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RESEARCH ARTICLE

Nisin J, a Novel Natural Nisin Variant, Is Produced by *Staphylococcus capitis* Sourced from the Human Skin Microbiota

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ABSTRACT The skin microbiota is thought to play a key role in host protection from infection. Nisin J is a novel nisin variant produced by *Staphylococcus capitis* APC 2923, a strain isolated from the toe web space area in a screening study performed on the human skin microbiota. Whole-genome sequencing and mass spectrometry of the purified peptide confirmed that *S. capitis* APC 2923 produces a 3,458-Da bacteriocin, designated nisin J, which exhibited antimicrobial activity against a range of Gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA) and *Cutibacterium acnes*. The gene order in the nisin J gene cluster (*nsjFEGBTCJP*) differs from that of other nisin variants in that it is lacking the nisin regulatory genes, *nisRK*, as well as the nisin immunity gene *nisI*. Nisin J has 9 amino acid changes compared to prototypical nisin A, with 8 amino acid substitutions, 6 of which are not present in other nisin variants (Ile4Lys, Met17Gln, Gly18Thr, Asn20Phe, Met21Ala, Ile30Gly, Val33His, and Lys34Thr), and an extra amino acid close to the C terminus, rendering nisin J the only nisin variant to contain 35 amino acids. This is the first report of a nisin variant produced by a *Staphylococcus* species and the first nisin producer isolated from human skin.

IMPORTANCE This study describes the characterization of nisin J, the first example of a natural nisin variant, produced by a human skin isolate of staphylococcal origin. Nisin J displays inhibitory activity against a wide range of bacterial targets, including MRSA. This work demonstrates the potential of human commensals as a source for novel antimicrobials that could form part of the solution to antibiotic resistance across a broad range of bacterial pathogens.

KEYWORDS antimicrobial agents, antimicrobial peptides, antimicrobial structure, bacteriocins, microbiota, natural antimicrobial products, skin microbiota, nisin

The human skin microbiome is home to $\sim 10^{12}$ bacteria (1), and interest in the potential of skin bacteria to produce antimicrobials is growing, given the spread of antibiotic resistance (AR). *Staphylococcus capitis* is a member of the resident skin microbiota. First isolated from human skin in 1975, it has since been regarded as an opportunistic pathogen and has been associated with sepsis in neonates, meningitis, and endocarditis (2). Little is known about the inhibitory nature or antimicrobial activity of *S. capitis,* with only one report of *S. capitis* EPK-1 producing the glycylglycine endopeptidase ALE-1, an enzyme that targets the cell wall of *Staphylococcus aureus* (3). More recently, genomic analysis of an *S. capitis* strain isolated from the skin of a human toe revealed the presence of gene clusters capable of encoding gallidermin, epidermin, and phenol soluble modulins, highlighting its potential to produce antimicrobial

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peptides (AMPs) (4). In a recent study, our group detected antimicrobial activity by a number of *S. capitis* strains isolated from different areas of the human skin (5) and highlighted the potential for *S. capitis* species to produce bacteriocins (small ribosomally synthesized peptides produced by a range of bacteria which kill other bacteria). Interestingly, bacteriocin production is considered to be a probiotic trait in that bacteriocins function in helping the producer strain to become established in a niche, by killing off competitors and interacting with the immune system. Although the impact of nisin on immune systems has not yet been completely elucidated, this peptide stimulates a wide array of effects, and it influences various populations of cells involved in immunity (6–12).

One of the oldest known and most intensively studied bacteriocins is nisin, which was first described in this journal by Rogers and Whittier in 1928 (13). Nisin has been used in food preservation since 1953 (14) and was granted generally regarded as safe (GRAS) status in 1988 by the Food and Drug Administration (FDA). It is also approved by the World Health Organization (WHO) as a food additive and has been assigned the E number E234. Since the discovery of nisin, interest in bacteriocins has grown rapidly. Nisin A, composed of 34 amino acids, is produced by several strains of *Lactococcus lactis* (15). Nisin is a lantibiotic and thus a member of the class I bacteriocins (16). Lantibiotics are small peptides (<5 kDa) and are produced by many Gram-positive bacteria to inhibit or kill other Gram-positive bacteria (17). Production of other lantibiotics is common among commensal coagulase-negative staphylococci. For example, *Staphylococcus gallinarum*, *Staphylococcus epidermidis*, and *Staphylococcus hominis* produce the lantibiotics gallidermin, epidermin, and hominicin, respectively (18–20). Class I bacteriocins consist of posttranslationally modified bacteriocins which are subdivided into 4 classes, as follows: class Ia, lanthipeptides (of which nisin is the most prominent member); class Ib, head-to-tail cyclized peptides; class Ic, sactibiotics; and class Id, linear azol(in)e-containing peptides (8, 21). Lantibiotics are characterized by the presence of lanthionine/β-methyllanthionine residues and are produced through the dehydration of serine and threonine residues to form dehydroalanines and dehydrobutyrines, respectively. These dehydrated residues in turn react with cysteine thiols, forming lanthionine bridges (22, 23). The lantibiotics are subdivided based on the enzymes catalyzing the formation of lanthionines. Subclass I requires two distinct enzymes, LanB and LanC, whereas subclass II is modified by a single enzyme, LanM. Subclass III has no associated antimicrobial activity and is modified by a single enzyme, LanKC, while subclass IV is modified by LanL (24). Studies have revealed that nisin and other structurally related lantibiotics use the membrane-bound peptidoglycan precursor lipid II as a docking molecule, which consequently promotes two bactericidal activities, pore formation and inhibition of peptidoglycan biosynthesis (25). Significantly, lantibiotics have been shown to possess activity against antibiotic-resistant targets such as vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) and may have the potential to mitigate the looming global AR crisis (26).

A number of nisin variants have been discovered since the original nisin A was characterized (Fig. 1A). Nisin variants of lactococcal origin are more similar to each other than to variants from other genera such as *Streptococcus* (Fig. 1B). Nisin Z is the most closely related nisin variant to nisin A, with only a single amino acid substitution, His27Asn. Nisin U, U_2, and P each contain 31 amino acids, nisins O_{1-3} contain 33 amino acids, and nisin O_4 contains 32 amino acids, making them shorter than other previously described nisin variants. Here, we describe nisin J, produced by the *S. capitis* strain APC 2923, isolated in a screening study of the human skin microbiota. At 35 amino acids, nisin J is the longest nisin variant identified to date and has antimicrobial activity against significant human pathogens, including staphylococci, streptococci, and *Cutibacterium acnes*.

RESULTS

A nisin-like gene cluster exists within the *S. capitis* APC 2923 genome. *S. capitis* APC 2923 was previously isolated from the toe web space area in a screening study of

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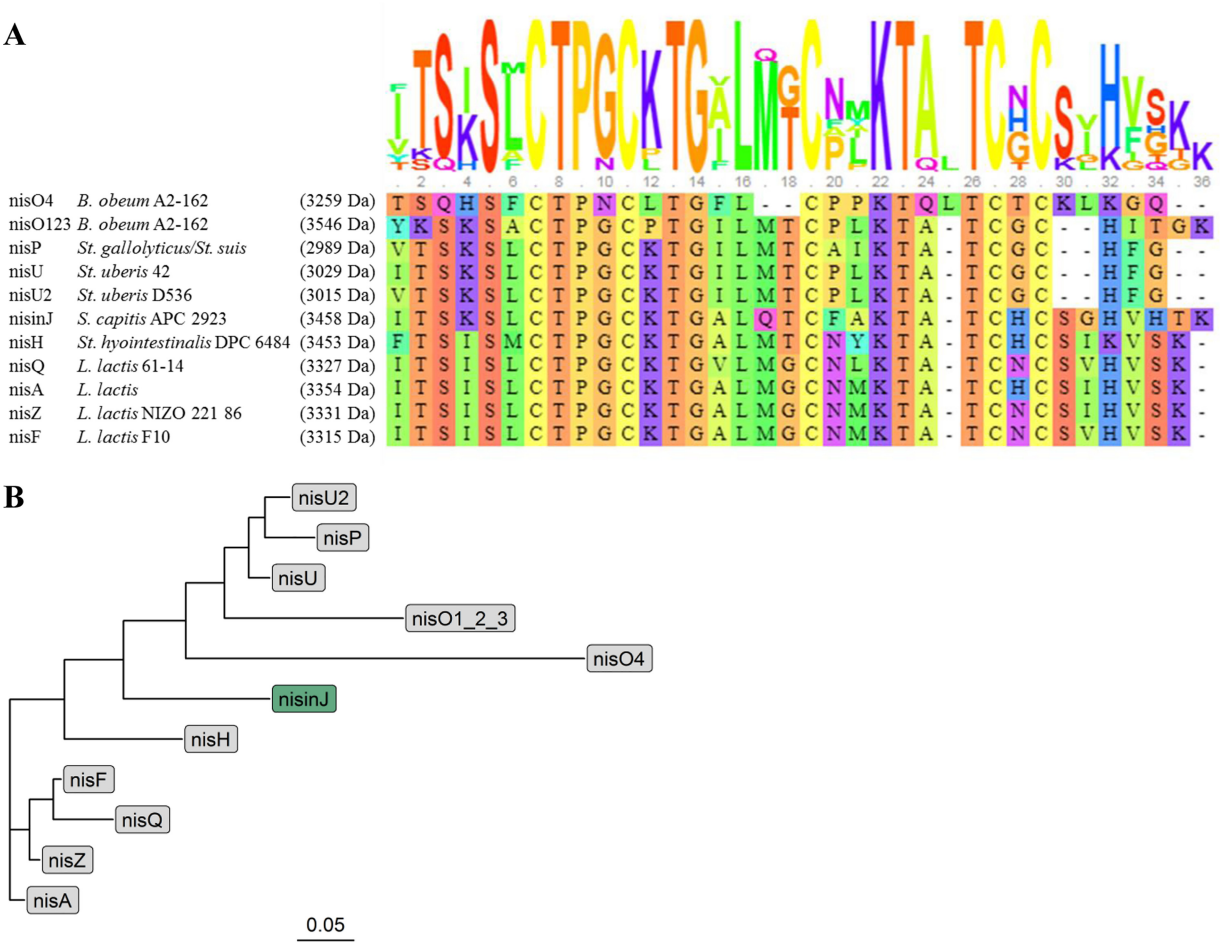


FIG 1 (A) Visualization of the multiple-sequence alignment from MUSCLE (plotted using http://msa.biojs.net/app/) of all natural nisin (nis) variants aligned with strain origin. The total height of the sequence logo at each position reflects the degree of conservation at that position in the alignment, while the height of each letter in that position is proportional to the observed frequency of the corresponding amino acid at that position. Nisin A (13), nisin Z (48), nisin F (49), nisin Q (50), nisin H (27), nisin J (5), nisins U and U_2 (51), nisin P (52, 53), and nisins O_1 to O_4 (54) are shown. *L.*, *Lactococcus*; *S.*, *Staphylococcus*; *B.*, *Blautia*; *St.*, *Streptococcus*. (B) Dendrogram showing phylogenetic relatedness in primary structures of all known natural nisin variants, suggesting the possible existence of an evolutionary link between the nisin-producing species. The order in which they branch shows the relatedness between them, and the branch length represents phylogenetic distance (0.05 represents a scale for the phylogenetic distance).

the human skin microbiota that sought to identify novel antimicrobial-producing strains (5). This strain was of particular interest due to its potent activity against the indicator strain *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901 and its broad inhibitory spectrum against a panel of *Staphylococcus*, *Streptococcus*, and *Corynebacterium* species and against *Cutibacterium acnes*. Whole-genome sequencing of this strain revealed a nisin gene cluster of ~9.78 kb compared to ~13.3 kb for nisin A. The structural gene *nisJ* encodes a peptide with the following eight amino acid variations compared to nisin A: Ile4Lys, Met17Gln, Gly18Thr, Asn20Phe, Met21Ala, Ile30Gly, Val33His, and Lys34Thr. Nisin J also contains an extra amino acid at the C terminus, making nisin J the longest nisin variant identified to date (Fig. 1A). A dendrogram of the natural nisin variants (Fig. 1B) demonstrates that peptides which have a closer common ancestor are more similar than are peptides than have more distant branching points. Lactococcal nisin variants are structurally distinct from all other nisin variants. Staphylococcal nisin J groups in the middle of the tree and appears to be more similar to streptococcal nisin than to lactococcal nisins. Nisins of *Blautia* origin appear to be more phylogenetically distinct due to longer branching. Streptococcal nisins H and J are more closely related to lactococcal nisins than to other streptococcal nisins, U, U_2, and P. The gene order of the nisin J cluster (*FEGBTCJP*) also differs from that of the nisin A

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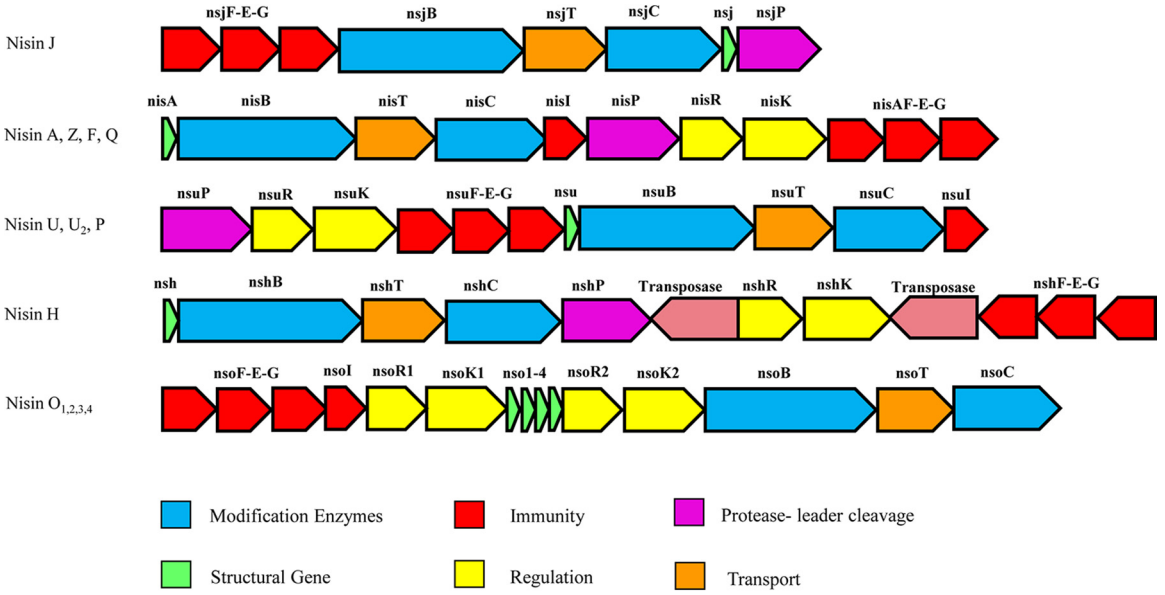


FIG 2 Comparison of bacteriocin gene clusters of different nisin variants.

in that it contains eight as opposed to the 11 genes within the cluster (Fig. 2). The BAGEL4 bacteriocin genome mining tool predicted that the nisin J prepeptide is composed of 61 amino acids with a leader sequence consisting of 26 amino acids. Overall, the nisin J mature peptide has 62.5% identity to the nisin H structural peptide produced by *Streptococcus hyointestinalis* (27). The identity and function of features of the nisin J operon are listed in Table 1.

Other genes contained in the *S. capitis* APC 2923 draft genome. In addition to the nisin J cluster, BAGEL4 and antiSMASH3.0 also highlighted a small gene cluster containing the *lanB* and *lanC* genes and a gene encoding a peptide with 93% identity to the gallidermin family in *S. capitis* APC 2923. These were located on a different contig from that of the nisin J gene cluster, and this mass was not detected from either the colony or purified cell free supernatants.

Purification and predicted structure of nisin J. Nisin J was purified in four steps using Amberlite XAD-16N solid-phase extraction (SPE), SP Sepharose cation exchange, C_{18} SPE, and reversed-phase high-performance liquid chromatography (HPLC). Antimicrobial activity correlated with the most dominant peak eluting at 24.5 min in the HPLC chromatogram, and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) revealed that the corresponding fractions had a mass of 3,458 Da (Fig. 3). This correlates with the predicted mass of the putative nisin J bacteriocin (following subsequent dehydration and ring formation reactions) as calculated from the draft genome sequence. Fractions deemed pure by MALDI-TOF MS were combined and lyophilized to give a yield of 3.00 mg/liter. Given that nisin J is a natural nisin variant with demonstrable conservation between key structural amino acids common to all natural nisin variants, it is predicted that the structure will be in line with those of other lactococcal nisins, as shown in Fig. 4.

Comparing the activities of purified nisins A, Z, and J. The spectrum of activity of pure nisin A, nisin Z, and nisin J, by means of a well diffusion assay (WDA), was performed on several target indicator strains. Nisin J was more active than nisin A against 12 of the 13 strains tested, while nisin J was more active than nisin Z for 7 of the target strains tested, including *Corynebacterium xerosis*, MRSA, *Streptococcus uberis*, and *S. aureus* (Table 2). However, in an MIC assay using *L. lactis* HP as the indicator, no difference was observed between nisins A, Z, and J, with all exhibiting MICs of 32 nM.

The nisin J-producing strain is cross-immune to nisin A and H but not to nisin U producers. Cross-immunity assays were performed to investigate whether the nisin

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TABLE 1 Identity and function of features of the draft pJOS01 plasmid sequence[a]

Feature name	Position of: Start codon	Stop codon	Size (aa)	E value	Putative function (conserved domain)	% identity to best match
J1	3	518	172	3E−124	DDE_Tnp_IS*240* superfamily; Rve transposase	100 to IS*6* family transposase of *S. epidermidis*
J2	562	1221	220	6E−149	ABC2_membrane superfamily; NosY ABC-type transport system involved in multicopper enzyme maturation, permease component	100 to ABC transporter permease subunit of *S. capitis*
J3	1749	1874	125	3E−17	DUF2648 superfamily; unknown function	100 to multiple species; DUF2648 domain-containing protein
J4	1886	3385	500	0	NADB_Rossmann superfamily; MqO malate: quinone oxidoreductase	100 to multiple species; malate dehydrogenase (quinone) (*Staphylococcus*)
J5	3446	5050	535	0	L-Lactate permease superfamily (energy production and conversion)	100 to *L. lactate* permease (*Staphylococcus*)
J6	5085	5789	235	6E−171	Alpha-acetolactate decarboxylase superfamily (secondary metabolite biosynthesis, transport, and catabolism)	100 to alpha-acetolactate decarboxylase
J7	5823	7487	555	0	Acetolactate synthase superfamily (PRK08617) (amino acid transport and metabolism, coenzyme transport, and metabolism)	100 to acetolactate synthase
J8	8213	8413	67	8E−39	CspA family (transcription) DNA binding domain	100 to cold shock protein (*Staphylococcus*)
CdR	8807	9424	206	1E−139	Cadmium resistance transporter superfamily; CadD protein, predicted permease (inorganic ion transport and metabolism)	100 to cadmium resistance transporter (*Mycobacteroides abscessus* subsp. *massiliense*)
J10	9442	9789	116	4E−74	Arsenical resistance operon repressor family; DNA-binding transcriptional regulator (transcription)	100 to HTH transcriptional regulator (*Staphylococcus*)
J11	10002	10610	203	2E−144	Serine recombinase family	100 to recombinase family protein (*Staphylococcus*)
J12	10716	11276	187	1E−124	None detected	100 to hypothetical protein (*Staphylococcus*)
J13	11884	12369	162	2E−112	None detected	100 to hypothetical protein (*Staphylococcus*)
J14	12632	13309	226	5E−166	NlpC/P60 family; the function of this domain is unknown; it is found in several lipoproteins	100 to hypothetical protein (*Staphylococcus*)
PSM	13578	13712	45	1E−22	*Staphylococcus* hemolytic protein	100 to beta class phenol-soluble modulin
J16	13944	14054	37	4E−17	DUF2648 superfamily; protein of unknown function	100 to multiple species; DUF2648 domain-containing protein (*Staphylococcus*)
J17	14064	15560	499	0	NADB_Rossmann superfamily; MqO malate: quinone oxidoreductase	100 to malate dehydrogenase:quinone (*S. capitis*)
J18	15780	16118	113	6E−75	DNA binding transcription regulator	100 to transcriptional regulator HXIR family (*Staphylococcus caprae*)
RepA	17825	18760	312	0	Replication initiator protein A (RepA) N terminus family; DNA replication initiator in plasmids	100 to replication initiator protein A (*Staphylococcus*)
J20	19190	19957	256	1E−178	Polar chromosomal segregation protein	100 to DUF536 binding domain (*Staphylococcus*)
J21	20132	20734	201	2E−140	NADB Rossmann superfamily; PRK07578 short-chain dehydrogenase	100 to short-chain dehydrogenase (bacteria)
J22	21220	21894	225	7E−165	DDE_Tnp_IS240 superfamily; Rve transposase	100 to IS*6*-like element IS*257* family transposase
nsjF	22148	22855	236	6.00E−119	ABC-type multidrug transport system, ATPase component (defense mechanisms)	75 to Lan protection ABC transporter ATP binding subunit in *Staphylococcus succinus*
nsjE	22857	23603	249	4E−85	Lantibiotic protection ABC transporter permease subunit, MutE/EpiE family; ABC-2 membrane superfamily	61.29 to hypothetical protein BU069_09230 in *S. succinus*
nsjG	23600	24337	246	1E−73	Lantibiotic protection ABC transporter permease subunit, MutG family; ABC-2 membrane superfamily	52.92 to hypothetical protein in *S. succinus*
nsjB	24362	27277	972	8E−90	Lantibiotic dehydratase C-terminal, thiopeptide-type bacteriocin biosynthesis domain	30.11 to lantibiotic dehydratase *Lactobacillus bombicola*

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TABLE 1 (Continued)

Feature name	Position of: Start codon	Stop codon	Size (aa)	E value	Putative function (conserved domain)	% identity to best match
nsjT	27450	29000	517	2E−120	MdlB: ABC-type multidrug transport system, ATPase and permease component (defense mechanisms)	40.95 to ABC transporter ATP-binding protein *L. bombicola*
nsjC	28993	30222	410	2E−40	LanC is the cyclase enzyme of lanthionine synthetase; LanC-like superfamily	29.31 to lanthionine synthetase family protein (*Bacillus nakamurai*)
nisJ	30263	30445	61	1E−09	Structural gene; lantibiotic precursor in gallidermin/nisin family	62.5 to nisin H structural protein (*Streptococcus hyointestinalis*)
nsjP	30565	31905	447	2E−58	Peptidase S8 family domain in lantibiotic-specific proteases	32.58 to peptidase S8 (*Bacillus endophyticus*)
J31	31962	32357	132	7E−88	None detected	99.24 to hypothetical protein (*S. epidermidis*)
J32	32449	33057	203	4E−144	Serine recombinase revolvase invertase superfamily; PinE	100 to multiple species; recombinase family protein (*Staphylococcus*)
J33	33277	33477	67	1E−39	Predicted transcriptional regulator; COG3905 superfamily	100 to plasmid replication-associated protein (*S. epidermidis*)
ParA	33483	34277	265	0	ParA family chromosomal segregation and plasmid partition; cellulose biosynthesis protein BcsQ	99.62 to ParA family protein (*S. epidermidis*)
J35	34343	34882	180			No significant similarity found
RepA	35097	36089	331	0	DNA replication initiator of plasmids; HTH superfamily	99.7 to replication initiator protein A (*S. capitis*)
J37	36119	36820	234	1E−173	Putative transposase (InsQ) DNA-binding domain; OrfB_Zn_ribbon superfamily	100 to transposase (*S. capitis*)
J38	36827	37102	92	8E−60	None detected	100 to hypothetical protein EQ811_12225 (*S. capitis*)
J39	37705	37857	51	1E−27	None detected	100 to transposase (*S. aureus*)
RepB	38075	38932	286	0	COG5527 superfamily	99.65 to RepB family plasmid initiator protein (*Staphylococcus*)
J41	39215	39664	150	2E−99	None detected	100 to hypothetical protein (*Staphylococcus*)
J42	39867	40565	233	3E−169	None detected	98.28 to hypothetical protein (*Staphylococcus*)
J43	40667	41023	119	5E−80	None detected	100 to hypothetical protein (*Staphylococcus*)
J44	41124	41618	165	1E−107	Asp_carb_tr superfamily; pyrimidine biosynthesis	99.38 to aspartate carbamoyltransferase (*S. epidermidis*)
J45	41674	41847	58	2E−22	None detected	93.48 to molybdopterin biosynthesis protein MoaB
HTH	41985	42665	227	3E−161	HTH superfamily	99.12 to "winged" HTH transcription regulator (*S. epidermidis*)
J47	42777	44150	458	0	Multidrug resistance MFS family permease; transport and metabolism	99.78 to MFS transporter (*S. epidermidis*)
J48	44846	45340	165	3E−109	None detected	98.78 to hypothetical protein (*S. epidermidis*)
J49	45337	46101	255	6E−176	None detected	100 to hypothetical protein (*S. epidermidis*)
HTH	46186	46455	90	2E−58	HTH XRE superfamily	100 to HTH transcription regulator (*Auricoccus indicus*)
J51	47042	47155	38	1E−15	None detected	100 to hypothetical protein UF66_0802 (*Staphylococcus cohnii* subsp. *cohnii*)
J52	48210	48476	89	8E−57	None detected	98.86 to multispecies hypothetical protein (*Staphylococcus*)
J53	48623	49843	407	0	None detected	100 to hypothetical protein (*S. capitis*)

[a]aa, amino acid. HTH, helix-turn-helix; XRE, xenobiotic response element; MFS, major facilitator superfamily.

J-, A-, H-, and U-producing strains were cross-immune to one another (Table 3). No zones were observed between nisins A, H, and J, indicating that these producing strains are all cross-immune. However, a zone was observed from the nisin J-producing strain against the nisin U producer (*S. uberis* strain 42), demonstrating that the strain is sensitive to nisin J.

Not all *S. capitis* strains contain a nisin-like gene cluster. The *nisJ* structural gene was amplified from nine antimicrobial-producing *S. capitis* strains isolated from human skin in a previous study by our group (5). Two of the nine *S. capitis* strains (APC 2918 and APC 2934) did not contain the *nisJ* structural gene. The other seven *S. capitis* strains tested positive for the *nisJ* structural gene, correlating with findings from our earlier

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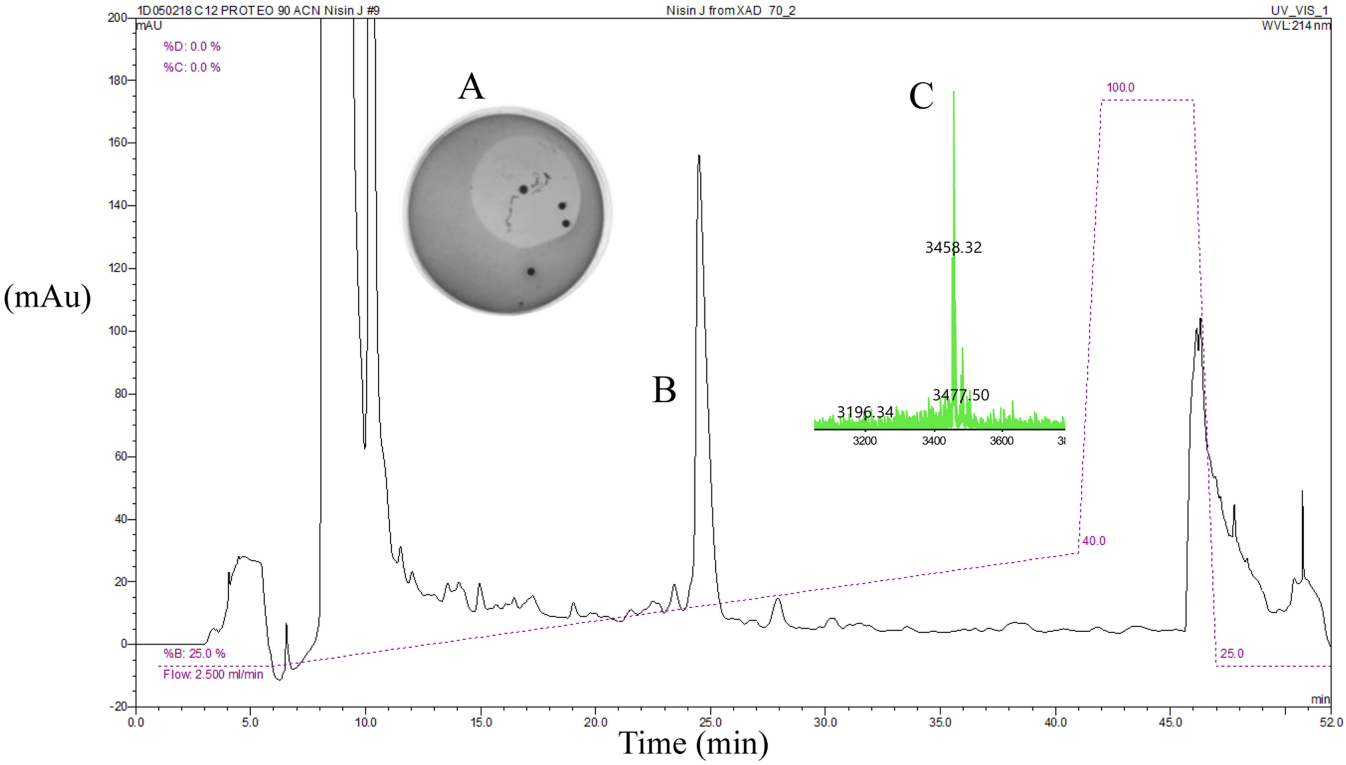


FIG 3 Purification of nisin J from *S. capitis* APC 2923 grown in XAD-BHI. (A) Original overlay plate where antimicrobial activity of the *S. capitis* APC 2923 strain was detected. (B) The RP-HPLC profile shows a peak at HPLC-active fraction of 24.5 minutes, which correlates with where pure nisin J elutes. (C) MALDI-TOF MS of active fraction. mAu, milli-arbitrary units.

study which found these strains to be cross-immune and to possess the same pulsotype, indicating that they were the same strain or very closely related strains and were therefore most likely producing the same bacteriocin (5). These 7 strains were isolated from 4 different subjects, indicating that the same pulsotype is shared across a number of individuals, implying that the ability to produce nisin J may be a dominant feature and thus an ecological advantage for this *S. capitis* strain.

The nisin J gene cluster resides on a plasmid. Analysis of the *S. capitis* APC 2923 contig harboring the nisin J gene cluster identified the presence of a plasmid replication protein A (RepA) and other plasmid replication-associated proteins, suggesting that it was of plasmid origin. Plasmid DNA was readily obtained from *S. capitis* APC 2923 using a commercially available plasmid maxi kit (data not shown). Short-read sequencing was performed on the plasmid DNA using the Illumina MiSeq platform to approximately 200-fold coverage. *De novo* assembly resulted in four contigs (Fig. 5), with a combined size of 49,951 bp. A plasmid map of pJOS01 (GenBank accession number MN602039) shows all of the genes encoding immunity and the biosynthetic machinery for nisin J (*nsjFEG*, *nsjB*, *nsjT*, *nsjC*, *nisJ*, and *nsjP*) reside on one of the contigs, supporting

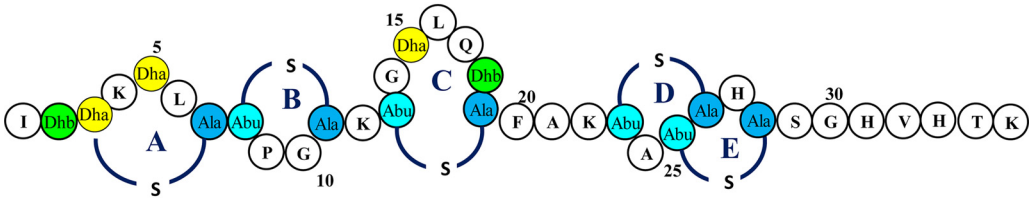


FIG 4 Proposed structure of the novel nisin variant nisin J. Residues are represented by the single-letter code. Posttranslational modifications are indicated as follows: Dha, dehydroalanine; Dhb, dehydrobutyrine; Abu, 2-aminobutyric acid; Abu-S-Ala, 3-methyllanthionine.

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TABLE 2 Inhibition spectra of purified peptides of nisins A, Z, and J against indicator strains using well diffusion assays and expressed as the area of the zone of inhibition

Target microorganism	Strain	Area of zone of inhibition[a] (mm^2) for nisin:		
		A	Z	J
Corynebacterium xerosis	DPC 5629	51.5	66.2	133.8
Cutibacterium acnes	LMG 16711	537	587.5	469
Lactobacillus delbrueckii subsp. *bulgaricus*	LMG 6901	555.7	672.7	651.44
Lactococcus lactis subsp. *cremoris*	HP	241.9	325.8	363
Listeria monocytogenes	WSLC 112	60	73	37
Enterococcus faecium	APC 852	93.2	120.6	170.9
Enterococcus faecalis	ATCC 19433	101.2	120.8	102.97
Methicillin-resistant *Staphylococcus aureus*	DPC 5645	77	115.9	135.8
Staphylococcus aureus	DPC 7016	109.4	143.4	153.1
Staphylococcus epidermidis	DPC 5990	136.8	180.3	159.5
Staphylococcus simulans	APC 3482	148.7	197.1	395.5
Streptococcus agalactiae	ATCC 13813	174.4	221.7	136.8
Streptococcus uberis	DPC 5344	98.5	153.9	248.8

[a]Calculated as the area of zone of inhibition (πr^2) − area of well (πr^2) in millimeters. Assays were carried out in duplicate; mean zone areas shown.

the plasmid association of the nisin J gene cluster (Fig. 5). Furthermore, three genes encoding plasmid replication functions (RepA and RepB) as well as genes encoding other nonessential plasmid-associated roles were present on the other contigs (Fig. 5 and Table 1). Restriction digestion with EcoRI yielded a profile comparable to the virtual digestion of the generated plasmid sequence, supporting the predicted size of ~50 kb (data not shown). Subsequent analysis revealed a GC content of ~28%, which is considerably lower than that of *S. capitis* chromosomal DNA (32 to 33%), a characteristic that has been observed for plasmids of many Gram-positive species (28).

Nisin J exhibits resistance to NSR. Deferred antagonism assays using *L. lactis* subsp. *diacetylactis* DRC3 (nisin resistance protein positive [NSR^+]) as a target indicator strain revealed that nisin J is partially resistant to NSR (result not shown). To establish if nisin J had increased inhibitory activity against NSR compared to that of nisin A, further WDAs were conducted using the NSR^+ and NSR^- strains *L. lactis* MG1614/pNP40 and *L. lactis* MG1614, respectively. While the inhibition zone of the nisin J producer is slightly decreased against the NSR-positive strain compared to the NSR-negative strain, it appears that nisin J is more active than nisin A and may be less susceptible to the proteolytic effects of NSR (Fig. 6A), which was also demonstrated in agarose assays (Fig. 6B). The analysis revealed a significant difference in the zones of inhibition between nisin A and nisin J against an NSR^+ strain (MG1614/pNP40), with a P value of 0.0001 compared to zone sizes against an NSR^- strain (MG1614), where no statistical difference ($P = 0.1701$) was observed (these data support Fig. 6).

DISCUSSION

As the burden of antibiotic resistance increases globally, there is an urgent need for novel therapeutic options. In addition to the well-established use of nisin as a food

TABLE 3 Cross-immunity of nisin A-, U-, H-, and J-producing strains using well diffusion assays and expressed as the area of the zone of inhibition

Target organism	Strain	Nisin produced	Area of zone of inhibition[a] (mm^2) against nisin:			
			A	U	H	J
Lactococcus lactis	NZ9700	A	0	0	0	0
Streptococcus uberis	42	U	0	0	0	85
Streptococcus hyointestinalis	DPC 6484	H	0	0	0	0
Staphylococcus capitis	APC 2923	J	0	0	0	0

[a]Calculated as the area of zone of inhibition (πr^2) − area of well (πr^2) in millimeters. Values are the means from triplicate assays. 0, no zone observed.

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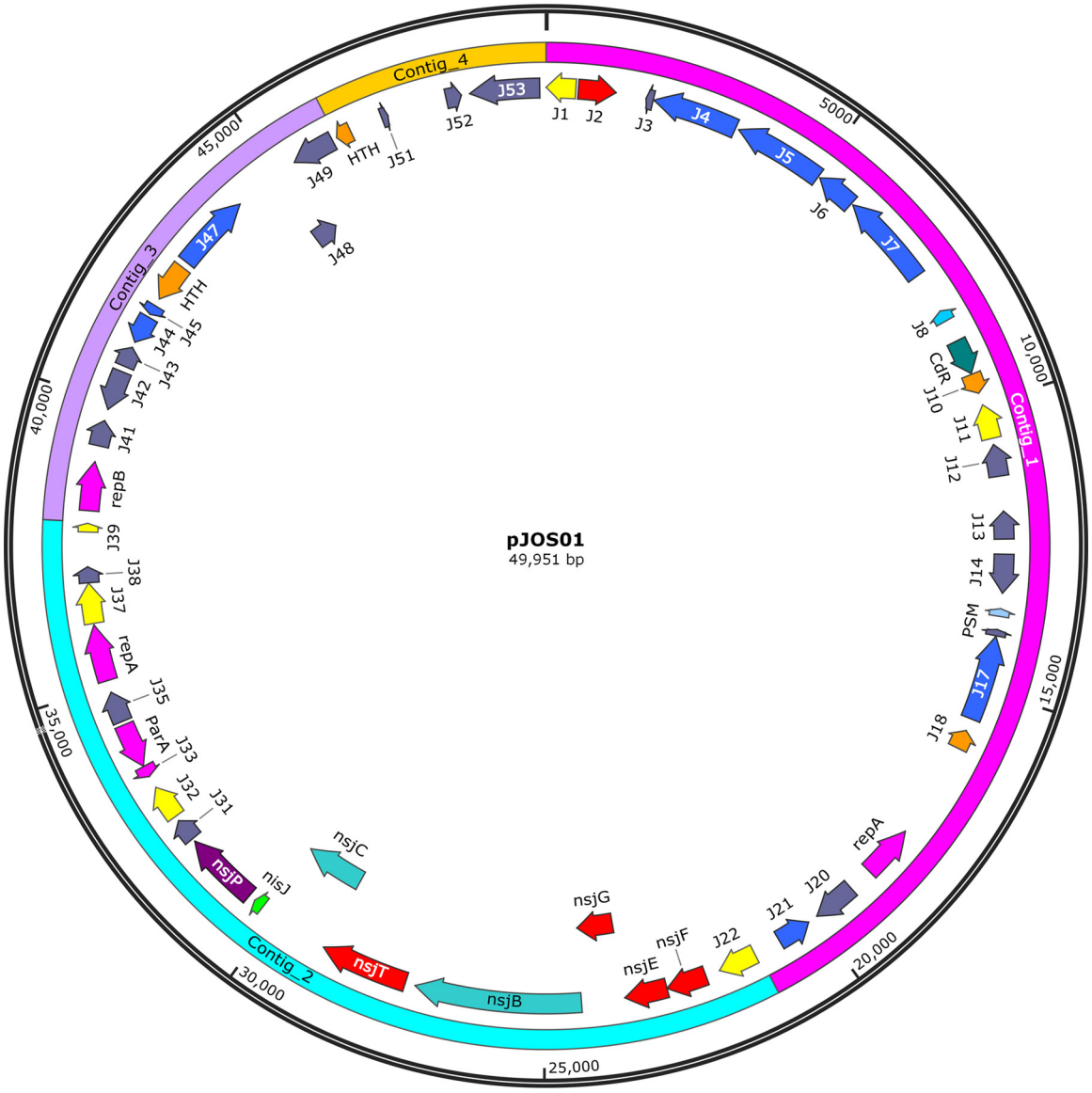


FIG 5 Plasmid map of pJOS01 draft sequence created on SnapGene version 2.3.2 (GenBank accession number MN602039).

preservative, many studies have focused on using nisin against drug-resistant pathogens in clinical or veterinary settings due to its high potency and multiple mechanisms of action (10–12). Nisin J is a novel nisin variant and the first such variant reported from a *Staphylococcus* species. A combination of whole-genome sequencing of *S. capitis* APC 2923 and peptide purification resulted in the identification of this broad-spectrum lantibiotic. The nisin J-producing *S. capitis* strain was isolated from the toe web space, an area associated with high microbial load. This suggests that the production of a broad-spectrum bacteriocin confers an advantage on this strain over competing commensal skin flora, as was also observed by O'Sullivan and colleagues (5) when four of the twenty subjects screened in the study exhibit the same pulsotype. The residence of the nisin J gene cluster on a plasmid is significant in that it may facilitate its dissemination to other skin microbes.

As mentioned previously, nisin J has eight amino acid changes and one extra amino acid near the C-terminal end compared to nisin A. Interestingly, six of the eight changes are unique compared to natural nisin variants. Natural nisin variants are tolerant to some amino acid changes at the N terminus, with Ile4 being the most commonly

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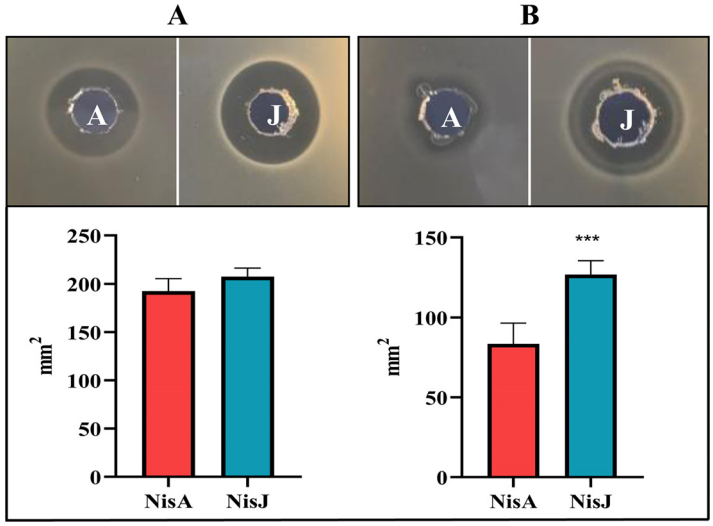


FIG 6 Activity of cell-free supernatant of nisin J- and A-producing strains as observed in WDA against MG1614 (NSR^-) (A) and MG1614/pNP40 (NSR^+) (B). The assay results are representative of triplicate experiments. The results reveal no significant differences in zones of inhibition against NSR^- (*P* value of 0.1701) (bar graph in panel A) but show a significant difference (***) against NSR^+ (*P* value of 0.0001) (bar graph in panel B).

substituted amino acid. Nisin J contains an Ile4Lys substitution which is also seen in nisins P, U, U_2, and O_{1-3} but remains unchanged in lactococcal nisins (A, Z, F, and Q) and nisin H. Nisin J differs most from other natural nisin variants in the center and at the C terminus of the peptide, which could be key to nisin J's enhanced activity. At the center of nisin J, amino acid positions 17 to 21, there are 4 amino acids that differ compared to nisin A. It contains a Met17Gln substitution which is unique, as all other natural nisin variants that demonstrate antimicrobial activity have Met at this position. The Gly18Thr change is also interesting, as it is observed in nisins H, U, U_2, P, and O_{1-3} and is proposed to be modified to dehydrobutyrine (Dhb), in light of the dehydration observed in a M17Q-G18T derivative of nisin Z (29). At position 20, nisin J has a highly hydrophobic residue, phenylalanine, compared to the polar asparagine in nisin A. Li et al. (30) found that extending the C terminus of nisin improves both its ability to permeate membranes and its inhibitory potential against Gram-negative bacteria. Therefore, nisin J's longer C terminus (compared to other nisin variants) could be more attracted to negatively charged cell membranes resulting in enhanced membrane insertion, which may be responsible for its broader host range. The skin origin of this nisin J producer suggests that its exposure to many competitors from the external environment may be responsible for the greater variation in the structure of nisin J.

Analysis of the nisin J gene cluster identified several key features associated with bacteriocin operons. These include a structural gene (*nisJ*), 2 genes associated with enzymatic modification (*nsjB* and *nsjC*), a gene involved in transport (*nsjT*), and immunity genes (*nsjFEG*) (Table 1 lists the identity and functions of features of the nisin J gene cluster). The arrangement of genes in the nisin J gene cluster differs from that of other nisin operons. Interestingly, the only conservation of gene order throughout all operons of natural nisin variants is *lanBTC*. Similarities in the structural peptides of different nisin variants from different origins indicate the possibility that an evolutionary link exists between lactococcal, streptococcal, *Blautia,* and now, staphylococcal species, a link previously mentioned by O'Connor et al. (27) with reference to streptococcal and lactococcal species. A dendrogram based on the primary structures of all known natural variants highlights the genetic relatedness between the nisin-producing species and further suggests the likelihood of this evolutionary link. The FEG locus is present in lantibiotic systems other than nisin, including subtilin (31) and epidermin (32), and has been linked to transport, immunity, and defense (33). Inactivation of these

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genes in the nisin A gene cluster decreased nisin production and immunity, confirming their role in immunity (34). Although the *nsjFEG* genes are present in the nisin J gene cluster, the absence of a specific immunity gene, *nsjI*, as well as the absence of an expression regulatory system, *nsjRK*, could explain why nisin J immunity mechanisms appear to be less able to protect the cell. It also further supports the finding that the producing strain was more sensitive to its own purified nisin J peptide than was a nisin A producer with a specific nisin immunity determinant.

The production of lantibiotics such as gallidermin and epidermin is associated with increased release of lipids and ATP and protein excretion, which are indicators of cell membrane damage (35). Thus, the production of these lantibiotics has been deemed a "burden" to staphylococci that produce them; therefore, the incomplete lantibiotic gene cluster, having only the *lanB* and *lanC* genes present, may be either an evolutionary feature of *S. capitis* genomes or may be an incomplete cluster of lantibiotic biosynthetic genes previously shown to occur in many microbes (35).

As previously discussed, the nisin J gene cluster resides on a plasmid, inviting the speculation that *S. capitis* acquired its antimicrobial ability through horizontal gene transfer. Indeed, residence on mobile genetic elements is a feature of natural nisin variants, as observed with nisins A and H, and may explain their presence in many different species.

Purification of nisin J resulted in a peptide with a mass of 3,458 Da. The mass of nisin J was predicted to be 3,622 Da, where the difference between predicted and observed masses can be accounted for by 9 dehydration reactions (−18 Da per loss of water residue) involved in the formation of lanthionine and β-methyllanthionine bridges (36). The predicted peptide structure was based on the nisin A template, with a lanthionine bridge likely to occur between Ser3 and Cys7 and four β-methyllanthionine bridges between Thr8 and Cys11, Thr13 and Cys19, Thr23 and Cys26, and Thr25 and Cys28.

True to all nisin variants, nisin J is a broad-spectrum lantibiotic with inhibitory activity similar to that of nisins A and Z, as can be seen in Table 2, inhibiting a wide range of bacterial genera with greater inhibition of staphylococcal targets than with nisins A and Z. This suggests that the nisin J-producing *S. capitis* strain may have naturally evolved to produce a nisin peptide with enhanced activity against other staphylococci in the skin microbiota (Table 2). Nisin J-, A-, and H-producing strains are immune to nisin peptides J, A, H, and U; however, the nisin U-producing strain is not immune to nisin J (Table 3). This may be due to the lack of the *nsjI* immunity gene in the nisin J cluster.

The nisin resistance protein (NSR) is a protease which cleaves nisin A at Ser29, significantly reducing the activity of the peptide. Employing a bioengineering strategy, Field et al. (37) demonstrated that the substitution of residues 29 and 30 with proline and valine, respectively (derivative designated S29PV), rendered the peptide resistant to proteolytic digestion by NSR. In this study, we found that the nisin J producer displays a higher resistance to NSR proteolytic enzymes than does nisin A, which is possibly due to a glycine residue at position 30 instead of the isoleucine as found in nisin A. Interestingly, a study carried out by Simões et al. (38) involving a multidrug-resistant *S. capitis* clone, NRCS-A, a major pathogen involved in sepsis in preterm neonates, demonstrated the presence of an NSR-encoding gene. PCR analysis failed to detect the presence of any *nsr* gene in any nisin J-producing *S. capitis* strain from our previous study (5).

Nisin J may have evolved to be more potent against specific competing organisms in a particular niche environment such as the skin. Employing a bioengineering strategy, Rink et al. (39) demonstrated that the replacement of residues I, S, and L at positions 4, 5, and 6 in nisin A with the residues K, S, and I, respectively, resulted in enhanced bioactivity. Notably, the residues K-S-L are naturally present in nisin J at the same positions. In a separate bioengineering study, Kuipers et al. (29) generated a novel nisin variant (M17Q/G18T) exhibiting enhanced bioactivity. It is interesting that both of these mutations are naturally present in nisin J. Furthermore, Field et al. (40) reported

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TABLE 4 Growth conditions of the bacterial strains used in this study

Species	Strain[a]	Growth conditions		
		Temp (°C)	Atmosphere[b]	Growth medium(a)[c]
Corynebacterium xerosis	DPC 5629	37	Aerobic	BHI
Cutibacterium acnes	LMG 16711	37	Anaerobic	mRCM and RCA
Enterococcus faecalis	ATCC 19433	37	Anaerobic	MRS
Enterococcus faecium	APC 852	37	Anaerobic	MRS
Lactobacillus delbrueckii subsp. *bulgaricus*	LMG 6901	37	Anaerobic	MRS
Lactococcus lactis	NZ9700	30	Aerobic	GM17
Lactococcus lactis subsp. *cremoris*	HP	30	Aerobic	GM17
Lactococcus lactis	MG1614	30	Aerobic	GM17
Lactococcus lactis	MG1614/pNP40	30	Aerobic	GM17
Lactococcus lactis subsp. *diacetylactis*	DRC3	30	Aerobic	GM17
Listeria monocytogenes	WSLC 1211	37	Aerobic	BHI
MRSA[d]	DPC 5645	37	Aerobic	BHI
Staphylococcus aureus	DPC 7016	37	Aerobic	BHI
Staphylococcus capitis	APC 2923	37	Aerobic	BHI
Staphylococcus epidermidis	DPC 5990	37	Aerobic	BHI
Staphylococcus simulans	APC 3482	37	Aerobic	BHI
Streptococcus agalactiae	ATCC 13813	37	Aerobic	BHI
Streptococcus hyointestinalis	DPC 6484	37	Anaerobic	GM17
Streptococcus uberis	DPC 5344	37	Aerobic	BHI
Streptococcus uberis	Strain 42	37	Anaerobic	GM17

[a]ATCC, American Type Culture Collection; APC, APC Microbiome Ireland Culture Collection; DPC, Teagasc Culture Collection; WSLC, Weihenstephan *Listeria* Collection; LMG, Laboratorium voor Microbiologie.
[b]Anaerobic conditions, where appropriate, were achieved through the use of anaerobic jars and Anaerocult A gas packs (Merck, Darmstadt, Germany).
[c]MRS, de Man-Rogosa-Sharpe; mRCM, modified reinforced *Clostridium* medium (made following the ATCC medium: 2107 modified reinforced clostridial agar/broth [prereduced] protocol); RCA, reinforced *Clostridium* agar; BHI, brain heart infusion; GM17, 0.5% glucose added to M17 agar.
[d]MRSA, methicillin-resistant *S. aureus*.

that a nisin A derivative, M21A, demonstrated enhanced bioactivity. Remarkably, alanine is naturally present at position 21 in nisin J.

In conclusion, we have identified a new natural nisin variant, nisin J, produced by *S. capitis* APC 2923, which was isolated from the human skin microbiota. Nisin J represents the first nisin variant isolated from *Staphylococcus* species and the first to demonstrate partial recalcitrance to NSR. Indeed, the enhanced activity of nisin J compared to that of nisin A and Z as observed against all staphylococcal strains utilized in this study is notable. The production of bacteriocins such as nisin J from skin bacteria highlights the potential of bacterial strains of skin origin to be used as live biotherapeutics.

MATERIALS AND METHODS

The antimicrobial-producing strain *S. capitis* APC 2923 was isolated in a previous screening study of the human skin microbiota by our group (5).

Bacterial strains and culture conditions. The growth conditions of the bacterial strains used in this study are listed in Table 4. Anaerobic conditions, where appropriate, were attained using anaerobic jars and Anaerocult A gas packs (Merck, Darmstadt, Germany).

Draft genome sequence of *S. capitis* APC 2923 and *in silico* analysis of the nisin J gene cluster. Bacterial DNA was extracted using the GenElute kit, as described by the manufacturer (Sigma-Aldrich Ireland Limited, Arklow, County Wicklow, Ireland), and was prepared for sequencing following the Nextera XT DNA library prep reference guide (Illumina, Inc.). A Qubit 3.0 fluorometer (Thermo Fisher Scientific, MA) was used for DNA quantification. Sequencing was performed at the Teagasc/APC Microbiome Ireland Sequencing facility, Teagasc Food Research Centre, Moorepark, Fermoy, County Cork, Ireland. In total, 94 contigs, including 16 large contigs, were revealed by *de novo* assembly using SPAdes (version 3.10.0). A total of 2,453 open reading frames (ORFs) and 60 tRNAs were detected and subsequently annotated using Prokka (version 1.11). The online tools Bacteriocin GEnome mining tooL (BAGEL4) and antiSMASH 3.0 were employed to identify bacteriocin operons/gene clusters in the genomes of interest, and by combining these software programs with the ARTEMIS genome viewer, the presence of the nisin J gene cluster was confirmed.

Evolutionary links between natural nisin variants. The European Bioinformatics Institute toolkit (https://www.ebi.ac.uk/services) was used to investigate the evolutionary relationships between the nisin structural variants. A multiple-sequence alignment was generated using MUSCLE (version 3.8) and visualized on a neighbor-joining tree without distance corrections. This tree was visualized using the ggtree package (version 1.10.5) in R (version 3.4.4).

Purification of the antimicrobial produced by *S. capitis* APC 2923. To purify the antimicrobial produced by *S. capitis* APC 2923, the culture was grown in a shaking 37°C incubator overnight in 1,800 ml

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of brain heart infusion (BHI) which had been passed through an XAD column to remove hydrophobic peptides before autoclaving (XAD-BHI). The culture supernatant was applied to an Econo-Column containing 60 g Amberlite XAD-16N beads (Sigma Aldrich, Arklow, Co. Wicklow, Ireland). The column was then washed with 350 ml of 30% ethanol, and the antimicrobial activity was eluted with 70% propan-2-ol (IPA) containing 0.1% trifluoroacetic acid (TFA) (Sigma Aldrich). The IPA was removed from the active column eluent and the pH adjusted to 4.4 with 7.5 N NaOH. The sample was then applied to an Econo-Column containing 90 ml SP Sepharose beads preequilibrated with 20 mM sodium acetate buffer (pH 4.4) (buffer A). The column was washed with 50 ml of buffer A and the antimicrobial activity eluted in 250 ml buffer A containing 1 M NaCl. The salt-containing eluent was applied to 60 ml of a 10-g C_{18} solid-phase extraction (SPE) column (Phenomenex, Cheshire, United Kingdom) preequilibrated with methanol and water. The column was washed with 60 ml of 25% ethanol, and nisin was eluted in 60 ml IPA (0.1% TFA), which was subjected to reversed-phase high-performance liquid chromatography (RP-HPLC). The sample was applied to a semipreparative Proteo Jupiter (250 mm [length] by 10 mm [inside diameter], 90 Å [pore size], 4 μm [particle size]) RP-HPLC column (Phenomenex) running a gradient of 25 to 40% acetonitrile and 0.1% TFA, where buffer B was 90% acetonitrile and 0.1% TFA. The resulting eluent was monitored at 214 nm, and fractions were collected at 1-min intervals. Column eluents and HPLC fractions were assayed for antimicrobial activity by well diffusion assays (WDAs), according to the method of Parente and Hill (41), using *L. delbrueckii* subsp. *bulgaricus* LMG 6901 as the target organism. Column eluents and HPLC fractions displaying antimicrobial activity were assayed for the nisin J molecular mass by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) on an Axima TOF^2 MALDI-TOF MS in positive-ion reflectron mode (Shimadzu Biotech, Manchester, United Kingdom). Fractions containing pure nisin J were pooled and lyophilized in a Genevac lyophilizer (Suffolk, United Kingdom). Pure nisin A peptide was prepared from *L. lactis* NZ9700 as described for nisin J but excluding the SP Sepharose step. Nisin Z pure peptide was sourced from Handary (Fleurus, Belgium).

Comparison of the inhibitory spectra of nisins A, Z, and J. Pure nisins A, Z, and J were resuspended in RNase-free water to a final concentration of 1 mg/ml and subsequently assayed by WDA against a range of target indicator strains (Table 2). Zone diameters were measured in millimeters using Vernier calipers (DML-Digital Micrometers Ltd., Sheffield, United Kingdom) and recorded in Table 2 as area of the zone (πr^2) minus the area of the well (πr^2) in millimeters.

MIC determinations. MICs were determined in triplicate from pure nisins A, Z, and J against approximately 1×10^5 CFU/ml of the target indicator strain *Lactococcus lactis* subsp. *cremoris* HP using 96-well microtiter plates (Sarstedt, Co. Wexford, Ireland) and using a Libra S2 colorimeter (Biochrom Ltd., Cambridge, United Kingdom) to measure the optical density at 600 nm (OD_{600}) of the indicator strains. Peptide concentrations of 4× the test concentration (2,048 nM) were prepared in 400 μl RNase-free and DNase-free water. One hundred microliters of growth medium was added to all wells of the 96-well plate. One hundred microliters of 4× concentration was added to the first well, and subsequently, 2-fold serial dilutions were carried out. MIC readings were taken after 16 h at 30°C. The MIC was recorded as the lowest concentration of lantipeptide where no growth of the indicator was observed (42).

Cross-immunity of nisin J-producing *S. capitis* APC 2923 to other nisin-producing strains. To investigate if the nisin J-producing *S. capitis* APC 2923 strain was immune to other nisin-producing cultures (*L. lactis* NZ9700 producing nisin A, *Streptococcus hyointestinalis* DPC 6484 producing nisin H, and *S. uberis* strain 42 producing nisin U), cross-immunity assays were performed based on the WDA method, whereby each strain was tested as an indicator and a producer (43).

Determining if the nisin J structural gene is unique to *S. capitis* APC 2923. To determine if the nisin J structural gene was present in other *S. capitis* strains isolated from the study by O'Sullivan et al. (5), oligonucleotide primers designed to specifically amplify the nisin J structural gene (*nisJ* F, 5′-ACTT TATAACTAAGATTAGC-3′, and *nisJ* R, 5′-TCGCTTTATTATTTAGTATGCACG-3′) were used in a PCR under the following conditions: initial denaturation, 94°C for 5 min; 35 cycles of 94°C for 40 s, 52°C for 30 s, and 72°C for 1 min; and a final extension 72°C for 10 min. Sequencing was conducted by Genewiz (Essex, United Kingdom). Sequencing data were analyzed employing the Lasergene 8 software (DNAStar, Inc., Madison, WI) and subsequently input into the ExPASy online translate tool (https://web.expasy.org/translate/) to translate the nucleotides into amino acid sequences.

Sequence analysis of the nisin J plasmid pJOS01. To confirm that the nisin J gene cluster was plasmid associated, the plasmid DNA of *S. capitis* APC 2923 was extracted using the Plasmid maxi kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions following an adapted user-developed protocol specific to staphylococcal species (https://www.qiagen.com/ie/resources/resourcedetail?id=82ddd661-fbab-4d35-819c-defd6269fc64&lang=en), using lysostaphin (Sigma-Aldrich Ireland Limited, Arklow, County Wicklow, Ireland). The resulting DNA extract was sequenced by Illumina MiSeq technology (2 × 250-bp paired-end reads; GenProbio, Parma, Italy). *De novo* sequence assemblies and automated gene calling were performed using the MEGAnnotator pipeline (44) and assessed for predicted tRNA genes via transcend-SE version 1.2.1 (45). Predicted open reading frames (ORFs) were determined via Prodigal version 2.6 and Genemark.hmm (46). A BLASTP (47) analysis was performed to assign functional annotations to the predicted ORFs (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Table 1). PlasmidFinder (version 2.0) was employed to confirm that the generated assembled contigs were plasmid sequences based on the identification of Rep proteins. SnapGene version 2.3.2 was employed to generate a map of the plasmid harboring the *nisJ* gene cluster (designated pJOS01 here). In addition to the sequence data analysis to confirm the plasmid association of the nisin J cluster, PCR-based analysis was undertaken using the plasmid DNA extract as the template. Oligonucleotide primers designed to specifically amplify the nisin J structural gene (*nisJ* F, 5′-ACTTTATAACTAAGATTAG

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C-3′, and *nisJ* R, 5′-TCGCTTTATTATTTAGTATGCACG-3′) were used in a PCR using Phusion Green Hot Start II high-fidelity PCR master mix with the following conditions: initial denaturation, 98°C for 5 min; 30 cycles of 98°C for 10 s, 52°C for 30 s, and 72°C for 15 s; and a final extension of 72°C for 10 min. Validation of the amplicon was performed by Sanger sequencing of the generated product (Source BioScience, Waterford, Ireland). Furthermore, restriction digestion of the plasmid DNA was carried out using EcoRI in 10× CutSmart buffer (New England BioLabs, Herts, United Kingdom).

Investigation for the presence of nisin-resistant determinants in *S. capitis* APC 2923. To determine if the gene encoding the nisin resistance protein (NSR) was present in *S. capitis* APC 2923 and the 7 other *S. capitis* isolates previously identified from the O'Sullivan et al. study (5), PCR was employed using the primers and reaction conditions described by Simões et al. (38). To determine if the nisin J-producing *S. capitis* strain APC 2923 was cross-immune or sensitive to NSR-producing strains, bioassays were carried out by spotting 10 μl of the nisin J overnight culture onto 1.5% BHI agar (Merck, Darmstadt, Germany). Following overnight incubation at 37°C, the plates were then overlaid with soft (0.75%) GM17 agar (BD Difco Trafalgar Scientific Ltd., Leicester, United Kingdom) seeded with 0.25% of an overnight culture of the NSR-positive strain *L. lactis* subsp. *diacetylactis* DRC3. To directly compare the resistance levels of nisin A and nisin J to NSR, WDAs were carried out as previously described (43), employing *L. lactis* MG1614/pNP40 (NSR-positive strain) and *L. lactis* MG1614 (NSR-negative strain) as target indicators. All lactococcal NSR indicator strains were grown aerobically overnight at 30°C. Agarose assays were subsequently performed as outlined in reference 42. Data obtained from the agarose assays were subjected to normality tests prior to statistical analysis using the GraphPad Prism software (version 8.2.1). *P* values were calculated using an unpaired *t* test.

Data availability. The plasmid map of pJOS01 has been deposited in GenBank under accession number MN602039. This whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession number WHVU00000000. The version described in this paper is version WHVU01000000.1.

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We declare no competing interests.

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