point of view, the stimulatory effect of LF-NisI seems to be of minor importance *in vivo*. However, in circumstances where nisin concentration is locally decreased, for instance, because of nisin-adsorbing fat particles, the LF-NisI-mediated enhancement of nisin activity would be favorable. In conclusion, the results propose a dual role for NisI. As a lipoprotein it can protect the cell against nisin, and in soluble form it enhances the activity of nisin.

1.1.2. Effect of LF-NisI secretion on nisin immunity (II)

To study the role of LF-NisI secretion in nisin immunity, the protein should be completely secretable, i.e., the lipid-modificated fraction of the protein must be eliminated. Therefore, the *nisI* lipoprotein signal sequence and the site for lipid-modification (Cys1) were replaced by a signal sequence of the gene *usp45* encoding lactococcal secreted protein with an unknown function (van Asseldonk et al. 1990). The resulting hybrid gene was cloned into a plasmid vector, and transferred into *L. lactis* strains MG1614 and NZ9840. NisI protein was localized in the strains by Western analysis. It was found to exist merely as a secreted, soluble protein in the supernatant, which showed that the secretion of LF-NisI was efficient, and no lipoprotein form was present. The ability of the secreted LF-NisI to protect the cell against nisin was then tested. In the strain MG1614, which does not contain any nisin immunity factors, a slight increase in the nisin immunity level by LF-NisI secretion was detected. This indicated that the secreted LF-NisI could have a supportive role in nisin immunity in nisin producing cells. In contrast, the excretion of LF-NisI in the strain NZ9840, which expresses the NisFEG transporter complex for partial nisin immunity, the contribution to the immunity was remarkable. This suggests that LF-NisI co-operated with NisFEG in exporting nisin from cell surface to the external environment. The LF-NisI-mediated immunity level was, however, lower than the immunity level provided by lipoprotein NisI, affirming that membrane-bound NisI is needed for full nisin immunity.

1.2. Interaction between nisin and NisI (II)

It has been shown previously by surface plasmon resonance analysis that purified NisI and nisin interact physically (Qiao 1996), and later by mixing high concentrations of the proteins in a tube, followed by precipitation of the complex by centrifugation (Stein et al. 2003). The results in the latter suggested rather weak interaction, since the NisI:nisin complex was easily dissolved in water. A deeper understanding of the mechanism of nisin immunity would need more specific information in regard to the nature of the NisI:nisin interaction. Therefore, the kinetics of the interaction was studied by surface plasmon resonance analysis using BIACORE 2000. Nisin was immobilized onto the surfaces of flow channels in the CM5 sensor chip. Purified LF-NisI was then injected through the flow channels, and the association of LF-NisI to nisin was measured, as well as the dissociation of the complex. The injected LF-NisI was shown to bind to the immobilized nisin. The obtained value for the equilibrium dissociation constant K_D for NisI:nisin interaction was in the micromolar range (0.6-2 μM), which indicates a weak affinity, but in a range to be expected for an interaction that is reasonable to dissociate easily. As the produced nisin is targeted for killing competing cells and for cell signaling (Dunny and Leonard 1997), and not for forming stable complexes with an immunity protein, it is to be expected that NisI:nisin complex dissociates easily.

1.3. Nisin immunity mechanism (I, II)

Immunity proteins of lantibiotic producers can function either by direct physical interaction with the bacteriocin, or by binding to structures of the cell needed for bacteriocin activity (Saris et al. 1996; Hoffmann et al. 2004). NisI functions by specific binding to nisin, as also shown by Stein et al. (2003). They proposed that membrane bound NisI would intercept nisin at the surface of the cytoplasmic membrane, and by sequestering nisin, prevents it from inserting into the membrane and/or prevents high local density of nisin molecules necessary for pore formation. Our results about the kinetics of LF-NisI:nisin interaction support the hypothesis of an unstable NisI:nisin complex. A weak affinity indicates that the function of NisI involve binding, followed by liberation of nisin. Micromolar interaction of NisI:nisin is clearly too weak to prevent the nanomolar interaction between nisin and lipid II (Wiedemann et al. 2001). The immunity protein LtnI for the bacteriocin lactococcin A interacts with the receptor of the bacteriocin, thus blocking the binding of lactococcin A to the receptor (Venema et al. 1994). Added to this, the immunity protein PepI for the lantibiotic Pep5 produced by *Staphylococcus epidermidis* seems to bind to the target of the bacteriocin, which probably is a membrane-bound precursor of anionic cell wall polymer such as teichoic acid (Hoffmann et al. 2004). Since NisI does not protect cells against other lantibiotics, which also use lipid II as a target (Brötz et al. 1998a; Brötz et al. 1998b; McAuliffe et al. 2001), the NisI:lipid II interaction is not plausible.

Supplemented to the growth medium, LF-NisI did not protect cells against nisin, but did protect when it was secreted *in vivo*. Consequently, the secretion process from the membrane through the cell wall to the external environment, is critical. Since LF-NisI had no affinity to cell surface, the secreted protein is probably diffused relatively freely across the cell wall. In the cell wall, LF-NisI may intercept nisin molecules, and carry them further from the membrane. The increase in immunity level by LF-NisI secretion was better seen in the strain expressing NisFEG, which exports cell-associated nisin to extracellular space (Stein et al. 2003). Although it should not be excluded, the actual ability of NisFEG to pick nisin from the membrane has not been shown. Thus, the putative co-operation between NisFEG and LF-NisI can be seen in several ways: (i) NisFEG removes nisin from the membrane and exports it to the cell wall, where secreted LF-NisI intercepts it, and through diffusion, transports and releases it to the environment, or (ii) as previous, but more specifically, LF-NisI acts as a nisin recipient protein for NisFEG, facilitating the release of nisin from the transporter, or (iii) oppositely, LF-NisI picks nisin from cell membrane/surface and provides it to NisFEG, which exports nisin to the extracellular space. The last alternative would mean that LF-NisI acts as a binding protein for NisFEG, thus, functioning similarly as extra-cytoplasmic binding proteins of import systems in *Streptococcus pneumoniae* and *Mycoplasma hyorhinis* (Gilson et al. 1988). Such a function of LF-NisI would be the first example of a binding protein for an export system.

In conclusion, the results indicate that secretion of LF-NisI by the nisin producer has a biological function, and that NisI has a dual role, as an immunity protein and as a factor enhancing the activity of nisin. The mechanism by which NisI stimulates nisin activity is unknown and requires further study. Also, the mechanism of the putative co-operation between NisI and NisFEG remains to be elucidated.

2. Food-grade cloning systems for LAB (III, IV, unpublished)

2.1. Nisin immunity gene *nisI* as a selection marker (III)

Although the expression of the nisin immunity gene *nisI* in nisin sensitive background was shown to make cells tolerate higher nisin concentrations (Kuipers et al. 1993; Qiao et al. 1995), no attempts had been made for selecting the *nisI* expressing cells on nisin-containing media. As the nisin resistance gene *nsr* had been exploited as a food-grade selection marker (Froseth et al. 1988; von Wright et al. 1990), we investigated the possibility to use *nisI* in a similar way. The first attempt to select *L. lactis* MG1614 transformants with nisin was made with the *nisI* expression plasmid pLEB415 (Qiao et al. 1995). The selection was found to function well, which encouraged us to construct a real food-grade plasmid, with *nisI* as a sole selection marker.

2.1.1. Construction of the food-grade vector pLEB590 (III)

The food-grade vector was designed to contain only the *nisI* gene with a promoter, the area needed for the plasmid's own replication, and few restriction sites for gene cloning. A 1.7 kb fragment containing the origin of replication and the *repA* gene for rolling circle replication was amplified by PCR from the cloning vector pVS2 (von Wright et al. 1987). The replicon originates from the 2.1 kb indigenous lactococcal plasmid pSH71 (Gasson 1983). Even though theta replicative plasmids are generally considered more stable than RCR plasmids, the segregational stability of pSH71 has been reported to be high in *L. lactis* (de Vos and Simons 1994).

To make the construction of the food-grade vector easier, the erythromycin resistance gene *ermC* in pVS2 was amplified in the same PCR-fragment as the replicon. Fig. 5 displays the schematic representation of the construction of pLEB590. Since it is constructed entirely of lactococcal DNA, except for a multiple cloning site of synthetic DNA, and since it is devoid of antibiotic resistance genes, pLEB590 is regarded as a food-grade plasmid.

2.1.2. Properties of pLEB590 (III, unpublished)

Resistance to nisin. The resistance to nisin of *L. lactis* MG1614 carrying pLEB590 (LAC233) was determined in broth containing different concentrations of nisin (0-500 IU/ml), and compared with the nisin producer strain N8, the host strain MG1614, and the *nisI* expression strain LAC34 (MG1614 carrying pLEB415). The nisin producer strain N8 exhibits the wild type level of nisin resistance (approximately 1000 IU/ml = 100%), and the host strain MG1614 displays the sensitivity of a nisin negative strain (< 2%). The food grade strain LAC233 grew well with 250 IU nisin/ml (25% resistance). The NisI-mediated immunity level has previously been described to be 1-4% of that of the nisin producer strain (Kuipers et al. 1993; Qiao et al. 1995). To examine the correlation between the amount of NisI in cells and the tolerance to nisin, the NisI content in different strains was determined by Western analysis. Even though Western analysis is not fully quantitative method, the comparison of protein amounts in different strains is possible, if samples are applied into the same run, and membranes are exposed simultaneously. As presented in Fig. 6, the NisI-mediated nisin immunity level appears to be comparative to the amount of the protein. The 25% immunity level of LAC233 is thus explained by a higher expression level of the *nisI* gene than the previously constructed *nisI* expression plasmids, such as pLEB415 in the strain LAC34. The expression of *nisI* in LAC233 cannot still be considered as overexpression, since NisI content in the wild-type *L. lactis* N8 was at least at the same level (Fig. 6).

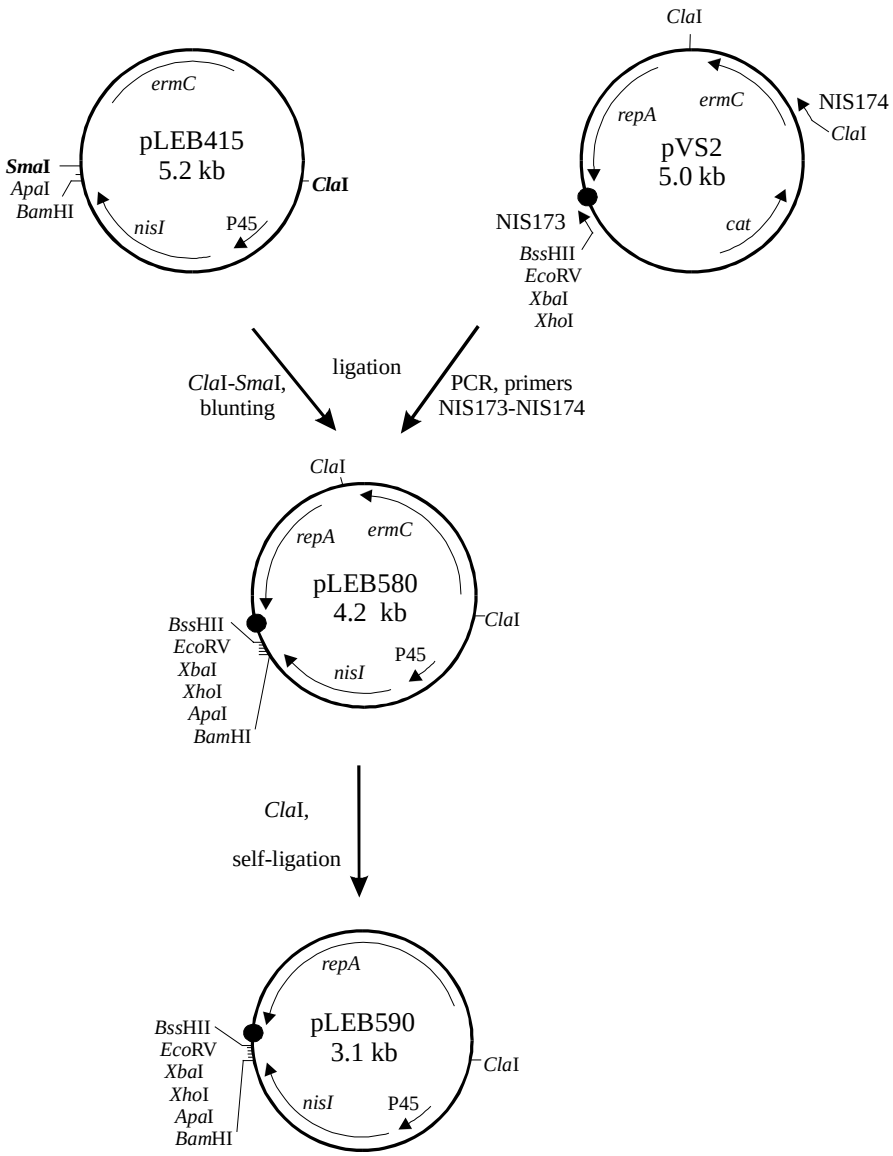

Fig. 5. Schematic representation of constructing the *nisI* food-grade vector pLEB590. Plasmid pLEB415 is lactococcal *nisI* expression plasmid (Qiao et al. 1995). Plasmid pVS2 is lactococcal RCR-type cloning vector (von Wright et al. 1987). P45 is lactococcal constitutive promoter showing a moderate level of gene expression (Koivula et al. 1991; Sibakov et al. 1991). The promoter P45-*nisI* fragment was restricted with *Cla*I-*Sma*I from pLEB415 and ligated with the PCR-fragment containing the replicon and *ermC* from pVS2. The ligation mixture was transferred into *E. coli* TG1 with erythromycin selection resulting in plasmid pLEB580 (strain LAC231). The *ermC* gene was then restricted from pLEB580 with *Cla*I, the remaining part was re-ligated, and electroporated into *L. lactis* MG1614 with nisin selection, resulting in the plasmid pLEB590. The black circle represents a transcription terminator.

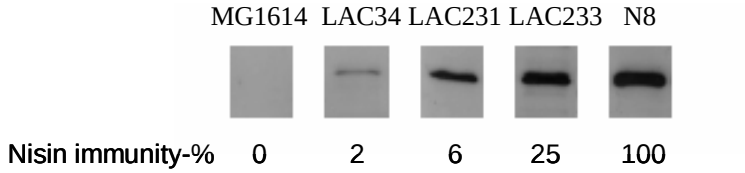

Fig. 6. Western analysis of NisI content in different *L. lactis* strains. Cells from 1 ml of overnight cultures were collected by centrifugation, and suspended in 100 µl of 50 mM Tris-HCl buffer pH 7.5. Ten µl of the suspensions were applied to SDS-PAGE followed by Western analysis with NisI antibody. The bands shown are from two gels in the same SDS-PAGE and Western runs. The two membranes were exposed simultaneously in the same exposure cassette. Strains: MG1614, nisin-negative host strain; LAC34, MG1614(pLEB415); LAC231, MG1614(pLEB580); LAC233, MG1614(pLEB590); N8, nisin-producing wild-type.

Expression of the nisin resistance gene *nsr* in the food-grade plasmids pFM011 and pVS40 caused approximately the same level of nisin resistance as pLEB590 (Froseth et al. 1988; Hughes and McKay 1992; Wessels 1993). Wessels (1993) reported *nsr*-mediated nisin resistance varying from 125 to 250 IU nisin/ml in different nisin sensitive host strains. Froseth et al. (1988) determined 160 IU nisin/ml MIC value for the *L. lactis* strain LM0230 carrying the plasmid pFM011. However, the same plasmid was later selected on agar plates with nisin concentration of 300 IU/ml (Hughes and McKay 1992). Apparently, the MIC values of nisin vary depending on the host strain and the growth conditions.

Transformation frequency. High transformation frequency is an important property for a cloning vector, as well as easy, cheap, and a well functioning selection. The transformation frequency of the food-grade vector pLEB590 was determined by electroporating 600 ng of plasmid DNA into *L. lactis* MG1614 and selecting transformants with 60 IU nisin/ml. Transformant colonies were tested for nisin resistance and plasmid contents. With 60 IU nisin/ml, no plasmid-free colonies were obtained, and the transformation frequency was calculated to be 3.6×10^5 colonies/µg plasmid DNA.

Stability. Plasmids replicating via RCR mechanism are considered less stable than those replicating via theta mechanism (Kiewiet et al. 1993). Segregational stability of pLEB590 was determined according to Wessels (1993) in a nisin-free medium to evaluate the applicability of the vector in non-selective conditions. Plasmid loss per cell per generation in M17G medium was calculated to be approximately 0.1%, which represents an average loss of lactococcal plasmids (Wessels 1993). After 18 generations in a non-selective medium, 97.8% of cells retained plasmid and showed nisin resistant phenotype on nisin-containing media. No structural rearrangements were observed in the restriction analysis of the plasmids isolated from 23 colonies after the cultivation in a non-selective medium. Hence, pLEB590 was shown to be relatively stable.

2.1.3. Nisin selection (unpublished)

The plating after transformation had, thus far, been performed by the pour plate method, in which melted M17GS agar was poured onto the electroporated cells on an empty sterile plate. The spread plate method, where electroporated cells are spread onto the solid agar, could be

more practical, since the agar plates can be made beforehand and stored for several days/weeks. Therefore, the two plating methods, pour plate and spread plate methods, were compared in nisin selection after transformation. Electroporated *L. lactis* cells were plated onto plates containing 60 IU nisin/ml. The plating method had a remarkable effect on the amount of background colonies. On the spread plates, nisin sensitive colonies covered the agar surface already after one day of incubation. On the pour plates, no background colonies were obtained in two days (Fig 7). Cells plated onto the surface of the agar could tolerate nisin better than cells growing inside the agar. Unlike the colonies growing on the agar surface, the ones obtained inside the nisin-containing agar carried, without exception, the correct transferred plasmid. This phenomenon is not fully understood, even though similar observations have been described. Harrington and Hill (1991) noticed that using the nisin resistance gene *nsr* as a marker, the primary selection with nisin was possible only in liquid but not on solid media. They did not state how the plating was actually performed on solid media, but it seems apparent that they used a spread plate method. Possibly, on the surface of the agar, cells growing on top of each other can form a diffusion barrier for nisin, and are thus not as susceptible to nisin as cells growing inside the agar, where nisin can diffuse from all directions and conditions are more humid. In conclusion, selection of pLEB590 transformants had to be made by the pour plate method.

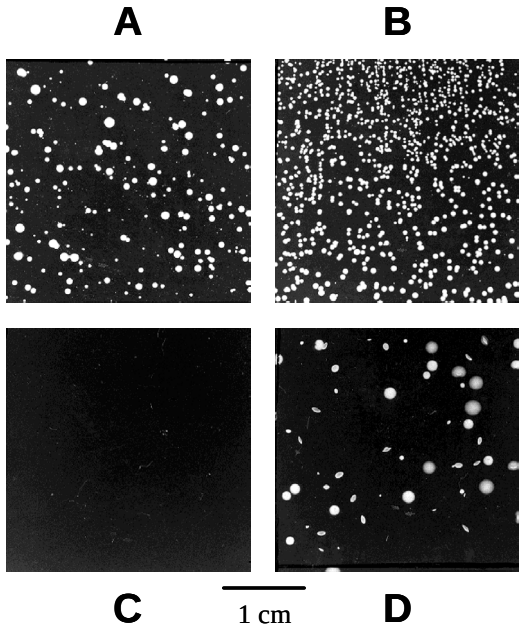

Fig. 7. Pour plate method versus spread plate method in nisin selection after electroporation of pLEB590 into *L. lactis* MG1614. **A** and **B**, Spread plates after 1 and 2 days, respectively. **C** and **D**, pour plates after 1 and 2 days, respectively. Colonies growing inside the agar in pour plates were without exception nisin resistant. The colonies on the agar of the spread plates were mostly nisin sensitive background colonies.

2.1.4. Nisin immunity in *Lactobacillus* (III, unpublished)

Nisin resistance or immunity genes have not previously been transferred into other LAB than *L. lactis*, despite that nisin sensitive species occur commonly in genera *Lactobacillus*, *Leuconostoc*, and *Pediococcus* (Radler 1990; Choi and Park 2000; Chung and Hancock 2000), and thus are potential hosts for nisin resistance vectors. To investigate if the nisin resistance in lactobacilli can be increased by *nisI* expression, pLEB590 was electroporated into the nisin sensitive *Lactobacillus plantarum* strain 755 with different concentrations of

nisin (25 to 200 IU/ml). Transformants were obtained with every concentration used, and they showed a stable Nis^r phenotype under nisin selection pressure. Transformation frequency was determined to be in the range of 10^3 to 10^4 transformants/µg DNA, depending on the nisin concentration used. Plasmid content was verified by plasmid isolation, restriction analysis, and agarose gel electrophoresis. The expression of *nisI* in *Lb. plantarum* was assessed by Western analysis to be at the same level as in *L. lactis*.

After the primary selection, transformants could be grown with higher nisin concentrations (200 IU/ml). However, selection with 200 IU nisin/ml resulted in a 10-fold decrease in transformation frequency (10^3 colonies/µg DNA). Wessels (1993) has described a similar phenomena in nisin selection with the plasmid pVS40 in *L. lactis*. Although the *nsr*-mediated resistance level was fairly high, the primary selection with higher nisin concentration was unsuccessful. Another remarkable phenomenon was observed with *Lb. plantarum*: it developed high resistance to nisin. If the cells had not been grown with nisin, they were sensitive to nisin. When the cells were grown on low or moderate nisin concentrations, they gradually became nisin resistant. Finally, when the strain was tolerated to nisin for a few days, not even the highest nisin concentration tested, 4000 IU/ml, retarded its growth. Similar observations have been described with other bacteria, such as *Lactobacillus casei* (Breuer and Radler 1996), and *Streptococcus bovis* (Mantovani and Russell 2001). In both cases, the cells were initially nisin sensitive, but acquired resistance when grown on sublethal concentrations of nisin. In *Lb. casei*, nisin induced the production of anionic, phosphate-containing polysaccharides, whereas in *S. bovis*, nisin resistance was connected with the increased level of lipoteichoic acids. In *S. bovis*, the resistance remain permanent after the cells had been tolerated with nisin, in contrast to *Lb. casei*, which became nisin sensitive if grown overnight in a nisin-free medium. The loss of resistance in *Lb. plantarum* observed here was not tested. However, the electroporation of pLEB590 into *Lb. plantarum* showed that NisI functions as an immunity protein in lactobacilli enabling nisin selection.

2.1.5. Transfer of pLEB590 into industrial *L. lactis* starter (III)

To evaluate the applicability of pLEB590, it was transferred into the industrial cheese starter strain *L. lactis* SSL110 (Valio Ltd.), which carries several indigenous plasmids. Plasmid DNA from transformant colonies was isolated and separated in agarose gel. The transferred pLEB590 displaced a small cryptic plasmid (approximately 2 kb), suggesting that they belonged to the same compatibility group (Fig. 8). No phenotypic effect was observed resulting from loss of the cryptic plasmid. The lost plasmid was the smallest among the indigenous plasmids, and it probably contained only DNA needed for its own replication. Since most wild-type LAB strains carry several plasmids (Davies and Gasson 1981; McKay 1983), incompatibility between foreign and indigenous plasmids is expected. Under the selection pressure, the foreign plasmid is more competitive and displaces the plasmids of the same compatibility group.

2.1.6. Cloning of chromosomal fragments (unpublished)

The suitability of pLEB590 for use as a cloning vector was examined. Plasmid pLEB590 provided a higher level of nisin resistance than the previously described *nisI* expression from plasmids pNZ9031 (Kuipers et al. 1993) and pLEB415 (Qiao et al. 1995), probably because of the higher plasmid copy number resulting in a higher gene dosage, thus increasing the expression level of the *nisI* gene.

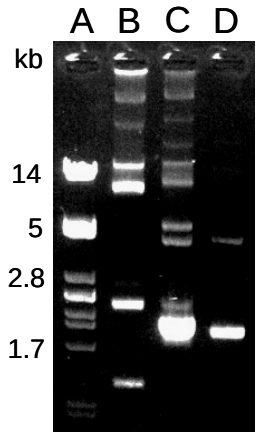

Fig. 8. Plasmid incompatibility caused by pLEB590 in the cheese starter strain *L. lactis* SSL110. Lanes: **A**, DNA size marker λ*Pst*I; **B**, Plasmid profile of *L. lactis* SSL110; **C**, Plasmid profile of *L. lactis* SSL110 transformed with pLEB590; **D**, Purified pLEB590 from LAC233. Transferred pLEB590 in lane C has displaced the smallest plasmid of SSL110 seen in lane B.

Copy number of RCR plasmids is inversely proportional to the plasmid size (Kiewiet et al. 1993; Smith and Bidochka 1998). Therefore, increasing the size of the plasmid by cloning a gene could decrease nisin resistance, thus, potentially making nisin selection impossible. To test the usefulness of pLEB590 for gene cloning, random *Sau*3A fragments from *L. lactis* N8 chromosomal DNA were ligated with *Bam*HI restricted pLEB590. Ligation mixture was electroporated into *L. lactis* MG1614, and transformants were selected on nisin plates. For plasmid/insert analysis, 100 transformant colonies were chosen randomly, plasmids were isolated and analysed in agarose gel. Larger plasmids than the vector pLEB590 were chosen for *Sau*3AI and *Bss*HII restriction analysis. Out of 100 colonies, 29 were found to contain inserts, sizes varying approximately from 0.2 to 2 kb. To sum up, pLEB590 is a suitable vector for gene cloning.

2.2. Complementary food-grade system based on lactose selection (IV)

Lb. casei is a major group of indigenous non-starter lactic acid bacteria (NSLAB) present in cheese (Jordan and Cogan 1993; McSweeney et al. 1993). Native NSLAB strains of *Lb. casei* as adjunct starters have been shown to have a favourable effect on cheese quality (Broome et al. 1990; Swearingen et al. 2001). The aim of this work was to construct a food-grade cloning system for NSLAB *Lb. casei* strain. Since the potential strains of NSLAB *Lb. casei* could not be transformed with the *nisI* vector pLEB590, a new food-grade cloning system had to be developed.

The objective was to construct a complementary system based on lactose selection. The work was preceded by the screening of several NSLAB isolated from high-quality cheese for their suitability as complementation hosts. A *Lb. casei* strain named “E” was found to be the most promising because of its strong and stable lactose utilization via a phosphoenolpyruvate-dependent phosphotransferase system.

2.2.1. Localization of lactose operon in *Lb. casei* strain E

The lactose phosphotransferase operon in the cheese isolate *Lb. casei* strains have been shown to be located either in the chromosome or in large plasmids (Chassy and Alpert 1989). To localize the lactose operon in *Lb. casei* strain E, total DNA was isolated and separated in agarose gel. DNA was transferred to a nylon membrane and hybridized with *lacG* gene as a probe. The *lacG* probe hybridized with a large plasmid and with the chromosomal DNA. The latter signal was probably due to the degradation of large plasmids during DNA purification steps. Thus, we concluded that the lactose utilization in *Lb. casei* E is a plasmid-associated trait. The size of the lactose plasmid was estimated to be at least 30 kb.

2.2.2. Construction of internal deletion in *lacG* by gene replacement

For constructing lactose-deficient complementation host from *Lb. casei* E, the objective was to inactivate the plasmid-encoded *lacG* gene by an internal 141-bp deletion via gene replacement. The integration plasmid was constructed in *E. coli* from the vector pLS19 containing a pUC19 replicon and the erm resistance gene *ermC*. The 1425-bp *lacG* gene was cloned into pLS19, and a 141-bp *Sph*I-*Cla*I fragment in the middle of the cloned *lacG* was then restricted. The *lacG* gene containing the internal deletion was subsequently designated Δ*lacG*. This integration plasmid, pVS88, was transferred into *Lb. casei* E. SCO recombinants were selected with erm. Since the cells of the integrant clone contained multiple copies of the lactose plasmid, the recombinant copy of the plasmid had to be enriched. Therefore, the cells were grown for approximately 100 generations in a medium containing erm. After enrichment, the lactose utilization of the integrant was determined. The integrants showed no acid production from lactose on a lactose-indicator plate, which indicated that there were no copies of the intact lactose plasmid left in the cells. Apparently, the integrated pVS88 in the lactose operon hampered the transcription of the lactose operon causing a Lac^- phenotype.

Next, the integrant was grown another 100 generations in a non-selective medium for the second crossing-over between the homologies of the Δ*lacG* and *lacG* genes, leading to gene replacement. Then, cells were plated onto a non-selective MRS agar and screened for Erm^S Lac^- colonies. The 141-bp deletion in the *lacG* gene of the Erm^S Lac^- colonies was confirmed by PCR (Fig. 9), restriction analysis, and Southern blotting with a *lacG* probe. The Δ*lacG* gene, generated into the plasmid-associated lactose operon, encoded an inactive phospho-β-galactosidase (P-β-Gal) enzyme, resulting in a lactose-negative phenotype. The Δ*lacG* strain was designated as EΔ*lacG*.

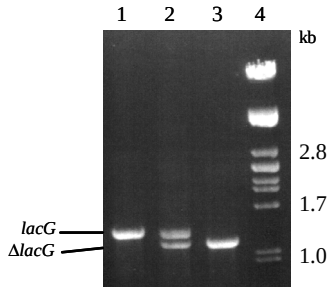

Fig. 9. Confirmation of *lacG* deletion by PCR with *lacG* primers in *Lb. casei* E variants. Lanes: **1**, *lacG* from *Lb. casei* E; **2**, *lacG* and Δ*lacG* from *Lb. casei* E with pVS88 integrated in the lactose plasmid; **3**, Δ*lacG* from *Lb. casei* EΔ*lacG*; **4**, DNA size marker λ*Pst*I.

Gene replacement in a multi-copy plasmid has several difficulties, since no selection method exists for the second homologous recombination, and the desired phenotype is not achieved if any copies of the intact plasmid is retained in the cell. This study showed for the first time that gene replacement is a possible method for the construction of knock-out mutations in multi-copy plasmids. Independently, at the same time (January 2003), another study using gene replacement in a plasmid was published (Cotter et al. 2003). In that study, lacticin 3147 gene in a 60-kb conjugative plasmid of *L. lactis* was deleted by DCO resulting in a strain unable to produce lacticin 3147, but containing the genes involved in the biosynthesis and immunity.

2.2.3. Complementation of the *lacG* deletion by food-grade vector

To complement the Lac$^-$ phenotype of the EΔ*lacG* strain, a *lacG* expression plasmid was constructed. The plasmid was constructed out of three DNA-fragments from different food-grade organisms: the *lacG* gene from *Lb. casei*, the pSH71 replicon from *L. lactis*, and the constitutive *pepR*-promoter (P_{pepR}) from *Lb. rhamnosus* for *lacG* expression. The fragments were ligated and transferred into *Lb. casei* EΔ*lacG* with lactose selection. Colonies obtained on lactose plates were picked, and the utilization of lactose was confirmed on indicator plates. The resulting plasmid was designated pLEB600. Construction of pLEB600 is shown in Fig. 10. The size of the plasmid is 3.6 kb, and it is constructed entirely of DNA from food-grade organisms, and is thus regarded as a food-grade plasmid.

The segregational and structural stability of pLEB600 was then evaluated. *Lb. casei* EΔ*lacG*(pLEB600) was grown in non-selective broth (MRS). After 170 generations in a non-selective medium, no plasmid loss was observed. Analysis of the plasmid content of 20 colonies showed no structural rearrangements. Thus, pLEB600 was shown to retain stably in *Lb. casei* EΔ*lacG* without selection pressure. Most if not all wild-type *Lb. casei* strains contain endogenous plasmids (Chassy et al. 1976; Lee-Wickner and Chassy 1985). Also *Lb. casei* E carries several plasmids of different sizes. Transformation with pLEB600 displaced the smallest (approximately 2 kb) cryptic plasmid. Apparently, the smallest endogenous plasmid of *Lb. casei* E belonged to the same compatibility group as the food-grade plasmid. Posno et al. (1991b) have also described plasmid incompatibility when small vectors were transferred into *Lb. casei*, *Lb. pentosus*, and *Lb. plantarum*. In their study, some of the introduced plasmids were completely lost after 100 generations in the absence of a selective agent, while some plasmids were perfectly stable.

Other food-grade cloning systems based on lactose selection have exploited *lacF* gene encoding lactose-specific phosphocarrier protein EIIA as a complementary selection marker (MacCormick et al. 1995; Platteeuw et al. 1996; Xiang et al. 2003). In lactose-PTS operon, *lacF* is the shortest gene (de Vos and Vaughan 1994), thus minimizing the size of the cloning vector. As a general rule, transformation frequency is inversely proportional to the size of the transferring DNA (Somkuti and Steinberg 1988; Gasson and Fitzgerald 1994). Hence, the 0.3 kb *lacF* would appear to be a more reasonable choise for a marker gene than 1.4 kb *lacG*. On the other hand, in some studies plasmid size from 4.4 to 30 kb had no effect on transformation efficiency (Powell et al. 1988; McIntyre and Harlander 1989).

The motives for preferring the *lacG* gene as a marker instead of smaller *lacF* were connected to the detection of the gene expression. When this work was started, there was no knowledge about promoter functions in *Lb. casei*. To ensure the functionality of the expressed protein and the promoter, the expression had to be possible to detect in some other species, preferably in *L. lactis*. With *lacF* this was not possible, since EIIA could not be conveniently detected in *L. lactis*. In contrast, the activity of P-β-gal encoded by *lacG* was simply detected with a chromogenic substrate. The lactococcal promoter P45 (Koivula et al. 1991; Sibakov et

al. 1991) was first used for the expression of *lacG*, but no P-β-gal activity could be detected in *Lb. casei*. Later it was ascertained that the promoter P45 is not functional at all in *Lb. casei* E (unpublished results). The promoter P45 was then replaced by the lactobacillar *pepR* promoter (Varmanen et al. 1998), which was found to function in both *L. lactis* and *Lb. casei* E.

The two previously published food-grade systems for *Lb. casei* were both integrative expression systems (Gosalbes et al. 2000; Martín et al. 2000). In these approaches, heterologous genes were integrated into the chromosome of *Lb. casei* via homologous recombination (Gosalbes et al. 2000) or via site-specific recombination (Martín et al. 2000). In integrative systems, a food-grade selection marker is not needed, because the marker gene is not present in the final strain construction, and the integrants can be selected with antibiotics. Thus, pLEB600 represents the first vector with a food-grade marker for *Lb. casei*. The superiority of replicative or integrative expression systems depends on the case. Generally, integrative systems are considered more stable, though chromosomes contain destabilizing elements as well, e.g., insertion sequences and group II introns, which render them susceptible to rearrangements (Davidson et al. 1996; Dunny and McKay 1999).

2.2.4. Lactose utilization

To examine the growth on different lactose concentrations simulating the conditions during cheese-making, *Lb. casei* strains E, Δ*lacG*, and Δ*lacG*(pLEB604) were grown for 48 hours in modified MRS broth supplemented with 0.5% glucose, or 0.1 to 1% lactose. The plasmid pLEB604 contains the proline iminopeptidase gene *pepI* cloned into pLEB600 (see next section 2.3. for construction of pLEB604).

Glucose supported growth of all three strains. The PepI expression strain and the wild type strain grew equally well on every lactose concentration examined. The Δ*lacG* strain did not ferment lactose, as already seen in previous experiments. Depending on the conditions in a cheese curd, e.g., salt concentration, lactose is either exhausted in approximately one week by starter bacteria, or else low concentration (0.1-0.5%) of lactose remain in the curd and is degraded later by NSLAB (Turner and Thomas 1980). The main energy source used by NSLAB for growth has not been clearly defined (Beresford et al. 2001). However, efficient utilization of lactose increases the competitiveness of the adjunct NSLAB and intensify their contribution to the ripening process. Therefore, the capacity to grow at low lactose concentration is a clear advantage compared to the previously published food-grade system for *Lb. casei*, which was unable to do so (Gosalbes et al. 2000).

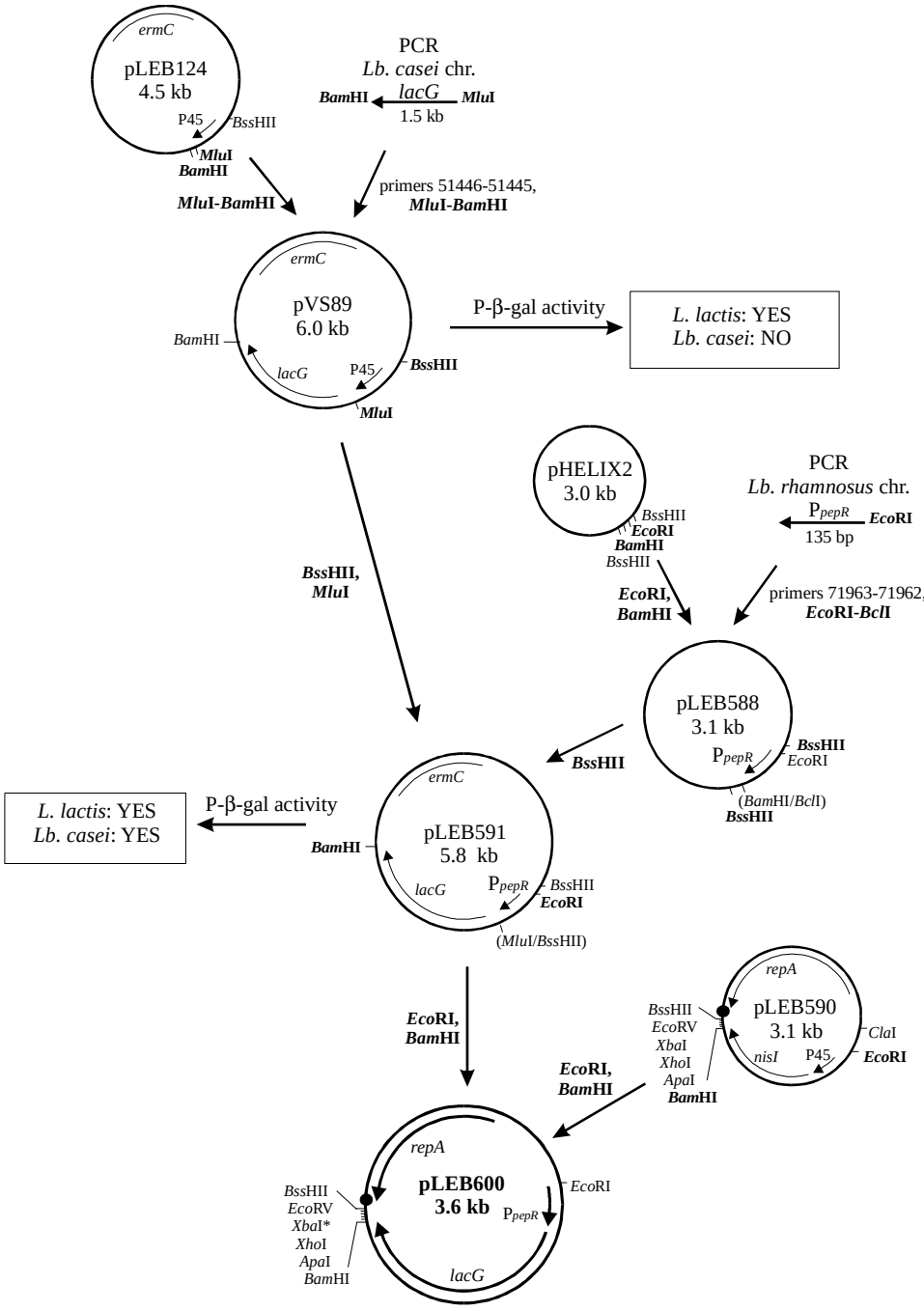

Fig. 10. Schematic representation of pLEB600 construction. Plasmids: pLEB124, lactococcal expression vector (Qiao et al. 1995); pHELIX2, *E. coli* cloning vector. The P*pepR* PCR-fragment contained an internal *Bcl*I restriction site. Black circle represents a transcription terminator. * = not a single site.

2.3. Applicability of the food-grade vectors: Expression of proline iminopeptidase gene *pepI* in *L. lactis*, *Lb. plantarum*, and *Lb. casei* (III, IV)

The applicability of the food-grade vectors pLEB590 and pLEB600 for expression of heterologous genes in LAB was demonstrated by cloning the proline iminopeptidase gene *pepI* from *Lb. helveticus* (Varmanen et al. 1996) as a 1-kb *Bam*HI-*Xho*I PCR fragment resulting in plasmids pLEB605 and pLEB604, respectively. The marker genes *nisI* in pLEB590 and *lacG* in pLEB600 lack transcription terminators allowing read-through expression of promoterless genes cloned downstream of the markers.

The *pepI* expression plasmid pLEB605 was introduced into *L. lactis* MG1614 and *Lb. plantarum* 755, and pLEB604 into *Lb. casei* EΔ*lacG*. The activity of PepI was determined from the transformants and compared with the plasmid-free host strains and the *pepI* origin strain of *Lb. helveticus*. The host strains were practically deficient in PepI. Expression of *pepI* from pLEB605 resulted in approximately 30 to 40 times higher PepI activity in *L. lactis* and *Lb. plantarum* compared to *Lb. helveticus* (Fig. 11). PepI production from pLEB604 in *Lb. casei* resulted in a 3 to 4 -fold higher PepI activity compared to *Lb. helveticus* (Fig. 11).

The higher PepI activity in pLEB605-transformed *L. lactis* and *Lb. plantarum* than in pLEB604-transformed *Lb. casei* is most probably explained by the higher expression level of the *pepI* gene. A possible reason for the difference in *pepI* expression levels is the copy numbers of the plasmids. The precise copy numbers of the food-grade plasmids were not determined, but according to gel electrophoresis after plasmid isolations, both plasmids appeared to have high copy numbers (data not shown). The copy numbers of pSH71-based high-copy plasmids of sizes between 4 to 6 kb have been shown to vary from 54 to 102 per cell in *L. lactis* (Gasson and Anderson 1985; de Vos 1986). Possibly, pLEB605 in *L. lactis* and *Lb. plantarum* had higher copy number than pLEB604 in *Lb. casei* resulting in higher gene dosage leading to the higher level of gene expression. Yet, the ten-fold difference in PepI activity between pLEB604 and pLEB605 most likely requires additional explanations. The P_{pepR} promoter may be weaker in *Lb. casei* than the promoter P45 in *L. lactis* and *Lb. plantarum*. Another explanation is that the *nisI-pepI* transcript is more stable than the *lacG-pepI* transcript, or that the *pepI* located downstream of *lacG* is not transcribed effectively. The *lacG* gene is twice as long as the *nisI* gene, which may affect mRNA stability or efficiency of *pepI* transcription.

Peptidases have a significant role in composing the combination of peptides and free amino acids in cheese, thus contributing to the flavour formation, texture, and ripening (Visser 1993; Christensen et al. 1999). Cheese maturation is a slow and expensive process, which could be accelerated by increasing the production of peptidases, or further, by lysing starter cells releasing the intracellular peptidases into the cheese matrix. Overexpression of several lactococcal peptidase genes in Cheddar starters, at least *pepN* and *pepC*, have been shown to have a positive effect on cheese flavor (Guldfeldt et al. 2001). In addition, lactobacillar peptidases, including PepI, have been successfully heterologously overexpressed in *L. lactis* (Wegmann et al. 1999; Luoma et al. 2001; Joutsjoki et al. 2002). The peptidases PepQ, PepX, and PepW have been identified to be efficient enzymes in increasing the level of free amino acids at the end of cheese ripening (Courtin et al. 2002). Lysis of the cheese starter cells have been shown to have a direct impact on the degree of proteolysis (Valence et al. 2000). Systems for enhanced lysis of starter cells have been developed, including overproduction of autolysin AcmA (Buist et al. 1997), use of a bacteriocin lacticin 481-producing adjunct culture in cheesemaking (O´Sullivan et al. 2003a), and nisin-inducible expression of enterolysin A in *L. lactis* (Hickey et al. 2004).

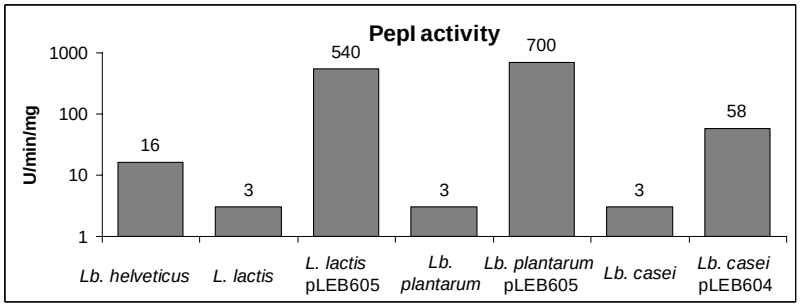

Fig. 11. Proline iminopeptidase activity in *Lb. helveticus*, *L. lactis*, *Lb. plantarum*, and *Lb. casei* strains. Cells were lysed by freezing in liquid nitrogen followed by sonication. Reaction mixtures (3 ml) containing 21 μg cell-free protein extract, and 0.55 mM L-proline-*para*-nitroanilide were incubated for 5 minutes at 40°C before measurement with spectrophotometer at 410 nm. Activities were calculated per minute per mg protein. One unit (U) is defined as the amount of enzyme activity producing a variation of 0.01 unit of absorbance at 410 nm.

Bitterness in cheese is connected with partial proteolytic digestion of proline-rich milk proteins α- and β-caseins (Sullivan and Jago 1972; Lemieux and Simard 1992; Broadbent et al. 1998; Broadbent et al. 2002). Proline-containing oligopeptides have been proposed as a cause for the bitter taste in cheese, whereas proline as free amino acid seems to have a sweet taste (Langler et al. 1967; Ishibashi et al. 1988). The liberation of proline from oligopeptides requires proline-specific peptidases (Christensen et al. 1999; Chen et al. 2003). Therefore, bitterness in cheese could be reduced by increasing the activity of proline-specific peptidases in cheese bacteria. The conditions, e.g., pH and salt concentrations in the ripening cheese are different than inside the cell. To have a greater effect on cheese, the peptidases should be functional after cell lysis, i.e., in conditions not optimal for the enzyme. PepI from *Lb. helveticus* has been shown to retain higher activity in conditions simulating cheese maturation than PepX, the other proline-specific peptidase tested (Joutsjoki et al. 2002). As shown here, the food-grade expression plasmids pLEB590 and pLEB600 are promising tools for overexpression of proline-specific peptidases in cheese bacteria.

CONCLUSIONS

In this study, the nisin immunity protein NisI conferring nisin resistant phenotype in *L. lactis* was shown to be secreted to the external environment, and to exist in a lipid-free form in a culture of the wild-type nisin producer *L. lactis* strain N8. The excretion of LF-NisI was demonstrated to increase nisin tolerance suggesting that the secreted form of the protein could have a supportive role in the nisin immunity mechanism. In a strain expressing NisFEG complex for partial nisin immunity, the LF-NisI-mediated nisin resistance was more evident, indicating that LF-NisI assisted the NisFEG transporter in exporting nisin from the cell surface. On the contrary, added exogenously to the growth medium, LF-NisI did not protect the cell against nisin, but it was able to stimulate nisin activity. The physical interaction between NisI and nisin was shown, as well as the weakness of the NisI:nisin complex. The observed enhancement of nisin activity by LF-NisI could be a result of hindering nisin from adsorbing surfaces, thus, increasing the concentration of free active nisin molecules. The mechanism of how NisI protects the cell is still not fully clear. Membrane-bound NisI may hinder nisin from inserting to the membrane, or NisI may assist the NisFEG complex to export nisin from the membrane. LF-NisI may help NisFEG complex as well, either by delivering nisin to the transporter, or functioning as a carrier transferring nisin from the transporter to growth medium.

Secondly, two food-grade expression systems were developed. The gene *nisI*, encoding NisI, was exploited as a dominant selection marker with nisin selection. The constructed plasmid pLEB590 consisting of merely lactococcal DNA could be introduced into an industrial *L. lactis* starter and into a *Lb. plantarum* strain with nisin selection, showing the functionality of nisin immunity in lactobacilli. Plasmid pLEB590 can thus be regarded as a food-grade vector for lactic acid bacteria.

A complementary food-grade expression system was constructed for a wild-type non-starter *Lb. casei* strain based on lactose selection. The plasmid-associated gene *lacG* encoding lactose-degrading enzyme phospho-β-galactosidase was inactivated by gene replacement through two homologous recombination events, resulting in a lactose-negative host strain. Gene replacement was shown to be a useful method for modifying genes in endogenous cryptic plasmids. The *lacG* complementation plasmid pLEB600 was constructed out of DNA from lactic acid bacteria, and is thus regared as a food-grade vector. The *lacG* expression from pLEB600 restored the lactose utilization to the wild-type level.

The applicability of the food-grade vectors was shown by cloning the proline-iminopeptidase gene *pepI* from *Lb. helveticus* into pLEB590 and pLEB600. The expression of *pepI* from the food-grade plasmids in *L. lactis*, *Lb. plantarum*, and *Lb. casei* resulted in high PepI activity in the PepI-deficient host strains. The results demonstrated that the food-grade expression vectors pLEB590 and pLEB600 show potential as tools for overexpressing useful genes in dairy starter bacteria.

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