

Nisin H Is a New Nisin Variant Produced by the Gut-Derived Strain *Streptococcus hyointestinalis* DPC6484

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Accumulating evidence suggests that bacteriocin production represents a probiotic trait for intestinal strains to promote dominance, fight infection, and even signal the immune system. In this respect, in a previous study, we isolated from the porcine intestine a strain of *Streptococcus hyointestinalis* DPC6484 that displays antimicrobial activity against a wide range of Gram-positive bacteria and produces a bacteriocin with a mass of 3,453 Da. Interestingly, the strain was also found to be immune to a nisin-producing strain. Genome sequencing revealed the genetic determinants responsible for a novel version of nisin, designated nisin H, consisting of the *nshABTCPRKGEF* genes, with transposases encoded between *nshP* and *nshR* and between *nshK* and *nshG*. A similar gene cluster is also found in *S. hyointestinalis* LMG14581. Notably, the cluster lacks an equivalent of the nisin immunity gene, *nisI*. Nisin H is proposed to have the same structure as the prototypical nisin A but differs at 5 amino acid positions—Ile1Phe (i.e., at position 1, nisin A has Ile while nisin H has Phe), Leu6Met, Gly18Dhb (threonine dehydrated to dehydrobutyrine), Met21Tyr, and His31Lys—and appears to represent an intermediate between the lactococcal nisin A and the streptococcal nisin U variant of nisin. Purified nisin H inhibits a wide range of Gram-positive bacteria, including staphylococci, streptococci, *Listeria* spp., bacilli, and enterococci. It represents the first example of a natural nisin variant produced by an intestinal isolate of streptococcal origin.

Bacteriocins of lactic acid bacteria have received extensive attention in recent years given their structural diversity and activity and their potential as biopreservatives and anti-infectives. Indeed, the production of bacteriocins by intestinal bacteria is considered a probiotic trait and has been shown to be associated with strain dominance, infection control, and host cell signaling (1). One of the oldest and undoubtedly the most extensively characterized bacteriocins is nisin A, which was discovered by Rogers in 1928 (2). Nisin A is produced by many strains of *Lactococcus lactis*, a species widely used for cheese manufacture. It has a broad antimicrobial spectrum against a wide range of Gram-positive genera, including staphylococci, streptococci, *Listeria* spp., bacilli, and enterococci (3). Nisin A has been used in the food industry as a biopreservative for more than 50 years without inducing widespread microbial resistance (4, 5). The bacteriocin has multiple antimicrobial actions; it binds to the precursor of peptidoglycan, lipid II, to inhibit cell wall biosynthesis and then forms pores in the cell membrane, leading to the release of essential ions and, ultimately, cell death (6–8).

The nisin gene cluster in *L. lactis* is associated with a conjugative transposon and consists of *nisABTCIPRKFEF*, where *nisA* encodes the nisin prepropeptide. Immunity to nisin is provided by a specific immunity protein, NisI, and a specialized ABC transporter, NisFEG (9). The lipoprotein, NisI, most probably orients to the outside of the cytoplasmic membrane and binds nisin, preventing it from binding to lipid II and forming pores in the cell membrane (10, 11). NisFEG are thought to transport nisin from the cytoplasmic membrane to the external environment, thus preventing the accumulation of the high number of nisin molecules necessary for pore formation (12, 13). The extent to which nisin is produced is affected by the level of immunity of the producing microorganism. For maximal nisin immunity, both the lipoprotein and the nisin transporters are required (12, 14–16).

To date, eight natural nisin variants have been discovered (Fig. 1).

These include nisins Z, F, and Q, which have been isolated from lactococci, nisins U and U2, from *Streptococcus uberis*, and nisin P, which is encoded on nisin operons present in both *Streptococcus gallolyticus* subsp. *pasteurianus* (17) and *Streptococcus suis* (18). Nisin Z producers are very common, and the amino acid sequence differs from that of nisin A at a single position (His27Asn) (19, 20), a change that improves the solubility of the peptide at a neutral pH (21). The operon encoding nisin F was found on a plasmid in *L. lactis* F10, isolated from the intestinal tract of a freshwater catfish in South Africa. Nisin F differs from nisin A at 2 amino acid positions: His27Asn, as seen in nisin Z, and Ile30Val (22). Nisin Q is produced by *L. lactis* 61-14, isolated from a river in Japan, and differs from nisin A at 4 amino acid positions, i.e., those observed in nisin F as well as Ala15Val and Met21Leu (23). The *Streptococcus*-associated variants differ more considerably from nisin A. The *S. uberis* producers of nisin U and U2 were isolated in cases of bovine mastitis in the United States. Nisin U differs from nisin A at 9 positions—Ile4Lys, Ala15Ile, Gly18Thr, Asn20Pro, Met21Leu, His27Gly, Ser29His, Ile30Phe, and His31Gly—and also lacks the three C-terminal amino acids of nisin A. In addition to these changes, nisin U2 contains a further Ile1Val change (24). Finally,

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A	ITSISLCTPGCKTGALMGCNMTATCNCSEIHSVSK	3352 Da
Z	ITSISLCTPGCKTGALMGCNMTATCNCSEIHSVSK	3331 Da
F	ITSISLCTPGCKTGALMGCNMTATCNCSEIHSVSK	3315 Da
Q	ITSISLCTPGCKTGALMGCNMTATCNCSEIHSVSK	3327 Da
H	FTSISMCTPGCKTGALMTCNYKTATCNCSEIKVSK	3453 Da
U	ITSKSLCTPGCKTGILMTCPLKTATCGCHFG	3029 Da
U2	VTSKSLCTPGCKTGILMTCPLKTATCGCHFG	3015 Da
P	VTSKSLCTPGCKTGILMTCALKTATCGCHFG	2989 Da
sald	FTSHSLCTPGCTGVLMGCHIQSIGCNVHIHSK	3468 Da
	** * ***** ** * ***** *	

FIG 1 Alignment of natural nisin variants, with amino acid changes in bold-face. Asterisks mark conserved amino acid residues.

and most recently, a phylogenetic study of lanthipeptide synthetases by Zhang et al. (2012) (17) revealed an *S. gallolyticus* subsp. *pasteurianus* strain that encodes a structural gene with the potential to produce a new nisin analogue, nisin P. Nisin P is closely related to nisin U2, differing with respect to just 2 amino acids: Phe20 and Leu21 in nisin U2 are changed to Ala20 and Ile21 in nisin P. The more distantly related nisin-like lantibiotic salivaricin D, isolated from *Streptococcus salivarius* 5M6c, a human isolate, differs from nisin A at 17 positions, with most differences seen at the C-terminal end of the molecule (25).

In this study, we have identified a new nisin variant, designated nisin H, produced by a strain of *Streptococcus hyointestinalis* isolated from the porcine intestine. The name *S. hyointestinalis* was first employed in 1988 to reassign a number of strains that had previously been classified as *Streptococcus salivarius* (26) and was derived from the Greek noun *hyos*, meaning pig, and the Latin adjective *intestinalis*, which reflects the association of the strains with the porcine intestine. Previously, an *S. hyointestinalis* isolate producing a broad-spectrum antimicrobial that inhibits bifidobacteria, lactobacilli, *Leuconostoc* spp., *Listeria* spp., *Staphylococcus aureus*, and *Streptococcus agalactiae* was isolated as part of a mammalian-gut-mining study by O'Shea et al. (2009) (27). Since this represented the first report of an *S. hyointestinalis* strain that produces an antimicrobial, we sequenced and analyzed the genome of this strain. Ultimately, this led to the isolation, characterization, and identification of a novel nisin variant, which we designate nisin H, produced by a gut-derived strain.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. Anaerobic conditions were generated through the use of anaerobic jars containing Anaerocult A gas packs (Merck, Darmstadt, Germany). Agar (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) was added (1%, wt/vol) to broth media when agar plates were required.

Isolation of DNA for PCR analysis. DNA was extracted from culture cell pellets for PCR analysis with a GenElute bacterial genomic DNA kit (Sigma-Aldrich, County Wicklow, Ireland), and molecular manipulation techniques from the work of Sambrook and Russell (2001) (28) were used when required. Oligonucleotide primers were synthesized by Sigma-Genosys (Poole, Dorset, United Kingdom), and purified PCR amplicons were sequenced by Beckman Coulter Genomics (Essex, United Kingdom). DNA was amplified with MyTaq DNA polymerase (Biolone, London, United Kingdom) according to the manufacturer's instructions. PCRs were carried out in a Techne TC-512 thermal cycler (Bibby Scientific, Staffordshire, United Kingdom).

Genome sequencing and analysis of the nisin H gene cluster. The sequence of the genomic DNA extracted from *S. hyointestinalis* DPC6484 was determined by 454 pyrosequencing (Beckman Coulter Genomics, USA). The resulting sequence reads were assembled into contigs using the Newbler package. Coding regions in the draft genome assembly were predicted using GLIMMER, version 2.0 (29), and annotation was subsequently determined using the GAMOLA software package (30). Sequence similarity analyses were performed using the gapped BLASTp algorithm and the nonredundant database provided by the NCBI ([ftp://ftp.ncbi.nih.gov/blast/db](http://ftp.ncbi.nih.gov/blast/db)) (31). By using the ARTEMIS genome viewer (32), components of the nisin H gene cluster were identified on two distinct contigs. PCR with the primer pair comprising 5' GTTGACTTATTGAGCGAGG 3' and 5' GCCAACTTATTACGTTCTTAC 3', designed to be specific to the sequences flanking the 3' and 5' termini of the respective contigs, confirmed the contiguous nature of this gene cluster. The annotation of the gene cluster was then verified manually. The sequence data were aligned and analyzed by using LASERGENE software (DNASar Inc., Madison, WI).

The genome was searched for the presence of a *nisI* immunity gene equivalent with primers designed to be specific to the *nisI* immunity gene of *L. lactis* NZ9700 and the *nsul* immunity gene of *S. uberis* 42 (Table 2).

Purification of the antimicrobial produced by *S. hyointestinalis* DPC6484. The antimicrobial was purified from the cell-free supernatant (CFS) of a 2-liter culture of *S. hyointestinalis* DPC6484 grown in tryptic soy broth (TSB) at 37°C overnight. The culture supernatant was applied to a 90-ml SP Sepharose column (GE Healthcare, Uppsala, Sweden) pre-equilibrated with 50 mM sodium acetate buffer, pH 4.4 (buffer A). The column was washed with 300 ml of buffer A, and the antimicrobial activity

TABLE 1 Bacterial strains and culture conditions

Strain	Growth medium ^a	Incubation:	
		Temp (°C)	Condition
<i>Streptococcus hyointestinalis</i> DPC6484	GM17 or TSB	37	Anaerobic
<i>Streptococcus hyointestinalis</i> LMG14579	GM17 or TSB	37	Anaerobic
<i>Streptococcus hyointestinalis</i> LMG14581	GM17 or TSB	37	Anaerobic
<i>Streptococcus hyointestinalis</i> LMG14582	GM17 or TSB	37	Anaerobic
<i>Streptococcus hyointestinalis</i> LMG14583	GM17 or TSB	37	Anaerobic
<i>Streptococcus hyointestinalis</i> LMG14585	GM17 or TSB	37	Anaerobic
<i>Streptococcus hyointestinalis</i> LMG14586	GM17 or TSB	37	Anaerobic
<i>Streptococcus hyointestinalis</i> LMG14587	GM17 or TSB	37	Anaerobic
<i>Lactococcus lactis</i> NZ9700	GM17	30	Aerobic
<i>Streptococcus uberis</i> strain 42	GM17	37	Anaerobic
<i>Lactococcus lactis</i> DPC3251	GM17	30	Aerobic
<i>Escherichia coli</i> DPC6912	LB	37	Aerobic
<i>Bacillus cereus</i> 9139	BHI	37	Aerobic
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> HP	GM17	30	Aerobic
<i>Enterococcus faecalis</i> 6307	LB	37	Aerobic
<i>Streptococcus agalactiae</i> ATCC 13813	BHI	37	Anaerobic
<i>Streptococcus agalactiae</i> DPC5338	BHI	37	Anaerobic
<i>Streptococcus bovis</i> DPC6491	BHI	37	Anaerobic
<i>Streptococcus gallolyticus</i> DPC6501	BHI	37	Anaerobic
<i>Listeria innocua</i> DPC3572	BHI	37	Aerobic
<i>Listeria monocytogenes</i> 1042	BHI	37	Aerobic
<i>Staphylococcus aureus</i> ATCC 25923	BHI	37	Aerobic
<i>Staphylococcus aureus</i> DPC5245	BHI	37	Aerobic
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> LMG6901	MRS	37	Aerobic

^a GM17, M17 medium with 5 g/liter glucose added (Difco Laboratories, Detroit, MI); TSB, tryptic soy broth (Difco Laboratories, Detroit, MI); BHI, brain heart infusion (Merck, Darmstadt, Germany); LB, Luria-Bertani medium or lysogeny broth (Merck, Darmstadt, Germany); MRS, de Man, Rogosa, and Sharpe medium (Difco Laboratories, Detroit, MI).

TABLE 2 List of primers used in this study

Gene	Primer pair sequence	Product size (bp)	Annealing temp (°C)
<i>nshH gap</i>	5' GTTGACTTATTGAGCGAGG 3' 5' GCCAACTTATTACGTTCTTCAC 3'	2,267	60
<i>nisI</i>	5' GGAATAAGTGGCTGTATACTGG 3' 5' GAGAGTAACTGTTGTGAATTTG 3'	903	50
<i>nshI</i>	5' TAGTTGCATGTAGATTGGTAG 3' 5' ATACGTGCTATTCTATATTCA 3'	619	48
<i>nshT</i>	5' CTCTCCTCTTGTATTATCCC 3' 5' GTAGAGAGCCATTAGATTGG 3'	702	53
<i>nshA</i>	5' CTAATAAGTAAACAGATTG 3' 5' GTTGTCATCTTCATATG 3'	481	50
<i>nshF</i>	5' GCTAGTCAGAATCGCCATAG 3' 5' GTCTGGCACTGTATGCCG 3'	324	60
<i>nshR</i>	5' TAGAGGAAAGAAGTGTATGTG 3' 5' TTGGGTTTACTTCATATGTAG 3'	177	53

was eluted in 300 ml of buffer A containing 1 M NaCl. The acetonitrile was removed by rotary evaporation, and the sample was applied to a 5-g, 20-ml Strata C₁₈-E solid-phase extraction (SPE) column (Phenomenex, Cheshire, United Kingdom) preequilibrated with methanol and water. The column was washed with 20 ml of 25% ethanol, and the antimicrobial activity was eluted with 20 ml of 70% 2-propanol–0.1% trifluoroacetic acid (TFA). The antimicrobial activities of cell-free supernatants and eluents from purification protocols were determined via the agar well diffusion assay described by Ryan et al. (1996) (33). *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG6901 was used as the indicator strain, and bioactivity was assessed following aerobic incubation of plates overnight at 37°C.

The 2-propanol was removed by rotary evaporation, and the sample was applied to a Phenomenex Jupiter Proteo reversed-phase high-performance liquid chromatography (RP-HPLC) column (length, 10 mm; inside diameter, 250 mm; particle size, 4 µm, pore size, 90 Å) running a 25-to-45% acetonitrile–0.1% TFA gradient over 35 min at 2.5 ml/min. The resultant eluent was monitored at 214 nm, and fractions were collected at 1-min intervals. Fractions were assayed for antimicrobial activity by a well diffusion assay with *L. delbrueckii* subsp. *bulgaricus* LMG6901 as the indicator strain, and those containing antimicrobial activity were analyzed via matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS) to determine the molecular mass of the antimicrobial peptide and to assess peptide purity. HPLC fractions deemed pure by MALDI-TOF MS were combined and were lyophilized in a Genevac (Suffolk, United Kingdom) lyophilizer. MALDI-TOF mass spectrometry was performed with an Axima TOF² MALDI-TOF mass spectrometer in positive-ion reflectron mode (Shimadzu Biotech, Manchester, United Kingdom). N-terminal sequencing (Edman degradation) of purified nisin H was performed by Abingdon Health Laboratory Services (Birmingham, United Kingdom).

Pure nisin A peptide was prepared from *L. lactis* NZ9700 as described for nisin H.

Cross immunity of *S. hyointestinalis* DPC6484 to other nisin-producing cultures. The cross immunity of *S. hyointestinalis* DPC6484 to the bacteriocins produced by *L. lactis* NZ9700 (nisin A), *S. uberis* strain 42 (nisin U), and *L. lactis* DPC3251 (lactacin 3147) and to *L. delbrueckii* subsp. *bulgaricus* LMG6901, a non-bacteriocin producer used as a bacteriocin-sensitive strain, was determined by spotting 50-µl aliquots of cell-free culture supernatants onto indicator plates seeded with 1% (vol/vol) of each of these strains. *L. delbrueckii* subsp. *bulgaricus* LMG6901, known

to be sensitive to each of these nisin variants and to lactacin 3147, was used as an indicator strain to confirm the production of nisins A, H, and U and lactacin 3147 by the respective strains.

Comparison of the inhibitory activities of pure nisin A and nisin H peptides. Purified nisin A and H peptides were resuspended at 0.22 mg/ml in 35% 2-propanol for optimum solubility. Aliquots (50 µl) of each peptide were tested for antimicrobial activity by well diffusion using the indicator strains listed in Table 4. The culture media and incubation conditions are outlined in Table 1. A 50-µl aliquot of 35% acetonitrile was assayed against each strain to ensure that it did not inhibit any of the test strains.

Genomic profiles of *S. hyointestinalis* strains. Molecular fingerprinting of *S. hyointestinalis* isolates was performed by pulsed-field gel electrophoresis (PFGE) as described by Simpson et al. (2002) (34) using SmaI restriction endonucleases and DNA molecular weight markers (9.42 to 242.50 kb; New England BioLabs, Beverly, MA). DNA fragments were resolved with a CHEF (contour-clamped homogeneous electric field) DRIII pulsed-field system (Bio-Rad Laboratories) at 6 V/cm for 18 h with a 1- to 30-s linear ramp time to resolve bands.

Assessment of the distributions of *nshA*, *nshF*, *nshR*, and *nshT* in *S. hyointestinalis* strains. The presence of the *nshA*, *nshF*, *nshR*, and *nshT* genes in *S. hyointestinalis* DPC6484 and *S. hyointestinalis* strains obtained from the BCCM/LMG culture collection were checked using gene-specific primer pairs. The primer pairs and the sizes of the expected gene products are given in Table 2.

Nucleotide sequence accession number. The sequence of the nisin H gene cluster of *Streptococcus hyointestinalis* DPC6484 is available from GenBank/EMBL under accession number KP793707.

RESULTS

Genome sequencing of *S. hyointestinalis* DPC6484 reveals a nisin-like gene cluster. In a previous study, which involved the screening of mammalian samples from the gastrointestinal tracts of humans, pigs, and cows, we identified *S. hyointestinalis* DPC6484, a strain that inhibits bifidobacteria, lactobacilli, *Leuconostoc* spp., *Listeria* spp., *S. aureus*, and *S. agalactiae* (27). Given that antimicrobial production has not been attributed to an *S. hyointestinalis* strain previously, the genome of DPC6484 was sequenced with a view to the identification of the gene cluster responsible for this phenotype. Analysis of the draft genome revealed the presence of a nisin homologue and of associated biosynthesis genes on two contiguous sequence regions. The assembled gene cluster, of ~15.8 kb (Fig. 2), was found to contain a putative nisin variant-encoding structural gene designated *nshA* (*nsh* for nisin from *S. hyointestinalis*, or nisin H) followed by homologues of *nisBTCP* (designated *nshBTCP*), a region encoding a streptococcal transposase, the equivalents of *nisRK* (designated *nshRK*), another region encoding a streptococcal transposase, and *nisFEG*-like genes (designated *nshFEG*). A notable feature was the absence of an equivalent of the *nisI* immunity gene. Further investigation of the *S. hyointestinalis* strain via a BLAST analysis on the draft genome sequence and PCR-based approaches suggested the absence of an obvious NisI homologue (data not shown).

The protein sequences ranged from 54% identity with the lactococcal equivalent for NshG to 82% identity for NshA. The predicted product of *nshA* is a 57-amino-acid peptide that, on the basis of comparison with other nisin peptides, is likely to consist of a 23-amino-acid leader and a 34-amino-acid propeptide. The putative propeptide differs from the corresponding nisin A peptide at five positions: Ile1Phe, Leu6Met, Gly18Thr, Met21Tyr, and His31Lys (Fig. 1).

Purification and predicted structure of nisin H. The nisin H

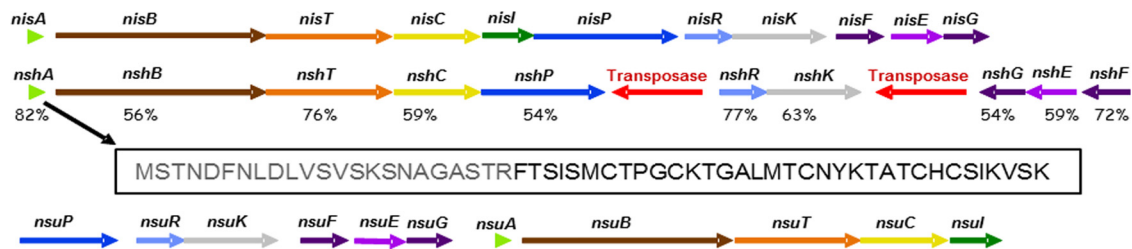


FIG 2 Representation of the bacteriocin-encoding *nshA* gene cluster as revealed by genome sequencing (center), compared with the *nisA* (top) and *nsuA* (bottom) gene clusters. The *nshA* gene cluster contains the nisin production genes *nshABT*, a gap region encoding a transposase, *nshRK*, a region encoding a second transposase, and *nshFEG*. For each gene, the percentage of amino acid identity to the protein encoded by the corresponding nisin A-associated gene is presented. The amino acid sequence of the unmodified NshA peptide is shown below the gene cluster. Residues predicted to be within the leader peptide are shown in gray, and those thought to correspond to the structural peptide are shown in black.

peptide was purified using SP Sepharose cation-exchange SPE, C_{18} SPE, and reversed-phase HPLC. The HPLC chromatogram (Fig. 3) shows a dominant peak corresponding to a fraction that inhibited the indicator strain, *L. delbrueckii* subsp. *bulgaricus* LMG6901. This purification strategy typically yielded 0.15 mg/liter, which is lower than the 0.50 mg/liter of nisin A recovered from a corresponding starting volume by using the nisin A producer *L. lactis* NZ9700.

The first 10 amino acids of the predicted NshA propeptide are FTSISMCTPG (Fig. 2). Lantibiotics can be difficult to sequence using Edman degradation, because the dehydrated amino acids and ring structures are not compatible with this technique. Nonetheless, Edman sequencing of the newly purified antimicrobial revealed a sequence consisting of F-X-X-X-M-X-X-P-G. This sequence conforms to the gene predictions of the identifiable residues at positions 1, 6, 9, and 10 and is consistent with the predicted presence of modified residues at positions 2, 3, 5, 7, and 8. MALDI-TOF MS analysis revealed a molecular mass of 3,453 Da, which is consistent with a modified form of the NshA peptide. MALDI-TOF MS also showed a difference of 101 Da between the molecular mass of nisin A (3,352 Da) and that of the antimicrobial produced by DPC6484 (3,453 Da) (data not shown). This difference precisely matches the molecular mass differences expected from the predicted amino acid changes and the likely dehydration of the additional threonine residue. Converting Ile to Phe, Leu to

Met, Gly to Thr, and Met to Tyr results in 34-, 18-, 44-, and 32-Da increases, respectively, and results in a total peptide mass of 3,480 Da. In addition, the alteration of His to Lys results in a 9-Da loss, giving a mass of 3,471 Da, 18 Da higher than the 3,453-Da mass for nisin H. However, in nisin molecules, threonine is always dehydrated to dehydrobutyryne (Dhb), thus accounting for this 18 Da. It is thus apparent that the purified antimicrobial, referred to below as nisin H, represents a modified form of the NshA peptide with 5 substitutions. The proposed structure of nisin H, modeled on known structures of nisin variants, is shown in Fig. 4.

Nisin A- and nisin H-producing strains are cross immune. The nisin H producer was tested to assess its cross immunity to CFSs from producers of nisins A and U. CFSs from nisin A-, H-, and U-producing cultures inhibited the growth of the indicator strain *L. delbrueckii* subsp. *bulgaricus* LMG6901, as expected (Table 3). Nisin A- and U-containing CFSs did not inhibit *S. hyointestinalis* DPC6484 or *L. lactis* NZ9700. However, CFSs from the nisin A and H producers inhibited the nisin U-producing *S. uberis* strain 42, suggesting that *S. uberis* is not cross immune to nisin A or nisin H. Further analysis with purified nisins revealed that at the concentrations used (0.22 mg ml^{-1}), the immunity mechanisms are overwhelmed, in that purified nisin A and nisin H generate zones of inhibition with areas of 0.32 cm^2 against the strains that produce these peptides and zones that are twice as large (0.69 cm^2) against the opposing producer. Notably, however, these peptides

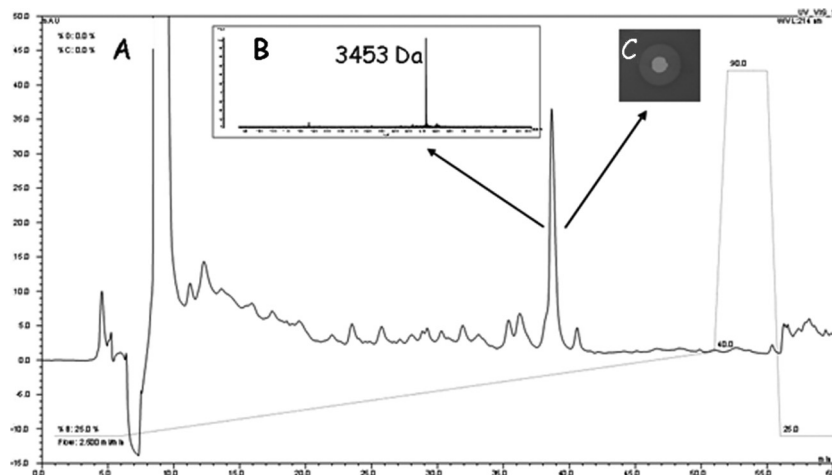


FIG 3 Purification of nisin H from *Streptococcus hyointestinalis* DPC6484 grown in TSB broth. (A) RP-HPLC chromatogram; (B) MALDI-TOF MS of the active fraction; (C) zone of inhibition of an aliquot of the HPLC fraction on an *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG6901 indicator plate.

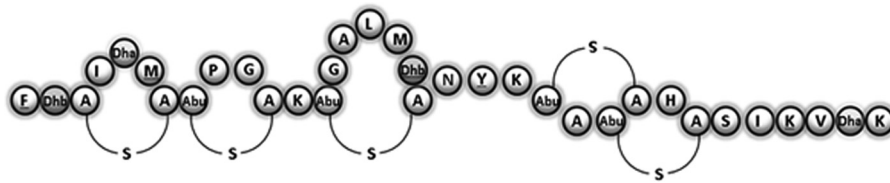


FIG 4 Proposed structure of the new natural nisin variant nisin H.

produce significantly larger zones, with areas of 3.39 and 2.38 cm², respectively, against *S. uberis* strain 42 (Table 4).

The activities of purified nisin A and nisin H peptides against *Escherichia coli* DPC6912, *Bacillus cereus* 9139, *L. delbrueckii* subsp. *bulgaricus* LMG6901, *Lactococcus lactis* subsp. *cremoris* HP, *Enterococcus faecalis* 6307, *S. agalactiae* ATCC 13813, *S. agalactiae* DPC5338, *Streptococcus bovis* DPC6491, *S. gallolyticus* DPC6501, *Listeria innocua* DPC3572, *Listeria monocytogenes* 1042, *S. aureus* ATCC 25923, *S. aureus* DPC5245, *S. hyointestinalis* DPC6484, and *S. uberis* strain 42 were assessed (Table 4). In the majority of instances, nisin A was more inhibitory than nisin H. However, nisin H was more effective than nisin A against one of the *S. aureus* strains tested, DPC5245, and the two peptides were equally effective against *L. innocua* DPC3572.

Not all *S. hyointestinalis* strains produce nisin H. PFGE of *S. hyointestinalis* DPC6484 and seven *S. hyointestinalis* strains from the BCCM/LMG culture collection confirm different banding patterns; thus, these strains are not clonal (Fig. 5A). Efforts to amplify each of the genes *nshA*, *nshF*, *nshR*, and *nshT* by PCR using specific primers (Table 2) resulted in the generation of amplicons of the appropriate sizes (Fig. 5Bi) for the nisin H producer *S. hyointestinalis* DPC6484 (positive control) and *S. hyointestinalis* LMG14581 but not for the other *S. hyointestinalis* strains. DNA sequencing of the *S. hyointestinalis* LMG14581 amplicons confirmed the presence of *nsh* equivalents in this strain. Although *S. hyointestinalis* LMG14581 contains *nsh* gene equivalents, this strain did not produce a zone of inhibition against *L. delbrueckii* subsp. *bulgaricus* LMG6901 (Fig. 5Bii).

DISCUSSION

Nisin H is of interest for several reasons. First, it is notable by virtue of being a nisin-like bacteriocin that is produced by an intestinal strain. As such, production of the bacteriocin has the

TABLE 3 Cross immunity of *Streptococcus hyointestinalis* DPC6484 to other nisin-producing strains

Target organism	Area of zone of inhibition (cm ²) ^a with CFS containing the following bacteriocin:			
	Nisin A	Nisin H	Nisin U	Lacticin 3147
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> LMG6901	11.41 ± 0.64	8.01 ± 0.00	8.17 ± 0.28	2.66 ± 0.00
<i>L. lactis</i> NZ9700	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.66 ± 0.14
<i>S. hyointestinalis</i> DPC6484	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.75 ± 0.30
<i>S. uberis</i> strain 42	0.32 ± 0.00	0.69 ± 0.00	0.00 ± 0.00	0.97 ± 0.24
<i>L. lactis</i> DPC3251	0.32 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

^a Calculated as $(\pi r_1^2) - (\pi r_2^2)$, where r_1 is the radius of the zone and r_2 is the radius of the well (in centimeters).

potential to give the strain a competitive advantage in the gut environment, either by directly inhibiting competitor bacteria or by facilitating communication with other strains or even the host. In addition, nisin H may also have a role in signaling to the host, since bacteriocin production has been associated with immunomodulatory effects often mediated through cytokine responses (35–37). Second, nisin H seems to represent an evolutionary link between lactococcal and streptococcal nisins in that, while it is quite different from nisin A by virtue of having 5 separate substitutions, it retains key features of the lactococcal peptides, including the three C-terminal amino acids, which are absent from the *Streptococcus*-associated nisin U. Third, the nisin H gene cluster is the only nisin gene cluster to lack an equivalent of the *nisI* immunity gene.

The lactococcal nisin A gene cluster (Fig. 2) encodes nisin production genes in the order *nisABTCIPRKFE*G, and this gene order is conserved in the corresponding nisin Z and nisin Q clusters (38). The gene order is different in streptococcal nisin gene clusters. In the nisin U gene cluster, *nsuPRKFE*G are at the start of the gene cluster, i.e., before the *nsuABTCI* genes, suggesting a rearrangement of *nsuABTCI* and *nsuPRKFE*G in *S. uberis* (24). Gene clusters containing structural genes for nisin analogues have also been identified in *S. gallolyticus* subsp. *pasteurianus* (17, 39), *S. agalactiae* (40), and, most recently, *S. suis* (18). The gene order in these gene clusters is identical to that for nisin U. It would seem

TABLE 4 Spectra of inhibition of purified nisin A and nisin H peptides against a range of strains

Target microorganism	Area of zone of inhibition (cm ²) ^a with purified peptides of:	
	Nisin A	Nisin H
<i>Escherichia coli</i> DPC6912	0.00 ± 0.00	0.00 ± 0.00
<i>Bacillus cereus</i> 9139	1.42 ± 0.14	1.03 ± 0.12
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> LMG6901	23.83 ± 0.89	16.64 ± 0.38
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> HP	9.47 ± 0.51	6.52 ± 0.25
<i>Enterococcus faecalis</i> 6307	6.24 ± 0.00	3.94 ± 0.00
<i>Streptococcus agalactiae</i> ATCC 13813	6.10 ± 0.25	2.66 ± 0.17
<i>Streptococcus agalactiae</i> DPC5338	4.06 ± 0.21	2.56 ± 0.17
<i>Streptococcus bovis</i> DPC6491	3.50 ± 0.38	1.75 ± 0.29
<i>Streptococcus gallolyticus</i> DPC6501	5.82 ± 0.00	3.94 ± 0.00
<i>Listeria innocua</i> DPC3572	1.50 ± 0.14	1.50 ± 0.14
<i>Listeria monocytogenes</i> 1042	2.10 ± 0.00	1.58 ± 0.00
<i>Staphylococcus aureus</i> ATCC 25923	3.28 ± 0.00	2.76 ± 0.17
<i>Staphylococcus aureus</i> DPC5245	3.39 ± 0.19	4.78 ± 0.21
<i>Lactococcus lactis</i> NZ9700	0.32 ± 0.00	0.69 ± 0.00
<i>Streptococcus hyointestinalis</i> DPC6484	0.69 ± 0.00	0.32 ± 0.00
<i>Streptococcus uberis</i> strain 42	3.39 ± 0.19	2.38 ± 0.28

^a Calculated as $(\pi r_1^2) - (\pi r_2^2)$, where r_1 is the radius of the zone and r_2 is the radius of the well (in centimeters). Peptides were used at a concentration of 0.22 mg/ml.

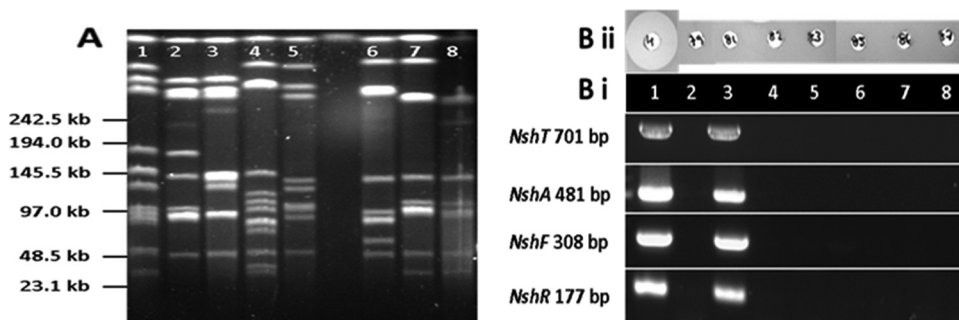


FIG 5 (A) PFGE macrorestriction patterns of *Streptococcus hyointestinalis* strains restricted with *Sma*I. Lane 1, *S. hyointestinalis* DPC6484; lane 2, *S. hyointestinalis* LMG14579; lane 3, *S. hyointestinalis* LMG14581; lane 4, *S. hyointestinalis* LMG14582; lane 5, *S. hyointestinalis* LMG14583; lane 6, *S. hyointestinalis* LMG14585; lane 7, *S. hyointestinalis* LMG14586; lane 8, *S. hyointestinalis* LMG14587. (B) (i) PCR amplification of strains of *S. hyointestinalis* template DNA with *nshT*-, *nshH*-, *nshF*-, and *nshR*-specific primers. Lanes correspond to those in panel A. (ii) Comparison of antimicrobial activities of *S. hyointestinalis* strains DPC6484 (H), LMG14579, LMG14581, LMG14582, LMG14583, LMG14585, LMG14586, and LMG14587 against the indicator strain, *L. delbrueckii* subsp. *bulgaricus* LMG6901. Wells are labeled with H (for nisin H) and with the last two digits of the strain designation for the other strains.

most likely that nisin-like clusters have moved between streptococci by horizontal gene transfer, as proposed by Richards et al. (2011) (40). Interestingly, the nisin H gene cluster differs from the *Lactococcus* and *Streptococcus* nisin clusters identified previously. Although the order of *nshABTCP* is the same as that for lactococci, the absence of a *nisI* gene between *nshC* and *nshP* is notable. Attempts to amplify a *nisI* gene equivalent using primers designed to be specific to the *nisI* gene of *L. lactis* and the *nsul* gene of *S. uberis* were unsuccessful. The absence of an obvious *nisI* gene in the remainder of the draft genome was confirmed by a comprehensive BLAST search; however, the presence of a novel immunity-like gene elsewhere in the genome cannot be ruled out. In addition, the nisin H gene cluster exhibits a number of other significant differences from previously described nisin clusters in gene order and orientation, most likely due to gene rearrangements brought about by the action of transposases.

The lack of an equivalent to the immunity protein, NisI, can have negative implications for bacteriocin production by the producing cell (41, 42). The yield of nisin H from the culture supernatant is low relative to that of nisin A, a finding initially attributed to the lack of a *nisI* immunity gene. However, further investigations revealed that poor bacteriocin production is most likely due to low cell numbers following 16 h of growth in TSB, typically 6×10^7 CFU/ml for *S. hyointestinalis* DPC6484 compared to 4×10^8 CFU/ml for *L. lactis* NZ9700. *S. hyointestinalis* strain LMG14587, which does not contain the nisin H gene cluster, grew to levels similar to those of DPC6484, suggesting that the absence of *nisI* was not responsible for a growth defect in DPC6484. In addition, previous *nisI* knockout studies have shown that a specific immunity gene is not necessary to confer full immunity (10), and Stein et al. (2003) reported that either *nisI* or *nisFEG* were able to confer immunity on nisin-sensitive *Bacillus subtilis* host cells (12). In this study, it would appear that the ABC transporter genes *nisFEG* are sufficient for self-protection in *S. hyointestinalis* DPC6484. Of note, other lantibiotic gene clusters, such as those of mersacidin and lactacin 481, also lack a specific immunity protein but possess NisFEG equivalents (13, 43).

The molecular mass difference of 101 Da between nisin A and nisin H can be accounted for by the amino acid differences. Given the highly conserved nature of nisin structures, we propose that nisin H has the same ring structure as nisins A, Z, F, and Q. It is

more similar in structure to the lactococcal nisins in that it contains the three terminal amino acids that are missing from the streptococcal nisins U, U2, and P.

Natural lantibiotic variants are likely to arise from point mutations in structural genes; by definition, they should have few amino acid differences and the same ring pattern, and the associated producers should exhibit cross immunity to other variants (44). Nisin H fits this definition in that it differs from nisin A with respect to 5 amino acids, is likely to have an identical ring pattern, and is immune to nisin A.

The lower antimicrobial activity of the CFS of DPC6484 than that of the control nisin A producer used in this study is not related exclusively to poor peptide production levels, since, at equal concentrations and purity, nisin A is more effective than nisin H against many of the target microorganisms investigated. Nisin has been extensively bioengineered in a quest to generate more-active peptides, and this strategy has provided information on the effects of specific amino acid changes (45). It has been stated that an unusual feature of nisin is the absence of aromatic residues and that, to date, any bioengineered nisins containing aromatic residues display reduced activity (46–49). Indeed, Field et al. (2008) (46) have shown that the bioactivity of a derivative of nisin A with Met21Tyr, a change that occurs naturally in nisin H, is reduced to 70% of that of the parental strain against *S. aureus* strains ST528 and DPC5245, and to 65% against *S. agalactiae* ATCC 13813. In addition, nisin H also contains a second aromatic residue, Phe1; thus, this is the first report of the presence of such aromatic amino acids in a natural nisin variant. Interestingly, salivaricin D also contains a phenylalanine at position 1. The presence of two aromatic residues may contribute to the potency of nisin H being lower than that of nisin A. It is also notable that the introduction of positively charged amino acids into nisin Z has had a beneficial impact on activity, in that the bioengineered Asn20Lys and Met21Lys variants were more active against the Gram-negative genera *Shigella*, *Pseudomonas*, and *Salmonella* (48). Although nisin H has a histidine-to-lysine change at position 32, this did not confer enhanced antimicrobial activity against *Escherichia coli*. However, since histidine is positively charged at low pHs, the addition of a lysine at this location does not constitute a significant change from a charge perspective. The Leu6Met amino acid change seen in nisin H is the first amino acid change at this posi-

tion reported for a natural variant. However, the specific impact of this change on activity will require further investigation.

Notably, nisin H appears as an intermediate between nisins of lactococcal and streptococcal origins, in that it retains the three terminal amino acids found in nisins A, F, Q, and Z while possessing other features, such as a Dhb at position 18, that are associated with nisins U, U2, and P. Alignment of the amino acid sequences of nisins A, Z, F, Q, H, U, U2, and P in Fig. 1 shows an increasing number of amino acid changes from the prototypical nisin A through to nisin P. Overall, nisin H aligns more closely with lactococcal than with streptococcal nisins, a fact that is reflected by amino acid identity.

Very few *S. hyointestinalis* strains have been deposited in culture collections. Since *S. hyointestinalis* was designated a new species in 1988, it is possible that isolates were previously catalogued as *S. salivarius*. PFGE analysis revealed that the seven strains of *S. hyointestinalis* obtained from the BCCM/LMG culture collection differ in their PFGE patterns (Fig. 5A). *S. hyointestinalis* LMG14581 conclusively contained the *nshA*, *nshF*, *nshR*, and *nshT* genes (Fig. 5Bii), but this strain did not display a bacteriocin-producing phenotype (Fig. 5Bi). A thorough analysis of the nisin H gene cluster in this strain would have to be carried out in order to determine the basis for this phenomenon.

In conclusion, we describe nisin H, a novel natural nisin variant produced by an *S. hyointestinalis* strain of porcine origin. The production of nisin H by a gut strain lends further support to accumulating observations suggesting that bacteriocin production may represent a potential probiotic trait for intestinal strains.

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