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 antimastitis 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 F (12) are produced by *Lactococcus lactis* strains, while nisin U, nisin U2, and nisin U3 are produced by *Streptococcus uberis* (41, 42). These variants differ from each other by as many as 11 residues across the 31- to 34-amino-acid peptides (17). The large differences between the three nisin U's and the other nisins is so significant that it could be argued that they are not, in fact, nisin variants but rather members of a distinct lantibiotic subfamily (35). As is the case with all lantibiotics, nisin producers possess immunity mechanisms which provide protection from autolethality. Despite the diversity of these peptides, the phenomenon of cross-immunity has been observed in some instances. For example, nisin U-producing strains are immune to nisins U, A, and Z (42). Similarly, the nisin A-producing strain is protected from the activity of nisin U (42). Lantibiotic immunity is provided by one or more systems consisting of a dedicated immunity peptide, LanI, or an ABC transporter which pumps the lantibiotic out of the cell, designated LanFE(G). A third immunity protein, LanH, is also present in some cases and acts as an accessory protein to the ABC transporter system (reviewed in references 7 and 15). In the case of the nisin family of lantibiotics, immunity is based on the action of both a LanFEG system and LanI protein. Despite the cross-protection referred to above, cross-immunity between lantibiotic producers is rare, with only a few exceptional examples (3, 22). Indeed, cross-immunity between producers of the closely related nisin A and subtilin peptides (63% identity) is not evident. Another unusual phenomenon is immune mimicry, whereby non-lantibiotic-producing strains express functional homologues of lantibiotic immunity systems. In the only study of this phenomenon to date, homologues of immunity genes associated with the

lantibiotic lacticin 3147 were identified in *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 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 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
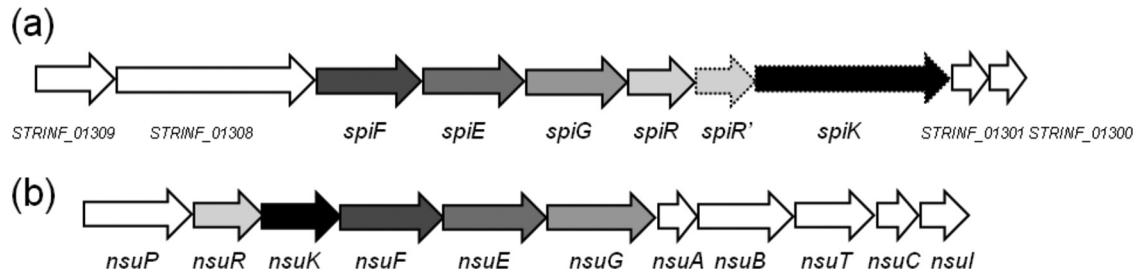
FIG 1 Genetic orientation of homologues of the nisin U (a) and nisin-associated immunity and regulatory (b) genes found in *S. infantarius* subsp. *infantarius*. The up- and downstream genes are included, and from BlastP analysis, their theoretical functions are as follows: STRINF_01300, UDP-*N*-acetyl-D-glucosamine 2-epimerase; STRINF_01301, hypothetical protein; STRINF_01308, hypothetical ATP-binding protein; STRINF_01309, hypothetical membrane protein.

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 NsuRK 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 NsuR, with an e value of 1e-50 (and here referred to as *spiR*), and the adjacent gene (STRINF_01303; *spiR′*) also encodes an NsuR-like protein. Although SpiR and SpiR′ are 67% identical to NisR, 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, mersacidin, 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 SpiR′. 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. uberis*, *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, on the basis of deferred-antagonism assays (for the method, see reference 17), less sensitive to nisin U than are a number of other *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 *nsuFEG* genes from *S. uberis* 42 (24, 42) served as a positive control. To facilitate this, genomic DNA was extracted from *S. uberis* as described previously (14). The *nsuFEG* genes were amplified using primers AA<u>ACTGCAG</u>AAGTAGCAACTAGAAAG and GG<u>GGTACCC</u>TTTTAGGTGGCTAGTATCGC, and the primers for *spiFEG* were AAA<u>ACTGCAG</u>AAAAGTTTGGGACTTCAATG and GG<u>GGTACCC</u>CTGTCACCTCAATTGTATTTG, 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 agars 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/pNZ44*nsuFEG*, 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 pNZ44*spiFEG* also provided

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[a]

Nisin variant	Avg zone size (mm) ± SD			
	HP (control)	HP pNZ44*nsuFEG*	HP pNZ44*spiFEG*	*S. infantarius*
A	18.6 ± 0.71	16.3 ± 0.71	17.85 ± 1.06	2.94 ± 0.18
F	20.6 ± 1.00	17.79 ± 0.27	19.67 ± 1.00	5.84 ± 0.50
Z	24.4 ± 0.36	20.2 ± 0.14	21.85 ± 1.62	4.9 ± 0.74
Q	16.08 ± 0.65	14.8 ± 0.28	15.05 ± 1.62	4.17 ± 0.75
U	8.4 ± 0.42	0	4.7 ± 1.27	0[b]
U3	16.06 ± 0.18	0	12.2 ± 2.97	0[b]

[a] Values are averages of triplicate experiments and represent zone sizes, i.e., diameter of zone minus diameter of bacterial growth.
[b] No distinct zone but some hazy growth adjacent to nisin-producing colony.

protection against nisin U, with zone sizes decreasing substantially. The SpiFEG system is thus capable of providing protection through immune mimicry. The abilities of pNZ44*nsuFEG* and pNZ44*spiFEG* 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 *nsuFEG* 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/pNZ44*nsuFEG*

to all forms of nisin and of HP/pNZ44*spiFEG* to nisins Z and U. However, in this instance, HP/pNZ44*spiFEG* 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/pNZ44*spiFEG* to this antibiotic was tested via antibiotic disc assays (10 IU; 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 β-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 C$_{18}$-E column (Phenomenex, Cheshire, United Kingdom) preequilibrated with methanol and water. The columns were 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 μm Proteo 90 Å; 250 by 10.0 mm, 4 μm) 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
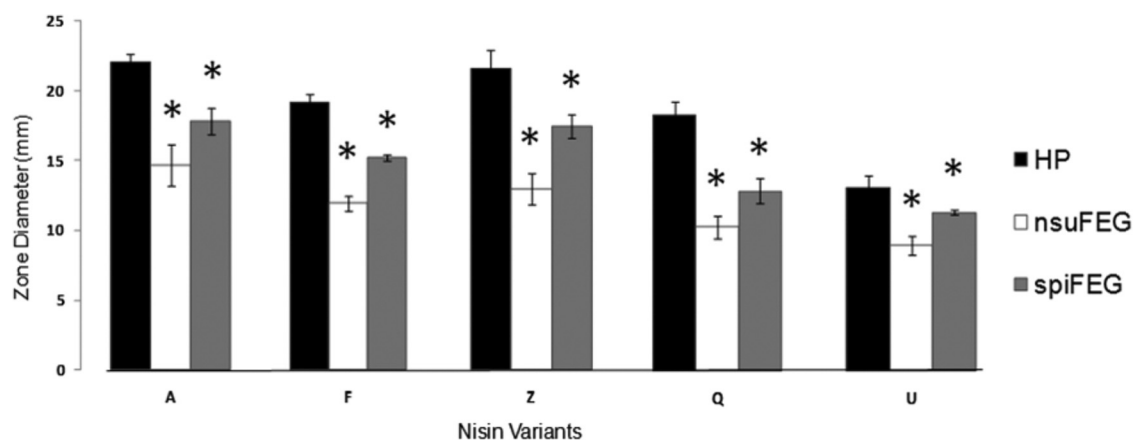

FIG 2 Agarose well diffusion assay, whereby *L. lactis* HP/pNZ44 and strains expressing *nsuFEG* and *spiFEG* were challenged under adverse growth conditions with nisins A, F, Z, Q, and U. Asterisks indicate zone diameters which were significantly smaller by Student's *t* test ($P < 0.0005$) than that found in *L. lactis* HP, hence implying the protective capabilities of these genes.
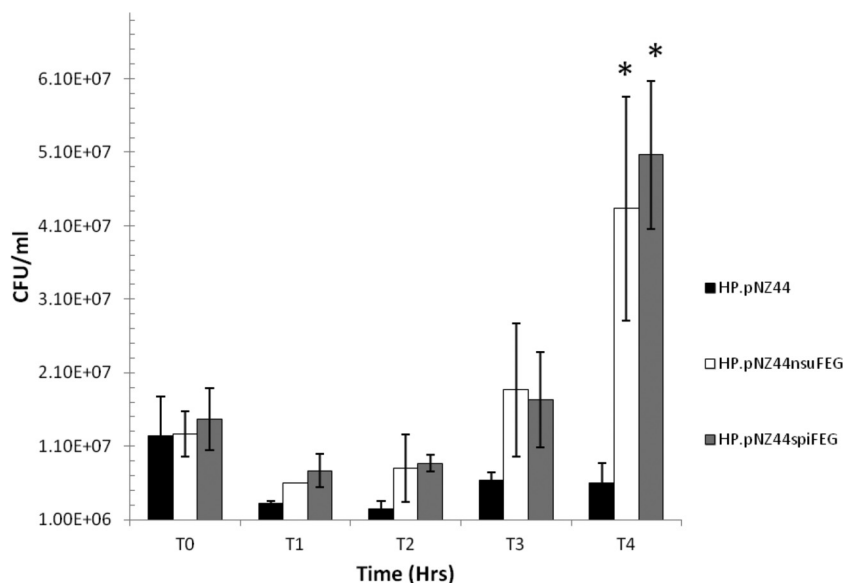
FIG 3 Survival and growth of *L. lactis* strain HP and strains expressing NsuFEG and SpiFEG when challenged with a sublethal level (416 nM) of nisin U. An asterisk at T4 indicates that the difference between strain HP/pNZ44 and the strains expressing immunity genes is statistically significant (Student's *t* test $P <$ 0.014).

with an Axima CFR plus matrix-assisted laser desorption ioniza-tion–time of flight (MALDI-TOF) mass spectrometer as previ-ously 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*nsuFEG*, and HP/pNZ44*spiFEG* 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 sig-nificantly ($P < 0.014$) greater than those of the corresponding HP control (Fig. 3).

Assessing the relative protection provided through heterol-ogous expression of *spiFEGRR′K*. We postulated that the prod-ucts 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_{spi}). To investigate this possibility, heterologous expression was again employed. P_{spi}-*spiFEGRR′K* was amplified using primers GGGGGTACCGAAGGTTGGACAGAAGTTTGG and GCTGCAG ACCATGTCGTAAATAGTCGTTTTTTC 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 elec-trocompetent *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$_{spi}$-*spiFEGRR′K* to provide pro-tection from a subsequent challenge with concentrated nisin. Spe-cifically, overnight cultures of *L. lactis* HP/pCI372 and HP/

pCI372P$_{spi}$-*spiFEGRR′K* were inoculated (3%) into fresh GM17 and incubated until they reached an optical density at 600 nm of 0.3, whereupon 1 ml of cells was exposed to a sublethal concen-tration (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 incubated in the wells for 3 h and subsequently overlaid with 2× GM17-agarose to allow growth of *L. lactis* strains (see refer-ences 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$_{spi}$-*spiFEGRR′K* to subsequent exposure to higher concentrations of nisin (Fig. 4). It should be noted, however, that this does not preclude the possi-bility 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 che-motherapeutic 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. Nonethe-less, 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 iden-tification of nisin immunity determinants in the genome of BAA-102 is noteworthy. On the basis of these findings, this phenome-
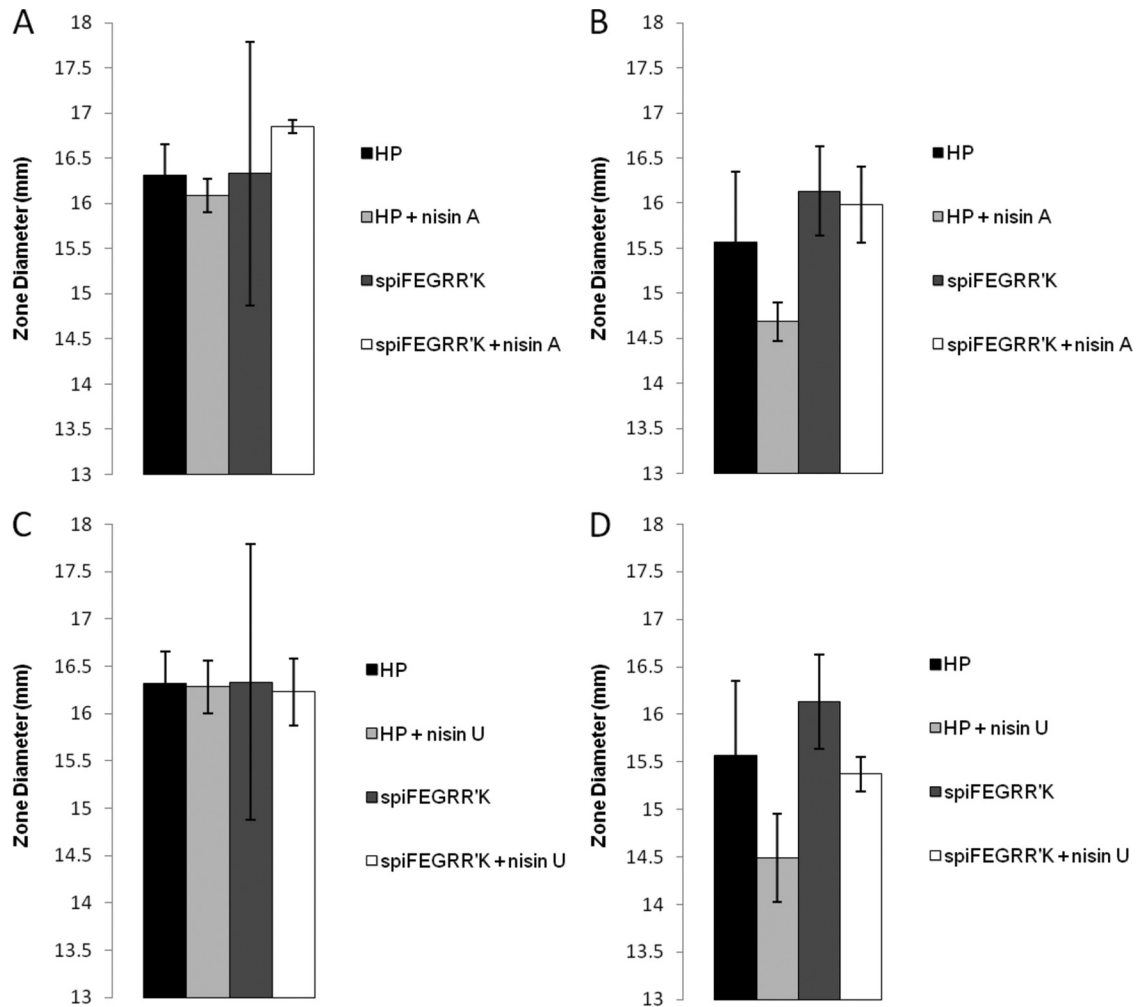
FIG 4 *L. lactis* HP expressing the genes *spiFEGRR′K* under the control of their native promoter in the vector pCI372 was assessed to discover if immunity could be induced in the presence of nisin. *L. lactis* HP was incubated in the presence of approximately 10 ng of either nisin A (A, B) or nisin 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.

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.

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