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Function of *Lactococcus lactis* Nisin Immunity Genes *nisI* and *nisFEG* after Coordinated Expression in the Surrogate Host *Bacillus subtilis**

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Nisin-producing *Lactococcus lactis* strains show a high degree of resistance to the action of nisin, which is based upon expression of the self-protection (immunity) genes *nisI*, *nisF*, *nisE*, and *nisG*. Different combinations of nisin immunity genes were integrated into the chromosome of a nisin-sensitive *Bacillus subtilis* host strain under the control of an inducible promoter. For the recipient strain, the highest level of acquired nisin tolerance was achieved after coordinated expression of all four nisin immunity genes. But either the lipoprotein NisI or the ABC transporter-homologous system NisFEG, respectively, were also able to protect the *Bacillus* host cells. The acquired immunity was specific to nisin and provided no tolerance to subtilin, a closely related lantibiotic. Quantitative *in vivo* peptide release assays demonstrated that NisFEG diminished the quantity of cell-associated nisin, providing evidence that one role of NisFEG is to transport nisin from the membrane into the extracellular space. NisI solubilized from *B. subtilis* membrane vesicles and recombinant hexahistidine-tagged NisI from *Escherichia coli* interacted specifically with nisin and not with subtilin. This suggests a function of NisI as a nisin-intercepting protein.

In recent years peptide antibiotics have gained increasing attention as therapeutics (1, 2) and food preservatives (3). Nisin represents the most prominent member of lantibiotics, peptide antibiotics with intramolecular lanthionine bridges (4–9). The nisin producer *Lactococcus lactis* 6F3 contains a gene cluster encoding proteins for the biosynthesis and transport (10–14), immunity (15), and regulation (16–18) of nisin. Subtilin (19, 20) and ericin S (21) produced by *Bacillus subtilis* ATCC 6633 and A1/3, respectively, are closely related lantibiotics. Lantibiotics form voltage-dependent pores in the bacterial cytoplasmic membrane that are lethal for the target cells but also for the producer. For nisin, the mode of action was investigated in several model systems such as black lipid bilayers and membrane vesicles (22–25). Recent findings demonstrated that specific binding of nisin to the cell wall precursor lipid II coincides with pore formation (26, 27). Specific self-protection (immunity) mechanisms are necessary to protect the lantibiotic-producing organisms from the action of their own lantibiotics. For nisin and subtilin (28) immunity is based on the expression of *lanFEG* encoding ABC transporter-homologous proteins (13, 15, 17; revised sequence of subtilin immunity genes, EMBL accession number U09819), and *lanI* encoding non-related lipoproteins with different sizes (for review, see Ref. 29). Only immunity transporters and no lipoproteins were found in the epidermin (30) or mersacidin (31) gene clusters. In contrast, for Pep5 (32), epicidin (33), and lactocin S (34) only lipoproteins and no transporters have been found.

Although numerous genes involved in lantibiotic immunity are known, the mechanism by which the encoded proteins confer immunity remain unclear. For full nisin or subtilin immunity, both are required, *i.e.* the lipoprotein as well as the immunity transporter. The lack of each component diminished the tolerance to nisin (35) or subtilin (28) significantly. Here we report for the first time on the establishment of nisin immunity in the heterologous host *B. subtilis*. Functional analyses of its different components provided evidence that NisI acts as a nisin-sequestering protein and that NisFEG acts as a nisin exporter that expels nisin molecules from the cytoplasmic membrane into the environment.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—*B. subtilis* MO1099 (36) and ATCC 6633 were grown at 37 °C on Difco sporulation medium or M9 medium (37) supplemented with 50 μg/ml phenylalanine, 20 μg/ml tryptophan, and 0.1% casamino acids. For subtilin production *B. subtilis* ATCC 6633 was grown at 37 °C in TY medium (0.8% tryptone, 0.5% yeast extract, 0.5% NaCl). Recombinant plasmids were amplified in *Escherichia coli* DH5α, TP611, or M15. *E. coli* strains were grown on Luria-Bertani medium (Invitrogen). Antibiotics were used in the following concentrations: 80 μg/ml ampicillin for *E. coli*, and 5 μg/ml chloramphenicol, 1 μg/ml erythromycin, and 25 μg/ml lincomycin for *B. subtilis*. The pDR67 vector with the IPTG[1]-inducible *Bacillus* promoter, P_{spac} (38) was used for chromosomal integration into the *amyE* locus of *B. subtilis* MO1099. Gene expression was induced with 1–2 mM IPTG.

Molecular Biology Techniques—Established protocols were followed for molecular biology techniques (37). DNA was cleaved according to the conditions recommended by the commercial supplier (Roche Molecular Biochemicals). DNA fragments were eluted from agarose gels by the Gene Clean Kit III (Bio 101, Vista, CA). The alkaline extraction procedure (39) was followed to isolate plasmids of *E. coli*. PCR was carried out according to standard procedures (37) in an Eppendorf Microcycler E. DNA was sequenced by Scientific Research and Development GmbH, Oberursel/Frankfurt, Germany. *B. subtilis* was transformed by the competence method (40) with slight modifications (20). Nisin immunity genes were amplified from *L. lactis* chromosomal DNA or the *nisIFEG*-containing plasmid pSI22 (41). A copy of *nisI* (*Eco*RV/*Xba*I) was cloned into pUC19 (42), resulting in pHZ39, and fused to *nisF* (*Xba*I/*Sph*I of

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[1] The abbreviations used are: IPTG, isopropyl-β-D-thiogalactopyranoside; RP, reversed phase; HPLC, high pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight.

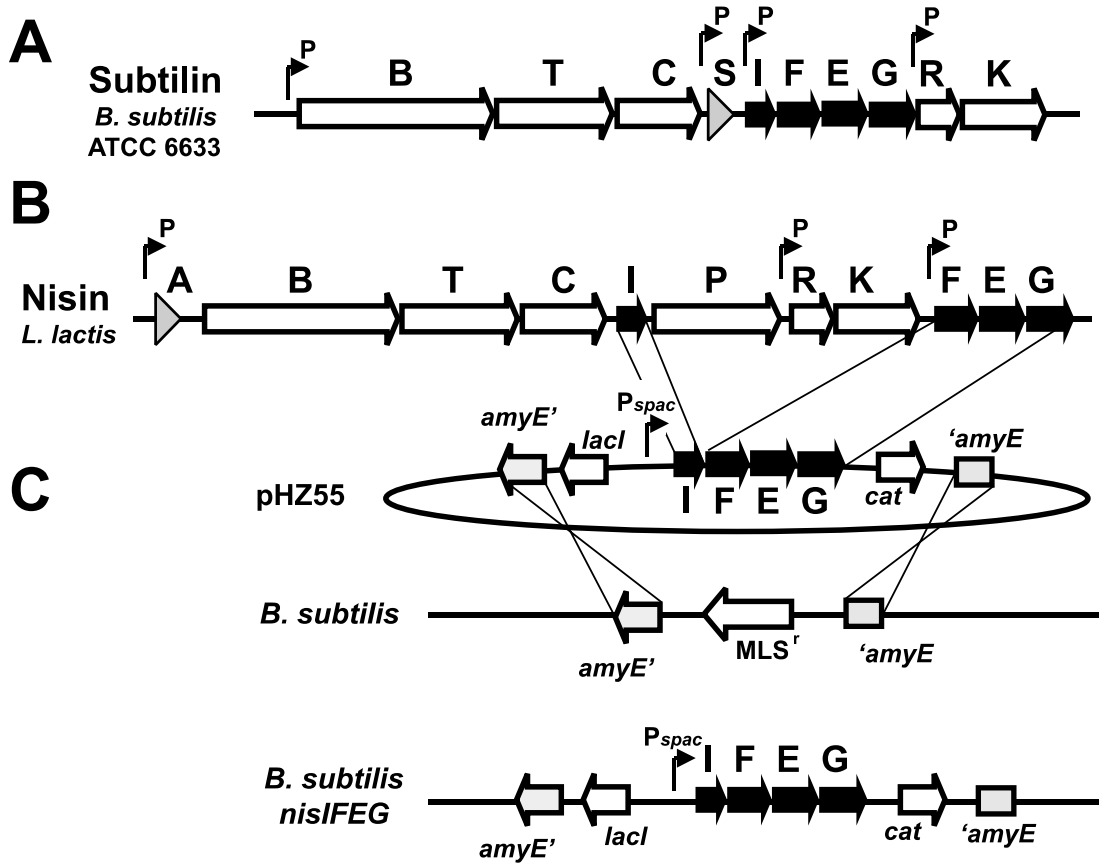

FIG. 1. **Lantibiotic gene clusters and transfer of nisin immunity genes.** *A* and *B*, genetic organization of the closely related subtilin (46) and nisin (47) gene clusters. Whereas *nisI* was found on a transcriptional unit *nisABTCIP* together with the structural gene (*nisA*) and genes involved in posttranslational modifications (*nisBC*), the processing (*nisP*) and transport (*nisT*) of nisin expression of *nisFEG* is controlled by its own promoter (47). *C*, *nisI* and *nisFEG* were fused into a single transcriptional unit under the control of the P_{spac} promoter (pHZ51) and integrated into the chromosome of *B. subtilis* MO1099. Double recombination is accompanied by resistance marker exchange, *i.e.* macrolide-lincomycin-streptogramin (*MLS*) *versus* chloramphenicol (*cat*). The incomplete *amyE* genes were represented as *amyE'* (5′-end) and *'amyE* (3′-end).

1214-bp PCR amplified with O1/2), resulting in pHZ40, and *nisEG* (*Sna*BI/*Sph*I of 908-bp PCR amplified with O3/4), resulting in pHZ41. Derivatives of pDR67 were constructed with *nisI* (*Bgl*II/*Sph*I of 936-bp PCR amplified from pHZ40 with O5/7), *nisFEG* (*Hin*dIII/*Sph*I from pHZ41), and *nisIFEG* (*Bgl*II/*Sph*I of 2070-bp PCR amplified from pHZ40 with O5/6 into pDR67 followed by integration of *Sna*BI/*Sph*I of 908-bp PCR amplified from pHZ41 with O3/4). The primers O1 (5′-GAATAGATTCTGAAACTAGTTTTATATAC-3′), O_2 (5′-AACAAATCAAGGCATGCGCAGCTAAC-3′), O3, (5′-GGAATGTGATCTGCAGAAATAATAGC-3′), O4 (5′-ATTAGGTCGAATTAGCATGCGAAAAAATAC-3′), O5 (5′-GTTACTTAGTCTTTGCTTGGAC-3′), O6 (5′-CGCCAAGCTTGCATGCGCAGC-3′), and O7 (5′-AATTTTTGCATGCATTATATTCCAG-3′) were purchased from ARK Scientific Biosystems (Darmstadt, Germany).

Subtilin Purification—Supernatants of stationary grown *B. subtilis* ATCC 6633 (in TY medium) were separated using semi-preparative RP-HPLC with a C-18 Lichrospher column (particle size 10 μm, 200 × 20 mm; Merck), an analytical ODS Hypersil column (particle size 5 μm, 250 × 2 mm; Maisch, Ammerbuch, Germany), and linear gradients of acetonitrile. Nisin was purchased from Sigma.

Nisin Sensitivity Assay—The nisin sensitivity of *B. subtilis* was determined using agar diffusion tests. Various amounts of nisin in a volume of 60 μl were poured into holes (1.6-mm diameter) of M9 test plates (20 ml). The plates were kept for 2 h at 4 °C for diffusion. 300 μl of stationary grown *B. subtilis* cultures were inoculated into 5 ml of M9 medium and grown to an A_{578} of 0.8. 100 μl of a 10^{-2} dilution were overlaid onto the test plates in 5 ml of molten M9 agar (50 °C) and incubated overnight at 37 °C.

Quantitative Nisin Transport Assay—To investigate the molecular mechanism of nisin immunity, a quantitative *in vivo* peptide release assay described by Otto *et al.* (43) was used with modifications. Stationary *B. subtilis* strains grown overnight in TY medium containing 1% (w/v) glucose were harvested and washed with 50 mM Tris-HCl (pH 8). The cell density was adjusted to an A_{578} of 10 with an incubation buffer (50 mM sodium phosphate (pH 7), 0.5 M NaCl, and 0.5% (w/v) glucose). 1-ml aliquots of the cell suspension were incubated with nisin for 30 min at 37 °C with gentle shaking. After centrifugation (10,000 × *g*, 10 min), quantitative HPLC analyses of the supernatants were performed on a Beckman Gold HPLC System using an analytical ODS Hypersil column (Maisch, Ammerbuch, Germany). Nisin was eluted using a linear gradient of 30–40% acetonitrile containing 0.1% trifluoroacetic acid (v/v/v) over 30-column volumes. Nisin was detected measuring the absorption at 214 nm. The flow rate (0.4 ml/min) was chosen so that a Gauss distribution of the nisin absorption peak was obtained that allowed a quantitative determination of the nisin amount after integration. Nisin attached to the cell sediment was extracted by gently mixing with 1 ml of 20% acetonitrile in water (0.1% trifluoroacetic acid) at room temperature for 5 min. After centrifugation (10,000 × *g*, 10 min), nisin was quantitatively determined in the supernatant.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analyses—SDS-PAGE and Western blot analyses were performed as described previously (14, 44); native PAGE was without SDS. Molecular weight standards for SDS-PAGE were purchased from Sigma.

Isolation of Membrane Vesicles—Membrane vesicles from *B. subtilis* were prepared as described previously (45).

Construction and Isolation of Hexahistidine-tagged NisI—A *nisI* copy was PCR amplified from pHZ39 with primers O8 (5′-GTTTATCAGGATCCTATCAAACAAGTC-3′) and O9 (5′-GAATTTTCTGCAGTCTAGTTTCCTAC-3′) (ARK) and inserted into the pQE9 vector (Qiagen, Hilden, Germany). The *E. coli* strain M15 (pREP4) was transformed with the resulting plasmid and grown in LB to an A_{600} of 0.5. After IPTG induction, the cells were incubated for additional 4 h. The cells were harvested, suspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), and disrupted by sonication. After removal of cell debris by centrifugation at 17,000 × *g* (30 min, 4 °C) the supernatant was incubated with nickel-nitrilotriacetic acid-agarose (Qiagen) with gentle shaking (1 h, 4 °C). The protein was eluted using the same buffer containing 100 mM imidazole and dialyzed against the storage buffer (20 mM Tris-HCl, 10% glycerol and 5 mM dithiothreitol, pH 6.5).

Interaction between NisI and Nisin—NisI membrane vesicles were

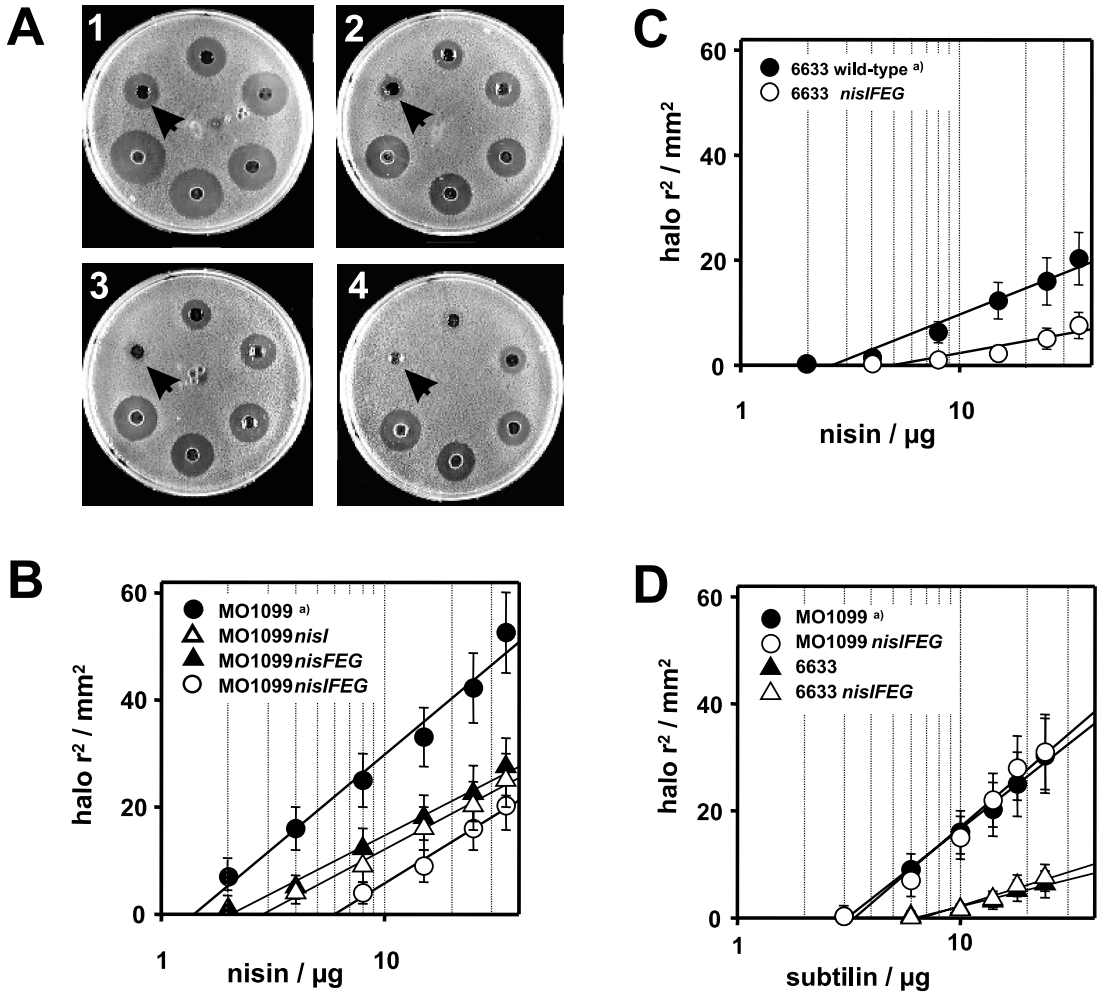

FIG. 2. **Functional analysis of nisin immunity in *B. subtilis* MO1099.** Lantibiotic sensitivity of *B. subtilis* strains expressing different combinations of nisin immunity genes. Strains were investigated in agar diffusion tests as indicated under "Experimental Procedures." *A*, nisin sensitivity of *B. subtilis* MO1099 transformed with the vector plasmid pDR67 without immunity genes (*plate 1*) and expressing *nisI* (*plate 2*), *nisFEG* (*plate 3*), and *nisIFEG* (*plate 4*). Starting from the *arrow* and moving clockwise, the applied amounts of nisin are 2, 4, 8, 15, 25, and 35 µg. According to the Second Law of Diffusion (also referred to as Fick's Law), the square of the diffusion distance of a given solute into a liquid is directly proportional to the natural logarithm of its initial concentration. Thus, using standard volumes (60 µl) and sufficient diffusion times, linear dependences between the square of the halos and the natural logarithm of the applied nisin amounts were obtained. *B*, *B. subtilis* MO1099 (*filled circles*) expressing *nisI* (*open triangles*), *nisFEG* (*filled triangles*), and *nisIFEG* (*open circles*). *C*, nisin sensitivity of *B. subtilis* ATCC 6633 (*filled circles*) expressing *NisIFEG* (*open circles*). *D*, subtilin sensitivity of *B. subtilis* MO1099 (*filled circles*) expressing *nisIFEG* (*open circles*) and *B. subtilis* ATCC 6633 (*filled triangles*) expressing *nisIFEG* (*open triangles*). S.E. was <15% for each given value in *panels B* and *C* (means of three independent assays).

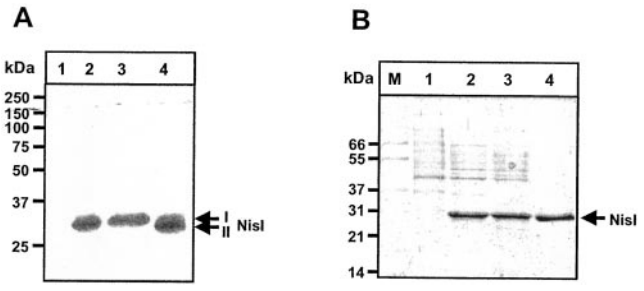

FIG. 3. **Expression of NisI in heterologous hosts.** *A*, NisI immunoblot of SDS-PAGE-separated *B. subtilis* MO1099 extracts. *B. subtilis* cells expressing NisI (*lane 2*) were disrupted by sonication. After centrifugation, the supernatant (*lane 4*) and the membrane pellet suspended into a comparable volume of lysis buffer (*lane 3*) were analyzed. *Lane 1*, *B. subtilis* MO1099 without NisI genes. *I* and *II*, NisI with and without lipo-modification, respectively. Approximately 40 µg of total protein was loaded onto each lane. *B*, bromphenol blue stain of SDS-PAGE-separated extracts of *E. coli* M15 (pREP4) cells overexpressing His_6-NisI without membrane anchor (*lane 1*) and after IPTG induction (*lane 2*). After lysis and centrifugation (17,000 × *g*, 30 min), His_6-NisI was found in the supernatant (*lane 3*) and was adsorbed to nickel-agarose. Purified His_6-NisI after elution from the nickel-agarose beads with 100 mM imidazole is shown in *lane 4*). *M*, molecular mass marker. The position of NisI is indicated by an *arrow*.

incubated under gently shaking with 0.17 volumes of 3% (w/v) laurylmaltoside (dodecyl-β-D-maltoside, Roche Molecular Biochemicals) and 0.17 volumes of 2 M 6-aminocaproic acid (Fluka) at room temperature for 2 h. After centrifugation (30 min, 18,000 × *g*, 4 °C), 15% (w/v) glycerol was added to the supernatant, which contained the dissolved membrane proteins. From this fraction, 30 µl were incubated with different amounts of nisin or subtilin (10 min, 37 °C). The samples were divided 70:30, and the larger sample was loaded onto 9% polyacrylamide gels without SDS, separated at 20 mA for 6 h, and electroblotted to nitrocellulose by using standard buffers with 0.1% SDS. The smaller sample was loaded onto a denaturing 14% SDS-PAGE, followed by electroblotting. Finally, NisI was visualized with NisI-antisera (41).

Different amounts of purified His_6-NisI (0–160 µg) were incubated with 20 µg of nisin in a final volume of 20 µl in 20 mM Tris-HCl, pH 6.5, and 1 mM dithiothreitol (37 °C, 30 min). After centrifugation (10 min, 13,000 rpm), the pellets were dissolved in 20 µl of 0.02 N HCl. 5-µl aliquots of the supernatants and solubilized pellets were analyzed in a agar diffusion tests using *Micrococcus luteus* ATCC 9341 as the test organism.

RESULTS

Fusion of nisI to nisFEG and Transfer to the Chromosome of B. subtilis—In contrast to subtilin immunity (Fig. 1*A*) (46), the *L. lactis* nisin immunity genes *nisI* and *nisFEG* reside on different transcriptional units (Fig. 1*B*) (18, 47). To study nisin

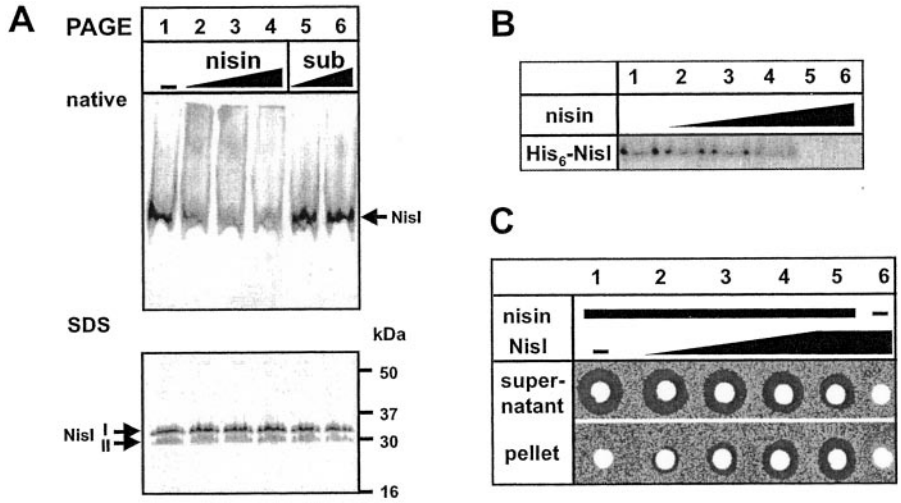

FIG. 4. **Functional analysis of NisI: Interaction with nisin.** *A*, protein fractions solubilized from membrane vesicles (60 μg) of NisI-expressing *B. subtilis* MO1099 were incubated with different amounts of nisin or subtilin. After splitting into two parts (70:30), the larger part was separated by native PAGE, and the minor part was separated by SDS-PAGE. NisI immunoblots of both gels are shown. The lanes identical for both gels. Solubilized membrane proteins were incubated with a culture supernatant of a subtilin-negative mutant (*lane 1*), 2, 3, or 4 μg of nisin (*lanes 2–4*), and 4 or 6 μg of subtilin (*lanes 5–6*). The position of NisI is indicated by *arrows*. *B*, NisI immunoblots of His_6-NisI (5 μg) after incubation with increasing amounts of nisin and subsequent native PAGE. *Lanes 1–6*, 0.05, 5, 10, 20, and 30 μg of nisin. *C*, after incubation of His_6-NisI with nisin, a pellet was formed. The antimicrobial activities within the supernatant and the solubilized pellet were analyzed using *M. luteus* as the test organism. The complete assays are described under "Experimental Procedures." Assays 1–5 contained 20 μg of nisin and 0, 20, 40, 80, and 160 μg of His_6-NisI, respectively; assay 6 contained 160 μg of His_6-NisI without nisin.

immunity without the influence of nisin production, we coexpressed different combinations of nisin immunity genes in the nisin-sensitive *B. subtilis* hosts MO1099 and ATCC 6633, as exemplified for the *nisIFEG* construct in Fig. 1*C*.

Effect of Nisin Immunity Genes in B. subtilis—The nisin sensitivity levels of the recipient *B. subtilis* strains were analyzed (Fig. 2). The highest level of tolerance was obtained for *B. subtilis* MO1099 containing all four immunity genes, *nisI*, and *nisFEG* (Fig. 2, *A*, *plate 4*, and *B*, semi-quantitative analyses). This strain tolerates 8 μg of nisin, a quantity that already induced large growth inhibition zones for the control strain without any immunity genes (Fig. 2*A*, *plate 1*). Remarkably, after expression of the lipoprotein NisI alone, a significant nisin tolerance level was obtained (Fig. 2*A*, *plate 2*) that was comparable with *B. subtilis* cells expressing *nisFEG* (Fig. 2*A*, *plate 3*). These data clearly showed that *B. subtilis* MO1099 represents a surrogate host for the functional expression of nisin immunity genes and that the action of two immunity systems, the lipoprotein NisI and the ABC transporter-homologous protein NisFEG, is needed to obtain full nisin tolerance.

Additionally, the nisin immunity components were transferred to the subtilin-producing *B. subtilis* strain ATCC 6633. After coordinated expression of *nisIFEG*, the recipient cells exhibited significant tolerance to nisin (Fig. 2*C*). This showed that the immunity system from *L. lactis* 6F3 is also effective in *B. subtilis* cells, which express two lantibiotic immunity systems, *nisIFEG* and *spaIFEG*. The different tolerance levels obtained for the *nisIFEG*-expressing *B. subtilis* strains MO1099 and the subtilin producer ATCC 6633 were based on the faster growth rate of the ATCC 6633 strain. Remarkably, the expression of nisin immunity genes *nisIFEG* (Fig. 2*D*) as well as other combinations of nisin immunity genes (not shown) in *B. subtilis* strains MO1099 and ATCC 6633 had no effect on subtilin tolerance.

Cellular Localization of NisI—NisI (25.8 kDa, calculated without signal sequence) expressed in *B. subtilis* was detected in both the membrane fraction (Fig. 3*A*, *lane 3*) and the soluble cell extract (*lane 4*). Two bands were obtained for NisI (shown in Fig. 3*A*, and more pronounced in Fig. 4*A*, see below), suggesting that the upper band (*I*) corresponds to membrane-associated NisI and the lower band (*II*) to NisI without the membrane anchor. Thus, at least approximately half of NisI seemed to be correctly anchored in the cytoplasmic membrane of *B. subtilis*. Probably, degradation and/or incomplete modification with the lipid moiety led to a protein with faster migration after SDS-PAGE. In accordance with this probability, prolonged incubation times of *nisI*-expressing cells led to the observation of NisI within the culture supernatant (not shown).

Construction and Isolation of Hexahistidine-tagged NisI—NisI is a typical lipoprotein, possessing both a N-terminal signal sequence and a membrane-anchoring Cys residue immediately proceeding the cleavage site. To avoid possible anchoring and export of NisI in *E. coli*, the NisI lipoprotein signal sequence and the anchoring Cys residue (amino acids 1–20) were substituted by the sequence MRSGSHHHHHH, resulting in the protein His_6-NisI. Recombinant His_6-NisI from *E. coli* (Fig. 3*B*, *lane 2*) was found in the soluble protein fraction (*lane 3*) after cell lysis. His_6-NisI was purified by nickel-agarose affinity chromatography (*lane 4*) and used for interaction studies with nisin (see below) and immunoaffinity purification of the polyclonal NisI antibody (48). The purified antibody showed no cross-reactivity with components of an SDS-PAGE-separated *B. subtilis* total cell extract (Fig. 2*A*, *lane 1*), demonstrating its high selectivity.

Specific Interaction between NisI and Nisin—To unravel the function of NisI in nisin immunity, we investigated possible interactions between NisI and nisin. NisI was solubilized from *nisI* expressing *B. subtilis* MO1099 membrane vesicles and incubated with different amounts of nisin and subtilin. Subsequently, the samples were analyzed by parallel native and SDS-PAGE (Fig. 4*A*). Incubation of NisI with nisin (*lanes 1–4*) led to a depletion of the NisI signal, which was not observed if subtilin was used (*lanes 5–6*). Regardless of a former incubation with nisin (*lanes 1–4*) or subtilin (*lanes 5–6*), all samples revealed the same NisI signal intensity after separation under denaturing conditions (SDS-PAGE in Fig. 4*A*). A similar signal depletion was observed for His_6-NisI isolated from *E. coli* (Fig. 4*B*), suggesting similar properties for NisI without a lipid anchor.

Remarkably, after incubation of nisin with His_6-NisI, a small

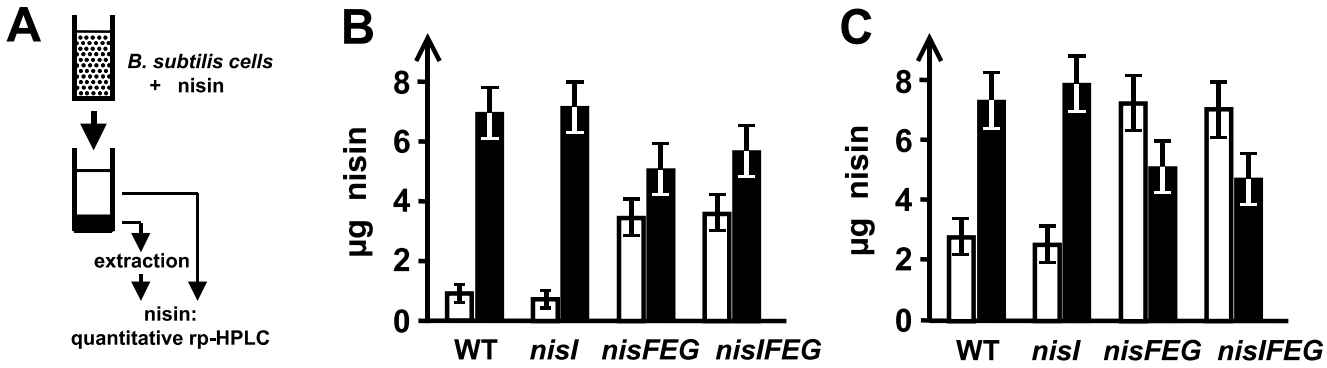

FIG. 5. **Functional analysis of NisFEG; quantitative nisin transport assay.** *A*, stationary grown *B. subtilis* cells were incubated with different amounts of nisin. After centrifugation, the quantity of nisin in the supernatant and the quantity of the cell-associated nisin were determined by quantitative RP-HPLC. *B* and *C*, the quantity of nisin determined by the nisin transport assay with 9 μg (*B*) and 12 μg (*C*) of applied nisin; *white bars*, supernatant; *black bars*, extracted from cells. The presented values represent the means of three independent assays for which all determinations were performed twice, respectively. S.E. of <20% was obtained for each given value.

pellet was observed. The pellet contained a portion of the nisin molecules that are easily solubilized under aqueous conditions as demonstrated by *M. luteus* agar diffusion tests (Fig. 4*C*, *lanes 2–5*, *pellet*). In accordance, after incubation of nisin with increasing amounts of His_6-NisI, a slight decrease of the activity in the supernatant was observed (Fig. 4*C*, *lanes 2–5*, *supernatant*). With equal amounts of subtilin, no pellet was formed (not shown). This argues for a specific complex formation between NisI and nisin that reduces the quantity of free nisin molecules.

The Function of NisFEG—The activity of NisFEG was investigated with a series of quantitative *in vivo* peptide release assays. After incubation of *B. subtilis* cells with nisin, the quantities of the nisin in the culture supernatant and the nisin associated with the cell-pellet were determined by quantitative RP-HPLC (Fig. 5*A*). For *B. subtilis* MO1099 and MO1099 expressing NisI, ~7.5 μg of nisin were found attached to the cells independently of the quantity of applied nisin (9 or 12 μg, Fig. 5, *B and C*). After expression of *nisFEG* or *nisIFEG*, the quantity of cell-associated nisin was significantly reduced to about 5 μg. In accordance, the nisin quantity in the supernatant increased about 4-fold if 9 μg of nisin was applied (Fig. 5*B*) and about 2.5-fold if 12 μg of nisin was applied (Fig. 5*C*), suggesting an export function of the transporter-homologous system NisFEG. Remarkably, in all experiments >90% of the applied nisin could be recovered. The structural identities of the nisin in the supernatant and the nisin attached to cells were verified after RP-HPLC and MALDI-TOF mass spectrometry analyses (*m/z* 3354, not shown). Thus, no nisin modification or degradation systems were present for lowering its toxicity. After application of subtilin (12 μg) in the peptide release assay, about two-thirds (7.5–8 μg) were found cell-associated and about one-third (3–4 μg) in the supernatant, regardless of whether *B. subtilis* MO1099 wild-type or *nisFEG*-expressing cells were analyzed.

DISCUSSION

To study the molecular mechanism of nisin self-protection provided by NisIFEG, different combinations of the immunity genes were integrated into the genome of nisin-sensitive *B. subtilis* strains. After expression of either the lipoprotein NisI alone or the ABC transporter-homologous system NisFEG, the recipient *B. subtilis* cells acquired a significant level of nisin tolerance. The strongest tolerance was obtained after coexpression of *nisI* and *nisFEG*, as previously suggested from deletion analyses in the nisin producer *L. lactis* 6F3 (15, 35). Although *B. subtilis* expressing the nisin immunity gene *nisIFEG* acquired a >3-fold nisin tolerance level as compared with wild-type *B. subtilis* (Fig. 2), only ~30% of the nisin immunity level of *L. lactis* 6F3 was achieved (15). However, in the nisin-producing *L. lactis* strain, the establishment of nisin immunity is based on two operons, *nisABTCIP* and *nisFEG*. The coordinated and IPTG-induced expression of *nisIFEG* in the *B. subtilis* host cells is quite different from the autoregulatory control of nisin immunity in *L. lactis* (18). Remarkably, the acquired nisin immunity levels of the heterologous host *B. subtilis* were comparable with the immunity level of nisin non-producing *L. lactis* strains expressing plasmid-encoded *nisI* and *nisFEG*. Also for these cells, an immunity level of only 20% compared with the nisin producer was obtained (49). The *B. subtilis* nisin tolerance level provided by the nisin immunity genes suggests the additive action of two independent systems, the lipoprotein NisI and the transporter NisFEG. Nevertheless, we cannot exclude the effect of additional factors that contribute to nisin immunity in the nisin producer *L. lactis*, for which a rather cooperative effect of NisI and NisFEG was discussed (35, 50). Remarkably, the transfer of all components of the nisin immunity system to a subtilin-producing *B. subtilis* host was successful. The functional expression of two closely related self-protection systems, *nisIFEG* and *spaIFEG* (Fig. 2, *C* and *D*), demonstrated that cross-immunity between both systems is less likely.

A typical lipoprotein signal sequence including the lipobox sequence LSGC (51) was found for NisI, suggesting that NisI becomes a peripheral membrane protein after lipid modification of the lipobox Cys residue, processing, and transport over the cytoplasmic membrane. Although it provides significant nisin tolerance, only 50% of NisI expressed in *B. subtilis* was localized in the membrane. The other portion of NisI was found in the soluble protein extract and showed a slightly faster migration after SDS-PAGE, suggesting that it represents an incompletely lipid-modified or degraded NisI species (Fig. 3*A*). Obviously, the lipid modification is not sufficient for complete attachment of the respective proteins to the cytoplasmic membrane. However, for non-lipo-modified NisI protein species significant activity is also conceivable. The essential major *B. subtilis* lipoprotein PrsA in *B. subtilis* showed at least partial activity without lipid modification (52).

To provide evidence for physical interaction between NisI and nisin, we used two different approaches. NisI could be efficiently extracted from *B. subtilis* membranes after treatment with laurylmaltoside and 6-aminocaproic acid. An interaction of NisI with nisin and not with the structurally closely related subtilin could be clearly monitored by the depletion of the NisI signal after native PAGE and immunoblotting (Fig. 4*A*). The detection of NisI signals not entering the native PAGE implies a low solubility of the NisI-nisin complex. A similar

behavior was obtained for recombinant His_6-NisI from *E. coli* (Fig. 4*B*), suggesting that the lipoprotein signal sequence and/or the lipid modification is not necessary for NisI activity.

Further evidence for specific interaction of NisI with nisin was provided after incubation of His_6-NisI with nisin (Fig. 4*C*), which resulted in the formation of an insoluble complex. This argues for an interception of a portion of soluble nisin molecules. Obviously, the complex is easily dissociated in aqueous conditions, thus fully recovering the activity of precipitated nisin.

The ABC transporter-homologous system NisFEG seems to work by expelling cell-attached nisin molecules into the environment, a mechanism that is similar to the one described for the epidermin transporter homologue EpiFEG (43). Nevertheless, the epidermin self-protection system lacks a lipoprotein LanI, implying a greater necessity for the epidermin immunity transporter in *S. epidermidis*. Our results suggest that the export capacity of NisFEG is independent of the lipoprotein NisI. After extraction, we could quantitatively recover cell-associated nisin. Even after prolonged incubation times (up to 60 min) of *nisIFEG*-expressing cells with nisin, approximately all applied nisin molecules could be recovered quantitatively. This argues against degradation or modification of nisin either by NisIFEG or other systems.

The lethal activity of nisin can be described with a four-step mechanism that includes membrane adhesion, membrane integration, pore formation, and pore dissociation (25). Specific binding of nisin to the cell wall precursor lipid II coincides with pore formation (26, 27). Our results provided experimental evidence that nisin immunity is based on two independently acting systems. The lipoprotein NisI is orientated to the outside of the cytoplasmic membrane. NisI would intercept nisin at the surface of the cytoplasmic membrane and, by sequestering nisin, prevent it from inserting into the membrane and/or prevent high local density of nisin molecules necessary for pore formation. The nisin-exporting function of NisFEG would diminish the quantity of nisin molecules that have already entered the cytoplasmic membrane before/during pore formation.

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