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Transcriptome Analysis Reveals Mechanisms by Which *Lactococcus lactis* Acquires Nisin Resistance

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Nisin, a posttranslationally modified antimicrobial peptide produced by *Lactococcus lactis*, is widely used as a food preservative. Yet, the mechanisms leading to the development of nisin resistance in bacteria are poorly understood. We used whole-genome DNA microarrays of *L. lactis* IL1403 to identify the factors underlying acquired nisin resistance mechanisms. The transcriptomes of *L. lactis* IL1403 and *L. lactis* IL1403 Nis^r, which reached a 75-fold higher nisin resistance level, were compared. Differential expression was observed in genes encoding proteins that are involved in cell wall biosynthesis, energy metabolism, fatty acid and phospholipid metabolism, regulatory functions, and metal and/or peptide transport and binding. These results were further substantiated by showing that several knockout and overexpression mutants of these genes had strongly altered nisin resistance levels and that some knockout strains could no longer become resistant to the same level of nisin as that of the wild-type strain. The acquired nisin resistance mechanism in *L. lactis* is complex, involving various different mechanisms. The four major mechanisms are (i) preventing nisin from reaching the cytoplasmic membrane, (ii) reducing the acidity of the extracellular medium, thereby stimulating the binding of nisin to the cell wall, (iii) preventing the insertion of nisin into the membrane, and (iv) possibly transporting nisin across the membrane or extruding nisin out of the membrane.

Nisin, a lanthionine-containing peptide produced by certain strains of *Lactococcus lactis* (24), is widely used in the food industry as a safe and natural preservative (11) because of its antimicrobial activity against a broad range of gram-positive bacteria, including *Listeria monocytogenes*. Nisin disrupts the cytoplasmic membrane of a bacterial cell through pore formation, which leads to the release of small cytoplasmic compounds, depolarization of the membrane potential, and, ultimately, cell death (4, 5). The lantibiotic uses lipid II as a docking molecule in the target membrane for efficient pore formation. By the binding of nisin to lipid II, bacterial cell wall synthesis is also inhibited (4, 5), which provides another mechanism of cell killing (59).

The efficiency of nisin as an antimicrobial agent could be seriously compromised by the occurrence of nisin resistance in spoilage or pathogenic bacteria. The generation of nonstable nisin-resistant (Nis^r) strains of *L. lactis* under laboratory conditions is achieved relatively easily by stepwise exposure of cells to increasing concentrations of nisin (20, 39, 42). Since nisin sensitivity and the lipid II content in the cytoplasmic membrane are not directly correlated (29), nisin resistance is expected to be achieved through another mechanism(s).

Nisin resistance in bacteria has been associated with an altered fatty acid composition of phospholipids (39, 42) or an altered phospholipid composition of the cytoplasmic membrane (41). Lipoteichoic acid (LTA) was shown to play an important role in the nisin resistance of *Staphylococcus aureus* and *Streptococcus bovis* (38, 45). An *S. aureus* strain containing several copies of the *dlt* operon (the gene products of which are involved in the synthesis of D-alanyl esters for substitution in LTA, resulting in the incorporation of positive charges) (10) was less sensitive to various antimicrobial peptides, including nisin (45). Nisin-resistant cells of *S. bovis* had more LTA in their cell walls than did nisin-sensitive cells (38). Another study showed that the cell wall can be a barrier for nisin to reach lipid II since protoplasts of a *Micrococcus flavus* Nis^r strain that is 125-fold more resistant than its parent were almost as sensitive to nisin as were protoplasts of the parental strain (29). In a spontaneous nisin-resistant strain of *L. monocytogenes*, the expression level of a putative penicillin binding protein (PBP) was significantly increased (18). This spontaneous Nis^r strain was sensitive to different beta-lactam antibiotics, which bind to PBPs, and also showed a slight decrease in sensitivity to mersacidin, a lantibiotic that, like nisin, binds to the lipid II molecule (18).

From the literature cited above, it can be concluded that nisin resistance in various organisms can be acquired through alterations in the expression of genes that are involved in cell wall and cytoplasmic membrane biosynthesis. However, a concise picture of all of the factors involved in nisin resistance in a single strain is not available, nor is it known whether the bacterial cell employs one or more strategies simultaneously to acquire nisin resistance.

In this study, we set out to investigate the nisin resistance mechanisms employed by the model bacterium *L. lactis* IL1403 by using DNA microarrays containing amplicons of 2,108 genes (>96% of all annotated genes) of *L. lactis* IL1403. Transcriptomes of *L. lactis* IL1403 and *L. lactis* IL1403 Nis^r, a nisin-adapted strain that is about 75 times more resistant to

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype or genotype	Source or reference
L. lactis IL1403	Plasmid-free laboratory strain	8
L. lactis IL1403 Nis^r	Nis^r	This work
L. lactis MG1363	Derivative of *L. lactis* NCD0712	14
L. lactis MG1363Δ*dltD*	Derivative of *L. lactis* MG1363 carrying a deletion in *dltD*	13
L. lactis MG1363Δ*ahrC*	Derivative of *L. lactis* MG1363 carrying a deletion in *ahrC*	34
L. lactis NZ9000	*L. lactis* MG1363 *pepN*::*nisRK*	31
L. lactis NZ9000Δ*galAMK*	Derivative of *L. lactis* NZ9000 carrying a deletion in *galAMK*	R. A. Neves and W. A. Pool lab collection
pNZ8048	Gene expression vector with nisin-inducible P_{nisA} promoter, Cm^r	11
pJK1	pNZ8048 derivative; *yneGH* controlled by P_{nisA}	This work
pFaB	pNZ8048 derivative; *ysaB* controlled by P_{nisA}	This work
pFaBC	pNZ8048 derivative; *ysaBC* controlled by P_{nisA}	This work

nisin than is its parent, were compared. The involvement of a selection of genes and their specific contribution to nisin resistance was further investigated and corroborated by analyzing knockout and overexpression mutants of these genes of *L. lactis*.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and molecular cloning techniques. The strains used in this study are presented in Table 1. All strains were grown at 30°C in M17 broth (52) with 0.5% glucose (GM17) and appropriate antibiotics (chloramphenicol at 5 μg/ml and nisin at 3,000 μg/liter when using the Nis^r strain). GM17 plates contained 1.5% agar. Molecular cloning techniques were performed essentially as described by Sambrook et al. (50). Restriction enzymes, deoxynucleotides, and T4 ligase were obtained from Roche Diagnostics (Mannheim, Germany) and used as specified by the supplier. *L. lactis* NZ9000 was transformed by electroporation by using a gene pulser (Bio-Rad Laboratories, Richmond, Calif.) as described earlier (35, 62). Plasmid isolation was performed according to the method described by Birnboim and Doly (2).

Generation of nisin-resistant variants of *L. lactis* IL1403. A nisin stock solution was derived from nisaplin (2.5% nisin; Aplin and Barrett, Danisco, Denmark) as described earlier (47). Nisin-resistant isogenic variants of *L. lactis* IL1403 were obtained by growing the strain in broth while stepwise increasing the nisin concentration as described before (29). The resulting strain lost its resistant phenotype almost completely after overnight growth in a medium without nisin.

Construction of the *L. lactis* IL1403 DNA microarray. DNA microarrays of *L. lactis* IL1403 used in this study were constructed essentially as described before (30, 56) and contained amplicons of 2,108 genes of *L. lactis* IL1403.

RNA isolation and cDNA labeling. Cells of a culture in mid-log phase (optical density at 600 nm [OD_{600}] of 0.7) were harvested by centrifugation at 8,000 × *g* for 1 min and immediately frozen in liquid nitrogen. Subsequently, cDNA was obtained and labeled as described before (56). Three biological replicates with dye swaps were performed under identical conditions (56). Hybridization was performed at 42°C as described before (56).

Bioinformatic analyses. Spots were quantified with Array Pro analyzer 4.5 (MediaCybernetics, Gleichen, Germany). Slide data were processed by using MicroPreP as follows (9, 57): (i) flagged (bad) spots were deleted, (ii) spot backgrounds in each grid for both channels were corrected for autofluorescence by subtracting the intensity of the weakest spot, and (iii) normalization (the ratios were made comparable across slides) was done using a grid-based Lowess transformation (61) (fraction of genes to use, 0.5). Differential expression tests were performed with a locally running copy of the Cyber-T implementation of a variant of the *t* test (37). False discovery rates were calculated by (i) ranking the genes by *P* value, (ii) multiplying the *P* values with the number of tests performed (Bonferroni correction), and (iii) dividing the resulting *P* values by the number of genes with lower *P* values. Genes were considered differentially expressed at *P* values of <0.01 and with a false discovery rate of <0.01.

Availability of array data. The TIFF files that were generated by the scanning of the hybridized slides and the tables containing expression data after normalization are available at http://molgen.biol.rug.nl/publication/nis_data/. The MicroPreP software used for normalization and data handling can be requested at http://molgen.biol.rug.nl/molgen/research/molgensoftware.php.

Construction of plasmids pJK1, pFaB, and pFaBC for expression of *yneGH* and *ysaBC*. The primers used for PCR amplification of *yneGH* and *ysaBC* from the genomic DNA of *L. lactis* IL1403 are presented in Table 2. PCRs were performed with Expand polymerase (Roche Diagnostics) in accordance with the manufacturer's instructions. DNA amplification consisted of 1 cycle at 95°C for 4 min and 30 cycles at 95°C for 1 min, 52°C for 2 min, and 68°C for 1 min, followed by an additional cycle of 10 min at 68°C. The PCR products were digested with BsaI and XbaI (Table 1) and ligated into pNZ8048 cut with the same enzymes. The resulting plasmids, pJK1, pFaB, and pFaBC, were introduced into *L. lactis* NZ9000, which was also used as the nisin-inducible expression host. The expression of the cloned genes from the nisin-inducible P_{nisA} promoter in pJK1, pFaB, and pFaBC after induction with subinhibitory amounts of nisin for 2 h (11) was verified by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (33).

Nisin sensitivity assays. Sensitivity to nisin was determined by using a microtiterplate assay essentially as described before (32) for the strains *L. lactis* NZ9000, *L. lactis* NZ9000Δ*galAMK*, *L. lactis* MG1363, and *L. lactis* MG1363Δ*dltD* (13). The cells of *L. lactis* NZ9000 (pNZ8048) and *L. lactis* NZ9000 (pJK1) were diluted 50-fold in fresh medium. At an OD_{600} of 0.2, the cells of both cultures were induced with a subinhibitory concentration of nisin (4 μg/liter) for 2 h, which is different from a normal sensitivity assay, to allow for the transcription of the genes of interest. Subsequently, nisin sensitivity was tested and outgrowth in all wells was determined at the moment when wells containing cells without nisin had reached an OD_{595} of

TABLE 2. Primers used in this study[a]

Primers	Sequence
yne forward	5′-CCCCGGTCTCCCATGATTAAAATCTACC-3′ (BsaI site is underlined.)
yne reverse	5′-CTAGTCTAGATTACTGACAAGAACAGTCC-3′ (XbaI site is underlined.)
ysa forward	5′-CCCCGGTCTCCCATGAATTTATCAAGAAAACATAAGG-3′ (BsaI site is underlined.)
ysaB forward	5′-CCCCGGTCTCCCATGCTTAGTTTAAAACTTGCGGC-3′ (BsaI site is underlined.)
ysaBC forward	5′-CATGCCATGGGCGCTTGCCTCCTTTATTCC-3′ (NcoI site is underlined.)
ysaC reverse	5′-GCTCTAGATTTAAACTAAGCATTTAAGG-3′ (XbaI site is underlined.)
ysa reverse	5′-CATGTCTAGACTTTATCTTTCAACAATTTTGTAGTAAACA-3′ (XbaI site is underlined.)

[a] The source or reference for each primer is this work.

TABLE 3. MICs for nisin and other bacteriocins of various *L. lactis* derivatives used in this study[a]

L. lactis strain/antimicrobial	MICs (μg/liter) for:			
	Nisin	Mersacidin	Vancomycin	Bacitracin
IL1403	40	2,500	600	4,000
IL1403 Nis^r	3,000	2,500	600	1,000
NZ9000[b]	40	–[c]	–	–
NZ9000 (pNZ8048) with induction	40	–	–	–
NZ9000 (pJK1) with induction	500	–	–	–
NZ9000 (pFaB)	320	–	–	–
NZ9000 (pFaBC)	320	–	–	–
NZ9000 (Δ*galAMK*)	27	2,500	–	–
NZ9000 (Δ*galAMK*, Nis^r)	400	–	–	–
MG1363	40	–	–	–
MG1363 (Nis^r)	3,000	–	–	–
MG1363 (Δ*dltD*)	8	2,500	–	–
MG1363 (Δ*dltD*, Nis^r)	400	–	–	–
MG1363 (Δ*ahrC*)	8	2,500	–	–
MG1363 (Δ*ahrC*, Nis^r)	200	–	–	–

[a] Standard errors never exceeded 10% of the given value.
[b] Strain *L. lactis* NZ9000 (MG1363::NisRK) is an isogenic derivative of *L. lactis* MG1363.
[c] –, not determined.

0.8, as measured in a microtiterplate reader (Spectramax plus 384; Molecular Devices, Wageningen, The Netherlands). MICs were calculated from the lowest concentration of nisin at which the growth of the test strain was inhibited (32).

RESULTS

Genome-wide identification of genes involved in nisin resistance in *L. lactis* IL1403. A nisin-resistant *L. lactis* IL1403 strain was obtained by consecutively growing the wild-type strain in the presence of increasing nisin concentrations in the medium. This approach was chosen to maintain high resistance since nisin resistance is easily lost. The final *L. lactis* Nis^r culture, which could grow in the presence of 3,000 μg nisin per liter, was colony purified, grown in the presence of the appropriate nisin concentration, and stored at −80°C. *L. lactis* IL1403 Nis^r was 75 times more resistant to nisin than was its parent (Table 3). In all experiments, this strain was grown in the presence of 3,000 μg nisin/liter since we observed a reversal to almost wild-type sensitivity after overnight growth without nisin.

Global gene expression patterns of *L. lactis* IL1403 Nis^r were compared to those of the parent strain using in-house-developed DNA microarrays of *L. lactis* IL1403 (30, 56). The data presented here are the means from three independent biological replicates. Various differentially expressed genes were identified in the Nis^r strain. Sixty-two genes were more highly expressed in the *L. lactis* Nis^r strain, corresponding to 2.9% of all genes (Table 4), and 31 were expressed to a lower extent in the *L. lactis* Nis^r strain, which corresponds to 1.5% of all genes (Table 5). The *P* values in a paired *t* test, which is a *t* test variant implemented in the Cyber-T software (1), ranged from 10^{-4} to 10^{-8} for the differentially expressed genes. The identified genes specify proteins that belong to seven functional groups, namely, cell wall synthesis, central and energy metabolism, fatty acid and phospholipid metabolism, regulatory functions, transport and binding proteins, stress-related proteins, and miscellaneous and unknown proteins (Tables 4 and 5). The finding that several genes involved in cell wall synthesis, such as those in the *dlt* operon, *nagA* and *pbp2A*, are expressed to a higher extent in the nisin-resistant strain supports our hypothesis (29) and existing literature (18, 45) stating that the constitution of the cell wall is very important in the development of nisin resistance in bacteria.

To understand the individual impact of a number of genes that are retrieved by the transcriptome analysis, these genes were either deleted or overexpressed and the resulting strains were compared to their parents with respect to nisin sensitivity. Moreover, some of the knockout strains were further tested for their ability to become resistant again to nisin by the same method as described above.

The importance of the *dlt* operon in the acquisition of nisin resistance in *L. lactis*. The *dlt* operon comprises *dltABCD* and is involved in the synthesis of D-alanyl esters for substitution in LTA (10). Only transcription of the *dltC* gene was nine times higher in *L. lactis* IL1403 Nis^r than in *L. lactis* IL1403. The other three genes were not tested because they were not present on the DNA microarray (Table 4). However, an upgraded DNA microarray containing all *dlt* genes revealed all *dlt* genes to be expressed to a significantly higher extent in the *L. lactis* Nis^r strain (data not shown). In the literature, a strain of *L. lactis* MG1363 has been described in which the *dltD* gene has been deleted (13). Nisin sensitivity of *L. lactis* MG1363Δ*dltD* was compared to that of *L. lactis* MG1363 to gain insight in the contribution of the gene *dltD* in acquired nisin resistance in *L. lactis*. The MIC of *L. lactis* MG1363Δ*dltD* was fivefold lower than that of *L. lactis* MG1363 (Table 3), showing the involvement of *dlt* expression in nisin resistance. To examine whether a strain carrying a mutation in *dltD* could still become resistant, we tried to make Nis^r derivatives of this strain by the method employed for *L. lactis* IL1403 (see Materials and Methods). As this was unsuccessful in the first trial, the procedure was altered to include successive steps of nisin at 10, 20, 60, and 80 μg/liter and then higher concentrations to try to adapt *L. lactis* MG1363Δ*dltD* to nisin. In three such independent trials, nisin resistance in *L. lactis* MG1363Δ*dltD* reached a plateau at 400 μg of nisin per liter, which is just 8 times the original MIC of *L. lactis* MG1363, in contrast to 75 times the MIC of the wild-type MG1363 Nis^r. This result clearly demonstrates the involvement of *dltD* expression in nisin resistance.

Characterization of novel factors involved in nisin resistance in *L. lactis*. The strains *L. lactis* MG1363Δ*ahrC* (34), *L. lactis* NZ9000Δ*galAMK*, and *L. lactis* NZ9000 (pJK1, overexpressing *yneGH*) were further examined with respect to nisin sensitivity.

The *arc* operon. Amplicons of the *arc* genes, which are involved in the breakdown of arginine via the arginine deiminase pathway (48) of both *L. lactis* IL1403 and *L. lactis* MG1363 were present on the *L. lactis* IL1403 DNA microarray used in this study. The obtained cDNA of *L. lactis* IL1403 Nis^r hybridized to the *arc* genes of both *L. lactis* strains, which is in agreement with the fact that the homology of the sequence in the amplicons ranges from 87 to 100%. A fourfold overexpression with both amplicon sets of the *arcAC1C2DT2* genes was recorded for the *L. lactis* Nis^r strain. As we expected the general mechanisms of acquired nisin resistance to be similar in

TABLE 4. Characteristics of significantly overexpressed genes of *L. lactis* IL1403 Nis^r relative to *L. lactis* IL1403[a]

Gene(s)	Avg regulation (*n*-fold)	*P* value	(Proposed) function
Cell wall related			
dltC in operon *dltABCD*[b]	9.4	10^{-8}	D-Alanine incorporation
pbp2A[b]	2.3	10^{-6}	Penicillin-binding protein 2 A
galMKT in operon *galMKTE*[b]	1.7	10^{-4}	Galactose pathway
Central and energy metabolism			
arcAC1C2TD2 in operon *arcABDC1C2TD*[b]	4.0	10^{-7}	Arginine pathway
ypbG next to *ypcABCD*	2.3	10^{-6}	Sugar kinase
nagA	2.2	10^{-7}	*N*-Acetylglucosamine-6-phosphate deacetylase
butA	2.0	10^{-8}	Putative acetoin reductase
lnbA	1.5	10^{-5}	Putative beta-*N*-acetylglucosaminidase
pta	1.9	10^{-7}	Phosphotransacetylase
galKMT in operon *galMKTE*[b]	1.7	10^{-4}	Galactose pathway
gidB	1.6	10^{-7}	Putative glucose-inhibited division protein
apI	1.6	10^{-6}	Alkaline phosphatase
glgD	1.5	10^{-5}	Glycogen biosynthesis protein
Membrane biosynthesis			
IpiL	1.9	10^{-4}	Lipoate-protein ligase A
Regulatory functions			
yecA	2.1	10^{-5}	Transcriptional regulator
rcfB	1.9	10^{-8}	Transcriptional regulator
kinD next to *llrD*	1.9	10^{-7}	Histidine kinase
llrD next to *kinD*	1.7	10^{-5}	DNA-binding response regulator
Transport and binding proteins			
ysaBCD in operon *ysaABCD*[b]	10.1	10^{-8}	Bacitracin resistance proteins
ynhCD	5.5	10^{-9}	Tellurite resistance protein
glpF1[b]	3.4	10^{-7}	Glycerol uptake facilitator
yhcA in operon *yhcACB*	3.4	10^{-4}	Putative ABC transporter
yrjBC in operon *yrjABCDEFGH*	1.9	10^{-7}	Transport permease
ptcA[b]	1.9	10^{-6}	Cellobiose PTS
ypcAGH[b]	1.8	10^{-8}	Sugar ABC transporter
ynaCD in operon *ynaABCDE*	1.6	10^{-4}	ABC transporter (homology to MDR *Enterococcus faecalis*)
glpF2[b]	1.5	10^{-5}	Glycerol uptake facilitator
Stress-related proteins			
ythBA in operon *ythCBA*	6.8	10^{-8}	Phage shock protein
dnaK	2.1	10^{-6}	Heat shock protein
grpE next to *dnaK*	1.8	10^{-6}	Heat shock protein
htrA	1.6	10^{-4}	Serine protease
Miscellaneous and unknown proteins			
yneGH in putative operon *yneBCDEGH*[b]	12.9	10^{-10}	ArsCD (pFam)
yajH in operon *yajFGH*	5.7	10^{-10}	Unknown
yniHIJ	3.5	10^{-4}	Unknown
ybfAC in operon *ybfADEBC*	2.8	10^{-6}	Unknown
yfhC in operon *yfhABCGHI*	2.6	10^{-4}	Unknown
yhjA in operon *yhjABC*	2.3	10^{-5}	Unknown
yvhB	2.2	10^{-4}	Putative acyltransferase
ymgGI	1.9	10^{-4}	Hypothetical proteins
ceo	1.7	10^{-4}	N_5-(1-carboxyethyl)-L-ornithine synthase
pydAB	1.7	10^{-6}	Dihydroorotate dehydrogenase
ispB	1.7	10^{-5}	Heptaprenyl diphosphate synthase
sbcCD	1.6	10^{-5}	Exonuclease

[a] Values represent higher (positive) expression in the *L. lactis* IL1403 Nis^r strain than that in the *L. lactis* IL1403 strain.
[b] Genes further investigated or discussed.

both *L. lactis* subspecies (see the results with the *dlt* mutation presented above), we decided to use knockout mutants in arginine metabolism in *L. lactis* MG1363 (34). The *ahrC* gene encodes a transcriptional regulator (repressor) of the *arc* operon, as was confirmed by DNA microarray analyses: deletion of *ahrC* resulted in only a low expression of the *arc* operon (reduced three- to fivefold) even at a high arginine concentration in the medium (34). Deletion of *ahrC* also resulted in an increase in the expression of the *arg* genes of four- to sixfold compared to that of the parent strain *L. lactis* MG1363 (34). *L.*

TABLE 5. Significantly down-regulated genes found in microarray expression analysis of *L. lactis* IL1403 and *L. lactis* IL1403 Nis^{r}[a]

Gene(s)	Avg regulation (*n*-fold)	*P* value	(Proposed) function
Energy metabolism			
yeeA[b]	−4.5	10^{-9}	Maltose hydrolase
pgmB	−3.4	10^{-7}	Beta-phosphoglucomutase
yedEF[b]	−3.0	10^{-8}	β-Glucoside-specific PTS system
gidA	−1.6	10^{-5}	Glucose inhibited division protein
Membrane biosynthesis			
fabDG1G2Z1Z2[b]	−1.5	10^{-5}	Fatty acid biosynthesis and substrate binding permease
Regulatory functions			
ybdA	−1.5	10^{-4}	Putative transcriptional regulator
glnR	−1.5	10^{-9}	Glutamine synthetase repressor
rmaG	−1.5	10^{-7}	Putative transcriptional regulator
Transport and binding proteins			
yriC in operon *yriDCBA*	−2.2	10^{-5}	Xanthine/uracil permeases
glnP	−2.1	10^{-7}	Glutamine ABC transporter
xpt	−1.7	10^{-9}	Putative xanthine phosphoribosyltransferase
potD	−1.6	10^{-8}	Putative ABC transporter
pbuX	−1.5	10^{-5}	Xanthine permease
dacA	−1.5	10^{-6}	Extracellular protein Exp2 precursor
plpABCD	−1.5	10^{-6}	Outer membrane lipoprotein precursors
Miscellaneous and unknown proteins			
hemH	−1.7	10^{-6}	Ferrochelatase
ribAH in operon *ribGBAH*	−2.7	10^{-6}	Riboflavin biosynthesis protein
rpsN	−2.0	10^{-6}	30S ribosomal protein
aroH	−1.8	10^{-7}	Tyr-sensitive phospho-2-dehydro-deoxyheptonate aldolase
phnA	−1.6	10^{-5}	Uncharacterized Zn-ribbon-containing protein involved in phosphonate metabolism
ynbE	−1.6	10^{-5}	Conserved hypothetical protein

[a] Values represent lower (negative) expression in the *L. lactis* IL1403 Nis^{r} strain than that in the *L. lactis* IL1403 strain.
[b] Genes investigated or discussed.

lactis MG1363Δ*ahrC* is fivefold more sensitive to nisin than is *L. lactis* MG1363 (Table 3), demonstrating the (in)direct involvement of arginine metabolism in nisin resistance. In three independent trials to make a Nis^{r} derivative of *L. lactis* MG1363Δ*ahrC*, the Δ*ahrC* mutant had already reached a plateau at 200 μg/liter nisin, showing the necessity of the presence of at least *ahrC* to reach the highest resistance levels.

The *gal* operon. The transcription of *galMKT* genes was 1.7 times higher in *L. lactis* IL1403 Nis^{r} than in *L. lactis* IL1403 (Table 4). The genes *galMKT* of *L. lactis* MG1363 have a high sequence homology to the genes of *L. lactis* IL1403, ranging from 80 to 93%. Therefore, the use of a deletion mutant in this strain seems justified. In accordance, *L. lactis* NZ9000Δ*galAMK*, an *L. lactis* MG1363 derivative, is twice as sensitive to nisin as its parent (Table 3), demonstrating the involvement of the *gal* operon in nisin resistance. *L. lactis* NZ9000Δ*galAMK* could be made resistant to maximally 400 μg of nisin per liter, which is only eight times the MIC of *L. lactis* NZ9000.

The putative arsenic resistance operon. The genes *yneGH* of *L. lactis* IL1403 have homology to *Escherichia coli arsCD*, which are involved in arsenic resistance. The protein YneG has homology to the *trans*-acting repressor ArsD from *Escherichia coli*, while YneH has homology to the arsenate reductase ArsC (49). Both genes were approximately 13-fold more highly expressed in *L. lactis* IL1403 Nis^{r} than in *L. lactis* IL1403. As it is not known whether they are essential, an *L. lactis* strain was constructed in which the *yneGH* genes could be overexpressed by using the nisin-inducible *nisA* promoter in pNZ8048, as confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (33). The expression of *yneGH* in *L. lactis* NZ9000 (pJK1) was induced by the addition of a subinhibitory concentration (4 μg/liter) of nisin, a concentration that does not lead to nisin stress or nisin resistance development. Sensitivity to nisin, after the induction of *yneGH* in *L. lactis* NZ9000 (pJK1), is clearly less than that of its parent *L. lactis* NZ9000 (Table 3), which shows that these gene products have a protective effect against nisin.

The putative bacitracin resistance (*ysaBCD*) operon. The genes *ysaBCD* of *L. lactis* IL1403 are part of a putative operon of four genes (3). The genes *ysaBC* are orthologs of genes found in *Streptococcus mutans*, namely, *mbrB* (*ysaB*) and *mbrA* (*ysaC*), which are involved in bacitracin resistance (53). Both genes were expressed at a 10-fold higher level in *L. lactis* IL-1403 Nis^{r} (Table 4). The expression of *ysaBC* in *L. lactis* NZ9000 (pFaB and pFaBC) was induced by the addition of a subinhibitory concentration (4 μg/liter) of nisin. Sensitivity to nisin after the induction of *ysaB* and *ysaBC* in *L. lactis* NZ9000 (pFaB and pFaBC, respectively) is clearly less than that of its parent *L. lactis* NZ9000 (Table 3), which shows that these gene products have a protective effect against nisin.

No cross-resistance of *L. lactis* IL1403 Nis^{r} to other lipid II binding peptides. Cross-resistance of *L. lactis* IL1403 Nis^{r} to

the globular lantibiotic mersacidin and to vancomycin was examined. Both peptides recognize specific regions of the lipid II molecule: mersacidin binds to the sugar-pyrophosphate moiety of lipid II (4, 7), while vancomycin binds to the L-Lys-D-Ala-D-Ala moiety of the pentapeptide side chain of lipid II. Mersacidin is a relatively small lantibiotic of 19 residues with no net charge (6), while vancomycin has one positive charge (40). No cross-resistance of the *L. lactis* Nis^r strain was observed for mersacidin and vancomycin (Table 3), indicating that none of the Nis^r mechanisms that are operative in *L. lactis* are involved in resistance against mersacidin and vancomycin. The *L. lactis* Nis^r strain, however, was more sensitive to bacitracin than was *L. lactis* (Table 3) (see Discussion).

DISCUSSION

We identified 92 genes as being directly or indirectly involved in the acquisition of nisin resistance. From these genes, 62 are more highly expressed and 30 are more lowly expressed in *L. lactis* IL1403 Nis^r. A part of the response we observed could be due to general stress rather than resistance, although preliminary studies indicate that most stress responses that were observed after sudden high and short exposures to nisin (<30 min) do not reveal many genes differentially expressed similar to those found in the current study (data not shown). Moreover, changing the expression of selected targets by either knockout mutation severely hampers subsequent resistance development, showing their true involvement rather than a general stress response.

No cross-resistance to vancomycin and mersacidin was observed in *L. lactis* IL1403 Nis^r, which could be explained by the fact that both antimicrobials are less charged than nisin. It is expected that these molecules have less electrostatic interactions with the cell wall than nisin and are probably able to reach lipid II more easily. This also indicates that none of the Nis^r mechanisms operative in *L. lactis* are directly involved in resistance against mersacidin and vancomycin.

The *dlt* and *gal* operons identified as being more highly expressed in *L. lactis* IL1403 Nis^r are both involved in the synthesis of lipoteichoic acid. LTA consists of a glycolipid anchor linked to the cytoplasmic membrane, a polyglycerol phosphate, and various substituents (10). A substituent can be a hydrogen atom, D-alanyl ester, α-glucosamine, or α-galactose. The gene *galE*, being part of the *gal* operon for the Leloir pathway (19), encodes a UDP-glucose 4-epimerase and is responsible for the synthesis of α-galactose. The α-galactose is transported over the membrane and substituted in the LTA. *L. lactis* MG1363Δ*galAMK* was twice as sensitive to nisin as the wild type, suggesting that α-galactose incorporation is important in nisin resistance in the Nis^r strain through its effect on the LTA structure. In fact, LTA of the *L. lactis* Nis^r strain contains twice as much α-galactose as LTA of the wild type, which could indicate a more densely packed LTA (28). The *dlt* operon is responsible for D-alanylation of LTA (10), a process through which positive charges are inserted in the negatively charged LTA in the net negatively charged cell wall (44, 45). The fact that *dltC* is more highly expressed in the *L. lactis* Nis^r strain and involved in nisin resistance is in agreement with the data that have been found for an *S. aureus* strain carrying additional copies of *dlt*: this strain was less sensitive to several antimicrobials, including nisin, than a strain with only one copy of *dlt* on its chromosome (45). The genes *dltA* and *dltC* encode the D-alanine-D-alanyl carrier protein ligase (Dcl) and the D-alanyl carrier protein (Dcp), respectively. DltB and DltD may function in transport and the actual esterification reaction, respectively, and thus introduce positive charges into the cell envelope (44). As nisin is a positively charged peptide, it could be repulsed when LTA becomes more positive and could thus be prevented from reaching the cytoplasmic membrane and lipid II. The LTA of the *L. lactis* Nis^r strain has indeed been shown to contain twice the amount of D-alanyl ester as that in the wild-type strain (28). The involvement of *dlt* in the Nis^r phenotype of *L. lactis* was proven when the first gene of the operon was deleted: *L. lactis* MG1363Δ*dltD* was five times more sensitive to nisin than was *L. lactis* MG1363. Generating a nisin-resistant *L. lactis* MG1363Δ*dltD* strain appeared to be difficult, indicating the importance of the *dlt* operon in the acquisition of nisin resistance.

PBPs are membrane-associated proteins which are present in all eubacteria and divided into two classes. The multidomain PBPs are associated with only transpeptidase (TP) activity (class B), while the bifunctional PBPs are catalyzing both glycosyltransferase and TP reactions (class A) (for a review, see reference 16). Class B PBPs, among which is PBP2A, have been found to play unique roles in the septation and regulation of the shape of bacterial cells (23). PBPs are involved in the second stage of peptidoglycan biosynthesis, following the formation of lipid II. This stage involves strand elongation and cell wall chain cross-linking. Strand elongation is a step that is catalyzed by glycosyltransferase enzymes (54), while peptidoglycan chain cross-linking is performed by TP enzymes via an interpeptide bridge formation (15). The data presented above suggest that *pbp2A* could be part of a common nisin resistance mechanism in bacteria. Altered expression of *pbp2A* may affect the composition of the bacterial cell wall, thereby altering the sensitivity of the bacterium to nisin, preventing nisin from reaching the lipid II molecule. Indeed, we have recently observed that the cell wall of the *L. lactis* Nis^r strain is locally thickened at cell division sites (28) and *pbp2A* expression was twice as high in this strain relative to *L. lactis* IL1403. In a previous study by Gravesen et al. (18), it was shown that the expression of a putative PBP was higher in a nisin-resistant mutant of *Listeria monocytogenes*.

The gene *nagA* is 2.2-fold more highly expressed in the *L. lactis* Nis^r strain than in its parent. The inactivation of *nagA*, *nagB*, or *glmS* affected the susceptibility of *S. aureus* to cell wall synthesis inhibitors, e.g., vancomycin, suggesting an interdependence between the efficiency of cell wall precursor formation and resistance levels (27). It is tempting to speculate that a higher expression of *nagA* in the *L. lactis* Nis^r strain might result in higher resistance to various cell wall synthesis inhibitors, such as nisin.

At least one operon involved in membrane synthesis is differentially expressed in the *L. lactis* Nis^r strain, namely, the *fabDG1 G2Z1Z2* operon, which is expressed to a lower extent in the *L. lactis* Nis^r strain than in *L. lactis*. The *fab* operon is involved in the saturation and elongation of fatty acids. The *L. lactis* genome contains two genes that are annotated as encoding β-ketoacyl-acyl carrier protein (ACP) reductases, namely, *fabG1* and *fabG2* (3). The *fabG1* gene encodes a protein that is 46% identical to *E. coli*

FabG, whereas the *fabG2*-encoded protein is 33% identical to *E. coli* FabG. The most clear-cut function for FabG2 could be to act as a β-hydroxyacyl-coenzyme A dehydrogenase, such as those functioning in β-oxidative fatty acid degradation pathways (58). The orthologue of *fabZ* in *E. coli* encodes a (3R)-hydroxymyristoyl acyl carrier protein dehydrase, an enzyme that converts β-hydroxyacyl-ACPs to *trans*-2 unsaturated acyl-ACPs (43). The *trans*-2 unsaturated acyl-ACPs that are produced are the substrates of enoyl-ACP reductases that catalyze the last step of each fatty acid elongation cycle (21). Nisin interacts with target membranes in four sequential steps: binding, insertion, aggregation, and pore formation. Alterations in cytoplasmic membrane composition might influence any of these steps. The lower expression of the *fab* operon, as observed in the *L. lactis* Nis^r strain, might lead to a lower amount of saturated fatty acids and less elongated fatty acids in the membrane, making it less densely packed. These results are in contrast to a study done in *L. monocytogenes*, where cells which were grown at 10°C displayed shorter fatty acids and an increase in the fluidity of the cytoplasmic membranes. Cells grown under these conditions were more sensitive to nisin than cells grown at 30°C (36). Thus, to obtain a complete understanding about the saturation and elongation of the phospholipids, further investigation is required.

Genes involved in energy metabolism such as the *arc* operon were found to be involved in the resistant phenotype. The genes *arcAC1C2DT2* were more highly expressed in the *L. lactis* Nis^r strain than in the wild type. When the repressor *ahrC* is deleted, the strain can be made resistant to only a relatively low concentration of nisin (200 μg/liter), whereas the *L. lactis* Nis^r strain can be made resistant to at least 3,000 μg nisin per liter. This result identifies the *arc* operon as being very important in acquired nisin resistance. The arginine deaminase pathway (ADI pathway) of *L. lactis* is responsible for the breakdown of arginine into ornithine, ammonium, and carbon dioxide. Three enzymes are responsible for the complete degradation of arginine, namely, arginine deiminase (ArcA), ornithine carbamoyltransferase (ArcB), and carbamate kinase (ArcC) (48). The degradation of arginine into ammonium might result in a locally less acidic pH at the outer side of the cytoplasmic membrane, preventing nisin from attaching to the lipid II molecule. It is known that, at neutral pH, nisin sticks tightly to the cell envelope, thereby preventing it from reaching the cytoplasmic membrane and lipid II (60). However, it is questionable whether this is the main cause for the observed resistance since the pH effect is not expected to be very strong, due to rapid diffusion processes. However, generating a highly nisin-resistant *L. lactis* MG1363Δ*ahrC* strain appeared to be impossible, indicating the importance of the *arc* operon in the acquisition of nisin resistance.

Various genes with unassigned functions were found, in this study, to be more highly expressed in the *L. lactis* Nis^r strain and involved in acquired nisin resistance. The protein YneG has homology to the *trans*-acting repressor ArsD from *Escherichia coli*, while YneH has homology to *E. coli* arsenate reductase ArsC. The *L. lactis* Nis^r strain grown in the presence of nisin is more sensitive for arsenate, while overproduction of YneGH resulted in a strain that is less sensitive to arsenate. It is tempting to speculate that a competition occurs between arsenate and nisin for the same transporters (e.g., high nisin-pumping activity would titrate away the transport activity for arsenate). This obviously would require further study to be confirmed.

The *ysaBC* orthologs of *S. mutans*, namely, *mbrB* and *mbrA*, respectively, are involved in bacitracin resistance (53). They encode ABC transporters MraBA and are presumed by us to either export a molecule that inactivates bacitracin or transport bacitracin into the cell to prevent it from binding to undecaprenol and stopping the cell wall synthesis. The genes are annotated as bacitracin resistance proteins, but they could be involved in a more general resistance mechanism for antimicrobials. Interestingly, bacitracin resistance proteins were also found to play a major role in LL-37 and Triton X-100 resistance in *Bacillus subtilis* (46). The way and nature of this transport activity deserves more attention, considering its key role in acquired antimicrobial resistance.

The reason why phosphotransferases (PTS) systems (*yedEF*) and maltose hydrolase (*yeeA*) are expressed at a lower level and the ABC transporter *ypcGH* is expressed at a higher level in the *L. lactis* Nis^r strain remains obscure. In studies on bacteriocin resistance in *L. monocytogenes* and *Enterococcus faecalis*, various (sugar) transport systems were also identified as being involved in bacteriocin resistance (17, 22). The mechanisms by which these transport systems contribute to nisin resistance remain to be elucidated. There could be an effect of sugar metabolism intermediates on cell wall biosynthesis, as has been shown for galactose (28). Another explanation as to why PTS systems may be involved in acquired nisin resistance could be that they support the probably energy costly nisin resistance mechanism(s).

Several genes involved in stress response were also expressed at a higher level in the *L. lactis* Nis^r strain. This indicates that even though the cells are adapted to levels of nisin that were 75 times the original MIC, they still undergo stress. The YthAB proteins are annotated as phage shock proteins. In *E. coli*, PspA appears to aid the maintenance of the proton motive force under stress conditions (26) and it can also stimulate the export of secreted proteins (25). This mechanism could also be operational in the *L. lactis* Nis^r strain.

Transcriptome analysis of *L. lactis* revealed many differentially expressed genes to be involved in nisin resistance. For some of these, a direct relation to the resistance mechanism could be indicated, such as for the *dlt*, *gal*, *ahrC*, and *fab* operon genes. Differential expression of several other genes, e.g., the "*y*" genes or stress-related genes, could be an indirect effect of the acquired nisin resistance.

We conclude that acquired nisin resistance of the *L. lactis* strain is a very complex trait. Most of the genes found are probably involved in nisin resistance since all knockout strains appeared to be more nisin sensitive and became less easily resistant to nisin. A putative nisin resistance pathway is presented in Fig. 1, in which four major mechanisms can be envisaged. The first and major mechanism seems to be the prevention of nisin from reaching the membrane and the lipid II molecule. The *L. lactis* Nis^r strain apparently does this by changing the cell wall make up. The cell wall of the *L. lactis* Nis^r strain is probably changed in three ways: (i) it is (locally) thicker (PBP2A), (ii) it becomes more densely packed (GalE, PBP2A), and (iii) it is less negatively charged (DltD). Second, the *L. lactis* Nis^r strain could change the local pH at the outer side of the cytoplasmic membrane by changing the expression

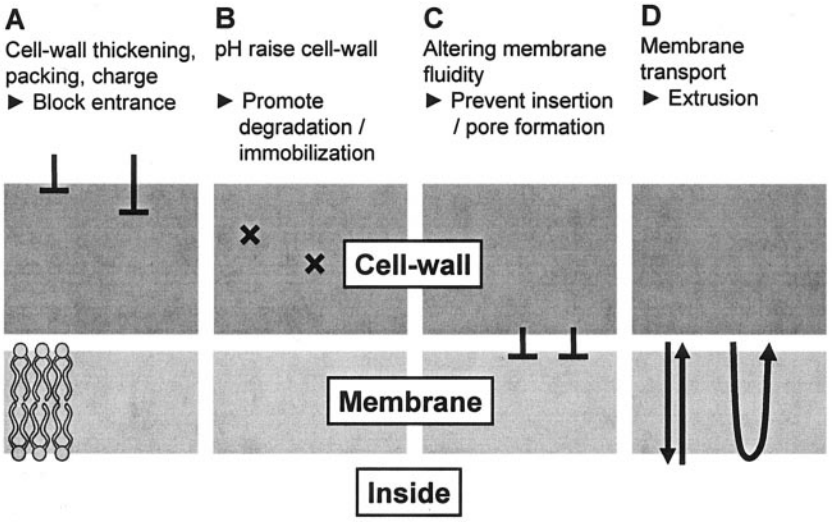

FIG. 1. Putative nisin resistance mechanisms. The putative nisin resistance pathway in *L. lactis* can be divided into four putative mechanisms. For each mechanism, the genes (putatively) involved, based on DNA microarray results, are indicated below between parentheses or in the text (Table 4 details their respective gene expressions). Dark squares, involvement of either the cell membrane or cell wall composition in the resistance mechanism. Mechanism A, cell wall changes, preventing nisin from reaching lipid II in the cytoplasmic membrane, by thickening the cell wall (*pbp2A*), by becoming more densely packed (*galE* and *pbp2A*) and by becoming less negatively charged (*dltD*). Mechanism B, the *L. lactis* Nis^r strain changes the local acidity on the outside of the membrane nisin by changing the expression of genes encoded by the *arc* operon. This results in an elevated pH, possibly promoting the degradation of the nisin molecule or forcing it to bind to the cell wall. Mechanism C, reduced levels of proteins encoded by the *fab* operon, which is involved in the saturation of the fatty acid chains in membrane phospholipids, might make the membrane more fluid, preventing nisin insertion into the membrane. Mechanism D, ABC transporters might be involved in transporting nisin out of the membrane, preventing nisin-lipid II binding.

of genes encoded by the *arc* operon. This would result in an elevated pH, probably causing nisin to stick more tightly to the cell wall and/or promote degradation of the molecule. A third mechanism of acquiring nisin resistance may be through changing the expression of the *fab* operon, which is involved in elongation and saturation of phospholipids (21). When phospholipids are less saturated and more loosely packed, insertion of nisin into the membrane is possibly hampered. This could also influence the insertion of other proteins in the membrane, and could perhaps explain the differential expression of various membrane proteins. As a fourth mechanism, ABC transporters might be involved in removing nisin from the cytoplasmic membrane, should it cross the cell wall, preventing nisin from binding to lipid II and/or to form pores.

The fact that alteration of the structure of the lipid II molecule does not occur as a resistance mechanism is not surprising in view of recent results showing binding of nisin to the pyrophosphate moiety of the lipid II molecule (55). The pyrophosphate moiety is early synthesized and indispensable for the complete functionality of lipid II. This study shows for the first time that bacterial cells employ diverse mechanisms simultaneously to defend themselves against increasing concentrations of the lantibiotic nisin. A similar stacking of mechanisms has been described before, e.g., for vancomycin-intermediate *Staphylococcus aureus* strains (51).

Specific food-grade inhibitors for some of the gene products that are involved in these mechanisms could now be developed. The feasibility of such an approach is already indicated by the fact that certain knockouts can no longer reach full nisin resistance. However, it remains to be determined whether similar resistance mechanisms operate in gram-positive food pathogens such as *Listeria monocytogenes* and *Bacillus cereus*.

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