The spiFEG Locus in *Streptococcus infantarius* subsp. *infantarius* BAA-102 Confers Protection against Nisin U

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Nisin U is a member of the extended nisin family of lantibiotics. Here we identify the presence of nisin U immunity gene homologues in *Streptococcus infantarius* subsp. *infantarius* BAA-102. Heterologous expression of these genes in *Lactococcus lactis* subsp. *cremoris* HP confers protection to nisin U and other members of the nisin family, thereby establishing that the recently identified phenomenon of resistance through immune mimicry also occurs with respect to nisin.

Lantibiotics are antimicrobial peptides that have been the focus of intense research in recent years. These ribosomally synthesized peptides undergo posttranslational modification, resulting in the presence of unusual amino acids such as the eponymous lanthionine residues, as well as a variety of other modified residues. The nisin family is the most studied of all lantibiotics. Nisin A was initially discovered in 1928 (36, 37), and it has been used commercially as a food preservative for over 50 years (31). The nisin family has also been investigated for potential applications in clinical and veterinary settings (6, 16, 43) since it is active against a wide range of pathogens, including many drug-resistant strains (40). Indeed, it is already commercially employed as an antimicrobial agent (39). To date, seven natural forms of nisin have been identified. Of these, nisin A (25), nisin Z (33), nisin Q (44), and nisin U (12) are produced by *Bacillus licheniformis* DSM13 and *Enterococcus faecium* DO. It was shown that heterologous expression of these homologues provided protection against lacticin 3147 (14). The identification of this phenomenon is a concern and may represent a means by which populations of bacteria could emerge with resistance to specific lantibiotics. Notably, while a number of systems involved in acquired (19, 27) and innate resistance to nisin have been identified (8, 9), systems capable of providing resistance to any of the nisin peptides through immune mimicry have not been discovered heretofore.

Here we identify the first incidence of resistance by means of immune mimicry with respect to the nisin family. More specifically, genes encoding a homologue of the nisin U immunity-providing ABC transporter (NsuFEG) were identified within the genome of a non-lantibiotic-producing pathogen, *Streptococcus infantarius* subsp. *infantarius* BAA-102. Although the BAA-102 strain was recalcitrant to genetic manipulation, and thus the creation of a knockout mutant was not possible, heterologous expression confirms that SpiFEG and NsuFEG can protect against the action of nisin U and other members of the nisin family.

**In silico screening for homologues of nisin U immunity determinants.** Immune mimicry is a recently identified phenomenon, and thus far, the only examples relate to the protection afforded against lacticin 3147 by homologues of its immunity proteins (14). To identify other examples of immune mimicry, a PSI-BLAST search (2) was undertaken using the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) to determine if genes encoding homologues of the nisin U immunity proteins could be identified in strains incapable of nisin production. This screening revealed the presence of the genes predicted to encode the components of an ABC transporter similar to that involved in nisin U immunity (NsuFEG [42]) within the genome of *S. infantarius* subsp. *infantarius* BAA-102 (38). The predicted product of STRINF_01307 (here referred to as...
 SpiF) resembled NsuF (67% identity, e value of 2e-83), while STRINF_01306 (here annotated as spiE) and STRINF_01305 (here referred to as spiG) are predicted to encode proteins that resemble NsuE (50% identity, e value of 4e-63) and NsuG (51% identity, e value of 5e-45), respectively. While the similarity between SpiFE and NsuFE is only marginally greater than that to NisFE, SpiG is only 35% identical to NisG. As a result of this discovery, other genes within this region of the BAA-102 genome were subjected to an in silico investigation to determine if other lantibiotic-associated genes might be present. Notably, the 3 genes immediately downstream from spiFEG all resembled those encoding the individual components of the nisin two-component system known as NisRK (or NisRK in the case of nisin U [42]), which are responsible for regulating nisin biosynthesis and immunity (28) (Fig. 1). More specifically, the gene product of STRINF_01304 is 70% identical to the response regulator NsrR, with an e value of 1e-50 (and here referred to as spiR), and the adjacent gene (STRINF_01303; spiR’) also encodes an NsrR-like protein. Although SpiR and Spi’ are 67% identical to NsrR, the two proteins are predicted to be quite different from each other, being only 11% identical. Furthermore, the predicted product of STRINF_01302 is 50% identical (e value of 4e-112) to the histidine kinase NisK and 43% identical to NsuK (e value of 7e-101) (Fig. 1). Although none of the nisin-like compounds have multiple LanR proteins associated with their regulation, multiple LanR proteins can be found in the lantibiotic operons of RumA, meracinidin, and cytolysin (11, 18, 20). The NisR binding motif, a defined sequence of nucleotides by which NisR binds to the promoters of NisF and NisA, is referred to as a nis box (26). A sequence with high similarity to a nis box is found in the region upstream of spiFEG and theoretically could act as a binding motif for SpiR and/or Spi’. It was noted that the percent GC content of spiFEGRR’ K (33.5%) is lower than that observed in the entire BAA-102 genome (37.7%), and thus, the possibility that these genes were acquired through horizontal transfer cannot be discounted. Analysis of up- and downstream genes reveals that none of these possess lantibiotic-associated features. Indeed, further analysis of the BAA-102 genome failed to identify any other lantibiotic-associated genes. Interestingly with respect to SpiF, a conserved domain, the BcrA subfamily (cd03268), was identified; homology between this bacitracin-associated transporter and SpiG was also revealed.

The presence of the spiFEGRR’ K genes in S. infantarius subsp. infantarius BAA-102 is noteworthy for a number of reasons. S. infantarius, referred to as S. bovis biotype II/1 before reclassification (5), has been isolated from the feces of infants, from clinical specimens associated with endocarditis, and from foods, including dairy products and frozen peas (1, 23, 38). This species can also contribute to the development of cancer, particularly in cases of chronic infection or inflammatory disease where the S. infantarius bacterial components interfere with cell function, leading to cell transformation and proliferation (4), and is most frequently associated with noncolonic digestive tract cancers (10). Notably, nisin U has been shown to be effective against a wide range of disease-associated streptococci, including Streptococcus pyogenes, Streptococcus salivarius, S. sberis, Streptococcus agalactiae, and Streptococcus dysgalactiae, and thus, the possibility that a potential target such as S. infantarius may be resistant to nisin as a consequence of immune mimicry is worthy of note.

**Heterologous expression of nsuFEG and spiFEG.** S. infantarius BAA-102 is based on the basis of deferred-antagonism assays (for the method, see reference 17), less sensitive to nisin U than any other number of Streptococcus species tested (S. pyogenes, Streptococcus mitis, and S. agalactiae; data not shown). However, despite several attempts, we were unable to successfully transform BAA-102 as a prelude to creating isogenic spiFEG knockout mutants. As a consequence, we used heterologous expression as a strategy to determine whether the newly identified spiFEG genes could encode protection against nisin U. The corresponding nsnFEG genes from S. sberis 42 (24, 42) served as a positive control. To facilitate this, genomic DNA was extracted from S. sberis as described previously (14). The nsnFEG genes were amplified using primers AA AACTGCAGAAATAGTCAAAGAAAGGGGTACCCCTT TAGGTTGCTGATGCG, and the primers for spiFEG were AAAACTGCAAAAGTTTGGGACTTCAATG and GGGGTA CCCCTGTCAACCTCAATTTGTATTTTG, where restriction enzyme sites are underlined. The resulting gene products and the shuttle expression vector pNZ44 (32) were digested with the relevant restriction enzymes, ligated, and introduced into electrocompetent L. lactis HP (via the intermediate host Escherichia coli TOP10) as described previously (14). Following confirmation of the integrity of the newly created vectors, deferred-antagonism assays were performed to provide an initial insight into the protection provided against the producer of nisin U and other nisins (A, Z, F, and Q [35]). These were carried out as described previously, using GM17 and TS agar plates for Lactococcus and Streptococcus, respectively (17). Relative sensitivity was assessed on the basis of zone size (Table 1). No inhibition was apparent when the nisin U producer was overlaid with L. lactis HP/pNZ44nsuFEG, thus establishing that the nisin U immunity proteins could provide protection when expressed heterologously to the otherwise nisin-sensitive HP strain. Notably, the presence of pNZ44spiFEG also provided
established that heterologous expression of pCI372nisQ (35), and nisin U3 (41) were also assessed. It was found that significantly enhanced resistance of HP/pNZ44 to all forms of nisin and of HP/pNZ44spiFEG to nisins Z and U. However, in this instance, HP/pNZ44spiFEG also displayed significantly enhanced resistance to nisins A, F, and Q (Fig. 2). These investigations are consistent with those of Wirawan et al., who previously noted cross-protection between nisin-producing strains (42). Given that SpiFEG also resemble transporters involved in bacitracin resistance, the relative resistance of L. lactis HP and HP/pNZ44spiFEG to this antibiotic was tested via antibiotic disc assays (10 U; Oxoid) (9). These assays revealed that spiFEG do not provide the HP strains with enhanced resistance to bacitracin (data not shown).

Antimicrobial activity assays with purified nisin U. To better assess the extent to which SpiFEG provide protection from nisin U, broth-based assays with purified nisin U were carried out. To facilitate this, the lantibiotic was purified using an approach previously employed to purify nisin A and derivatives (17) but with some slight modifications. Specifically, the tryptone-yeast extract-glucose growth medium was supplemented with higher levels of glucose (11 g liter\(^{-1}\)) and \(\beta\)-glycerophosphate (21 g liter\(^{-1}\)) before the nisin U present in cell-free culture supernatant was isolated by passage through 60 g XAD-16 beads (prewashed with water), washed with 30% ethanol, and finally eluted with 70% isopropanol. This was combined with the nisin-containing 70% isopropanol from the purification of cell-attached nisin as previously described (17). Subsequent purification was performed using a 10-g (60-ml) Strata C18-E column (Phenomenex, Cheshire, United Kingdom) prre-equilibrated with methanol and water. The column was washed with 30% ethanol, and the inhibitory activity was eluted in 70% isopropanol–0.1% trifluoroacetic acid (TFA). Aliquots (20 ml) were concentrated to 2 ml through the removal of propan-2-ol by rotary evaporation before being applied to a Phenomenex C\(_{12}\) reverse-phase high-performance liquid chromatography (HPLC) column (Jupiter 4 \(\mu\)m Proteo 90 Å; 250 by 10.0 mm, 4 \(\mu\)m) previously equilibrated with 25% acetonitrile–0.1% TFA. The column was subsequently developed in a gradient of 30% acetonitrile containing 0.1% TFA to 60% acetonitrile containing 0.1% TFA from 10 to 45 min at a flow rate of 2.0 ml min\(^{-1}\). Fractions containing nisin U were collected after HPLC, acetonitrile was removed by rotary evaporation, and the protein was lyophilized by freeze-drying. Mass spectrometry was performed protection against nisin U, with zone sizes decreasing substantially. The SpiFEG system is thus capable of providing protection through immune mimicry. The abilities of pNZ44nsuFEG and pNZ44spiFEG to protect HP against the actions of nisin A (produced by L. lactis NZ9700 [29]), nisin F (produced by L. lactis NZ9700/pCI372nisF [35]), nisin Z (produced by L. lactis NZ9700/pCI372nisZ [35]), nisin Q (produced by L. lactis NZ9700/pCI372nisQ [35]), and nisin U3 (41) were also assessed. It was established that heterologous expression of nusuFEG in strain HP provides protection against nisin U3 and to a lesser degree against nisin A, nisin F, nisin Z, and nisin Q, with zone sizes smaller than those observed when HP was used as the target (Table 1). The presence of pNZ44spiFEG also substantially reduced the sensitivity of the HP strain to nisin Z and nisin U3 (Table 1). To further assess the level of protection, the same collection of strains was employed to carry out a series of agarose-based well diffusion assays (Fig. 2) (13, 30). In this instance, the antimicrobials were present in the form of cell-free supernatant from overnight cultures of the nisin producers. The benefit of this approach is that the enhanced rate of diffusion of the antimicrobials through agarose (relative to agar) and the use of target cells in early-log-phase cells provide greater sensitivity. The results from these assays confirm the significantly enhanced resistance of HP/pNZ44nsuFEG

### Table 1 Deferred-antagonism assay analysis of the protective capabilities conferred by NsuFEG and SpiFEG when expressed in L. lactis HP, or the resistance of the natural S. infantarius isolate, against the action of a range of natural nisin variant producers

<table>
<thead>
<tr>
<th>Nisin variant</th>
<th>Avg zone size (mm) ± SD</th>
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<tbody>
<tr>
<td>HP (control)</td>
<td>HP pNZ44nsuFEG</td>
</tr>
<tr>
<td>A</td>
<td>16.8 ± 0.71</td>
</tr>
<tr>
<td>F</td>
<td>20.6 ± 1.00</td>
</tr>
<tr>
<td>Z</td>
<td>24.4 ± 0.36</td>
</tr>
<tr>
<td>Q</td>
<td>16.08 ± 0.65</td>
</tr>
<tr>
<td>U</td>
<td>8.4 ± 0.42</td>
</tr>
<tr>
<td>U3</td>
<td>16.06 ± 0.18</td>
</tr>
</tbody>
</table>

\(^\ast\) Values are averages of triplicate experiments and represent zone sizes, i.e., diameter of zone minus diameter of bacterial growth.

\(^\ast\) No distinct zone but some hazy growth adjacent to nisin-producing colony.

**FIG 2** Agarose diffusion assay, whereby L. lactis HP/pNZ44 and strains expressing nsuFEG and spiFEG were challenged under adverse growth conditions with nisins A, F, Z, Q, and U. Asterisks indicate zone diameters which were significantly smaller by Student’s t test \((P < 0.0005)\) than that found in L. lactis HP, hence implying the protective capabilities of these genes.
with an Axima CFR plus matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometer as previously described (17), which confirmed that the final purified product was nisin U. The purified nisin U was employed in studies to compare the growth of HP, HP/pNZ44, and HP/pNZ44spiFEG in the presence of 416 nM nisin U over 4 h in broth (Fig. 3). This was assessed by inoculating 1 × 10^7 CFU/ml of target cells into fresh broth containing nisin U, incubating them at 30°C, and at intervals removing aliquots, which were subjected to serial dilution in 1/4-strength Ringer’s solution and plated on GM17 agar. All growth experiments were performed in triplicate with samples from three separate overnight cultures and repeated on at least three different days. These assays revealed that after 4 h, the numbers of HP bacteria expressing nsuFEG and spiFEG were significantly (P < 0.014) greater than those of the corresponding HP control (Fig. 3).

Assessing the relative protection provided through heterologous expression of spiFEGRR K. We postulated that the products of spiRR K may sense and respond to the presence of nisin to further enhance the expression of spiFEG and nisin resistance. However, should such a phenomenon exist, it would be mediated through the nis box within the promoter upstream of spiFEG (P_nis). To investigate this possibility, heterologous expression was again employed. P_nis-spiFEGRR K was amplified using primers GGGGTACCGAAGGTTGGACAGAAGTTTGG and GCTGCAGACCATGTCGTAAATAGTCGTTTTTTC and digested with the appropriate restriction enzymes (Fastdigest; Fermentas) (21). This was ligated with similarly digested pCI372 (a shuttle vector) which, unlike pNZ44, does not contain a constitutive promoter to drive the expression of cloned genes) and transformed into electrocompetent L. lactis HP. A phenotypic assay was performed to assess if exposure to sublethal concentrations of nisin A or nisin U enhanced the ability of pCI372P_nis-spiFEGRR K to provide protection from a subsequent challenge with concentrated nisin. Specifically, overnight cultures of L. lactis HP/pCI372 and HP/pCI372P_nis-spiFEGRR K were inoculated (3%) into fresh GM17 broth and incubated until they reached an optical density at 600 nm of 0.3, whereupon 1 ml of cells was exposed to a sublethal concentration (0.3 μM) of nisin A or nisin U for 1 h in 1.5-ml tubes at 30°C. The cells were then washed in 10 mM sodium phosphate buffer and seeded in 1/100-strength GM17-agarose into which wells had been bored. Approximately 30 μM nisin A and nisin U were inoculated in the wells for 3 h and subsequently overlaid with 2× GM17-agarose to allow growth of L. lactis strains (see references 13 and 30 for the method used). Having determined relative sensitivity on the basis of zone size, we found that in no instance did exposure to a sublethal concentration of nisin significantly enhance the protection provided by pCI372P_nis-spiFEGRR K to subsequent exposure to higher concentrations of nisin (Fig. 4). It should be noted, however, that this does not preclude the possibility that SpiRR K sense and respond to the presence of nisin in their native background.

As a consequence of the continued emergence of antibiotic-resistant bacteria, the possibility of using ribosomally synthesized antimicrobial peptides such as the lantibiotics as alternative chemotherapeutic agents has received attention (34). Despite nisin having been used for over half a century for food applications, the development of resistance has not become a problem. Nonetheless, it has been established that some bacteria possess innate nisin resistance mechanisms and that others can become resistant upon exposure to nisin in the laboratory (8, 9, 27). It is thus a concern that the use of nisin and other lantibiotics for clinical applications could also result in the emergence of resistant strains. However, it is hoped that by developing a clearer understanding of the various different mechanisms by which resistance can emerge, it will be possible to develop strategies to counteract such occurrences. The phenomenon of resistance through immune mimicry has been described on only one previous occasion (14), and thus, the identification of nisin immunity determinants in the genome of BAA-102 is noteworthy. On the basis of these findings, this phenome-
non may be more common than has previously been appreciated, and the possibility that the presence and transfer of such genes could potentially lead to the emergence of lantibiotic-resistant strains needs to be considered carefully.

ACKNOWLEDGMENTS

This work was supported by the Irish Government under the National Development Plan through Science Foundation Ireland Investigator awards 06/IN.1/B98 and 10/IN.1/B3027.

REFERENCES


FIG 4  L. lactis HP expressing the genes spiFEGRR’K under the control of their native promoter in the vector pCI372 was assessed to discover if immunity could be induced in the presence of nisin. L. lactis HP was incubated in the presence of approximately 10 ng of either nisin A (A, B) or nisin U (C, D) prior to being challenged by agarose well diffusion assay with both nisin A (A, C) and nisin U (B, D). L. lactis HP/pCI372 alone was included as a control. The data show that the action of the spiFEG genes has not been induced and thus they are not active under these conditions.


