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RESEARCH ARTICLE

Human skin microbiota is a rich source of bacteriocin-producing staphylococci that kill human pathogens

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One sentence summary: In the search for novel topical skin therapies, we have examined skin-derived bacteriocins, produced by microbes which have evolved in such a way as to outcompete pathogens, and successfully colonise the skin environment.

Editor: Julian Marchesi

ABSTRACT

The demand for novel antimicrobial therapies due to the threat posed by antimicrobial resistance has resulted in a growing interest in the protective role of our skin bacteria and the importance of competition among bacteria on the skin. A survey of the cultivable bacteria on human skin was undertaken to identify the capacity of the skin microbiota to produce bacteriocins with activity against skin pathogens. Twenty-one bacteriocins produced by bacteria isolated from seven sites on the human body of each subject exhibited inhibition spectra ranging from broad to narrow range, inhibiting many Gram-positive bacteria, including opportunistic skin pathogens such as *Propionibacterium acnes* (recently renamed *Cutibacterium acnes*), *Staphylococcus epidermidis* and methicillin-resistant *Staphylococcus aureus* (MRSA). Sequencing indicated that the antimicrobial-producing isolates were predominately species/strains of the *Staphylococcus* genus. Colony mass spectrometry revealed peptide masses that do not correspond to known bacteriocins. In an era where antibiotic resistance is of major concern, the inhibitory effect of novel bacteriocins from the bacteria of skin origin demonstrates the antimicrobial potential that could be harnessed from within the human skin microbiota.

Keywords: skin pathogens; staphylococci; antimicrobial potential; bacteriocin; skin microbiota; skin microbiome

INTRODUCTION

The human skin microbiome is home to hundreds of diverse bacterial species from the four phyla Actinobacteria, Proteobacteria, Firmicutes and Bacteriodetes, which act as a part of the body's first line of defence against the external environment (Natsuga 2014). The commensal microbiota of the skin contributes to host health and is thought to play a role in protecting the host against a wide range of infections. One such defence mechanism is the production of bacteriocins. Bacteriocins are ribsosomally synthesised, heat-stable antimicrobial peptides that are produced by bacteria that can exhibit both

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broad and narrow range inhibition spectra. Lantibiotics belong to class I bacteriocins, which are heavily post-translationally modified—characterised by the presence of lanthionine/B-methyllanthionine residues (McAuliffe, Ross and Hill 2001). Bacteriocins may exert a diverse array of functions from colonising peptides, which help the producer to become established in a niche, to killing peptides for eliminating competitors or as signalling peptides through interactions with other bacteria and the immune system (Schauber and Gallo 2008; Bastos *et al.* 2009; Dobson *et al.* 2012). The importance of these traits can be seen where imbalances in the ratio or composition of skin microbiota have been linked to skin diseases/infections such as psoriasis, atopic dermatitis, acne and impetigo (Yamasaki and Gallo 2008; Otto 2009; Zeeuwen *et al.* 2013; Grice 2014). It has been hypothesised that restoration of this natural microbial balance could potentially alleviate or prevent such skin infections and would reduce the use of antibiotics to treat these conditions (Sanford and Gallo 2013).

Staphylococcus species are the dominant bacterial colonisers of the skin and are divided into two main groups on the basis of coagulase activity. Coagulase triggers the coagulation of soluble fibrinogen-forming insoluble fibrin, resulting in the formation of a clot. Thus, coagulase-positive staphylococci, for example *Staphylococcus aureus*, which can reside on the skin surface as a commensal organism, are potentially pathogenic and are capable of producing haemolysins and a range of heat-stable toxins. Conversely, the skin is also colonised by a diverse array of coagulase-negative *Staphylococcus* species, which form a part of the normal commensal bacteria of the skin. The production of lantibiotics is abundant within commensal coagulase-negative staphylococci; for example *S. gallinarum, S. epidermidis* and *S. hominis* produce the lantibiotics gallidermin, epidermin and hominicin, respectively (Götz *et al.* 2014). Studies have characterised the human skin microbiome in healthy and diseased states (Grice and Segre 2011), and while little has been done in screening the human skin specifically for novel bacteriocins, there is increasing interest in the human skin microbiome as a source of competitive strains and novel antimicrobials. This has led to the discovery of a novel antibiotic, lugdunin, isolated from a nasal strain of *Staphylococcus lugdunensis* (Zipperer *et al.* 2016). Furthermore, the application of human skin commensals as sources of protection against *S. aureus* and skin neoplasms (Nakatsuji et al., 2017, 2018) highlight the potential of human skin commensals as possible alternative solutions to antibiotics.

Here, we describe one approach targeted specifically at the isolation of coagulase-negative staphylococcal skin isolates and investigate their inhibitory activity against a range of skin pathogens associated with skin infections such as MRSA, atopic dermatitis and *Acne vulgaricus*, as well as the causative agents of mastitis.

MATERIALS AND METHODS

Isolation of antimicrobial-producing skin isolates

Twenty healthy volunteers, 18–65 years of age, 13 females and 7 males, were recruited for this study, which was approved by the Cork Clinical Research Ethics Committee under Protocol number APC071. Seven different areas of the body were swabbed, specifically the retro auricular crease (behind ear), the axillary vault (underarm), the inguinal crease (groin), the umbilicus (belly button), the gluteal crease (between lower back and buttocks), the alar crease (side of nose) and the toe web space (between toes). Each swab (Amies, PS, Viscose swabs; Sarstedt, Sinnottstown Lane, Drinagh, Wexford, Ireland) was dipped in sterile saline before swabbing the body area (Landers, Hoet and Wittum 2010) and swabs were stored for no more than 12 hr at 4°C before being homogenised in 2 mL of Maximum Recovery Diluent (MRD; Oxoid Ltd., Basingstoke, Hampshire, UK). Tenfold serial dilutions were performed in MRD and 100 μL aliquots were spread-plated onto Mannitol Salt Agar (MSA; Oxoid Ltd.) and Brain Heart Infusion (BHI) agar (Merck, Darmstadt, Germany). All plates were incubated aerobically at 37°C for 48 hr. After incubation, colonies were counted and colonies of different morphologies and colours were streaked for purity and grown aerobically in 10 mL of BHI broth (Merck) at 37°C in a shaking incubator (311DS Labnet) overnight.

Detection of antimicrobial activity

Spot bioassays for antimicrobial production were then carried out on the isolates by spotting 10 μL of the overnight culture onto BHI agar; the plates were incubated overnight at 37°C and then overlaid with MRS sloppy *Lactobacillus* MRS broth (BD™ Difco™ Trafalgar Scientific Ltd, Leicester, United Kingdom) containing 0.75% agar seeded with 0.25% of an overnight culture of *Lactobacillus delbrueckii* ssp. *bulgaricus* LMG 6901 and grown anaerobically overnight (see Table 1 for optimal growth conditions of bacterial strains used in this study). The remaining colonies on the BHI plates were also overlaid with *L. delbrueckii* ssp. *bulgaricus* LMG 6901. The MSA plates were overlaid with sloppy (0.75%) BHI agar seeded with an overnight inoculum of either 0.25% *Listeria innocua* DPC 3572 or 0.25% MRSA DPC 5645. Colonies from spot assays and plate overlays that exhibited zones of inhibition were then inoculated into BHI broth, grown overnight at 37°C and stocked in 20% glycerol at −80°C for further characterisation (Fig. 1).

Characterisation of antimicrobial activity of skin isolates

Well diffusion assays (WDA)

Cell-free supernatants (CFS) were prepared from 10 mL overnight cultures of putative antimicrobial-producing strains (isolated from BHI and MSA plates) by centrifugation (Sorvall Legend RT) at 4000 × *g* for 20 min following overnight growth in BHI broth in a shaking incubator at 37°C (Ryan *et al.* 1996). To eliminate inhibition due to acids, the CFS were neutralised to pH 6.9–7.2 using 1 M NaOH. Wells were bored in MRS agar plates, previously seeded with 0.25% inoculum of an overnight culture of *L. delbrueckii* ssp. *bulgaricus* LMG 6901, and 50 μL of each neutralised supernatant was added to the wells. The plates were incubated anaerobically at 37°C overnight and observed for zones of inhibition around the well (Ryan *et al.* 1996). Strains showing zones of inhibition were selected for further study.

16S rRNA sequencing

Genomic DNA was extracted from the antimicrobial-producing strains (grown overnight in 10 mL BHI broth at 37°C) using a Sigma-Aldrich DNA purification kit as described by the manufacturer (Sigma-Aldrich Ireland Limited, Vale Road, Arklow, Co. Wicklow, Ireland). For 16S rRNA sequencing, universal primers Uni F 5′-AGAGTTTGATCCTGGCTCAGG-3′ and Uni R 5′-ACGGCAACCTTGTTACGAGT-3′ were used for PCR reactions (run conditions: initial denaturation: 94°C × 5 min, cycling conditions: 30 cycles of 94°C × 40 s, 55°C × 30 s, 72°C × 1 min, final extension: 72°C × 10 min) to initially identify the

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Table 1. Growth conditions of the indicator strains used in this study.

Species	Strain	Growth conditions		
		Temp (°C)	Atmosphere	Growth media
Bifidobacterium longum subsp. *infantis*	ATCC 15697	37	Anaerobic*	mMRS
Bifidobacterium longum subsp. *longum*	DSM 20097	37	Anaerobic	mMRS
Corynebacterium xerosis	DPC 5629	37	Aerobic	BHI
Corynebacterium variablis	NCIMB 702097	30	Aerobic	BHI
Cutibacterium acnes	LMG 16711	37	Anaerobic	mRCM & RCA
Enterococcus faecalis	ATCC 19433	37	Anaerobic	MRS
Fusobacterium varium	DSM 19868	37	Anaerobic	BHI
Lactobacillus delbrueckii subsp. *bulgaricus*	LMG 6901	37	Anaerobic	MRS
Lactobacillus fermentum	APC 2582	37	Anaerobic	MRS
Lactobacillus rhamnosus	APC 3483	37	Anaerobic	MRS
Listeria innocua	DPC 3572	37	Aerobic	BHI
Listeria monocytogenes	DPC 5788	37	Aerobic	BHI
Listeria monocytogenes	DPC 6893	37	Aerobic	BHI
Listeria monocytogenes	NCTC 5348	37	Aerobic	BHI
Pseudomonas aeruginosa	NCIMB 8295	37	Aerobic	BHI
Pseudomonas aeruginosa	APC 2064	37	Aerobic	BHI
Pseudomonas fluorescens	DPC 6056	30	Aerobic	BHI
Pseudomonas putita	ATCC 17522	37	Aerobic	BHI
MRSA	DPC 5645	37	Aerobic	BHI
Staphylococcus aureus	ATCC 25923	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, APC 1055	37	Aerobic	BHI
Streptococcus bovis	DPC 6491	37	Aerobic	GM17
Streptococcus dysgalactiae	APC 3484	37	Aerobic	BHI
Streptococcus uberis	DPC 5344	37	Aerobic	BHI

mMRS = modified MRS, mRCM = modified Reinforced Clostridial Media (made following ATCC Medium:2107 Modified Reinforced Clostridial agar/broth (pre-reduced) protocol), RCA = Reinforced Clostridium agar.
*= Anaerobic conditions, where required, were achieved through the use of anaerobic jars and Anaerocult A gas packs (Merck, Darmstadt, Germany).
ATCC = American Type Culture Collection, APC = APC Microbiome Ireland Culture Collection, DPC = Teagasc Culture Collection, WSLC = Weihenstephan Listeria Collection, LMG = Laboratorium voor Microbiologie, Universteit Gent, Belgium.

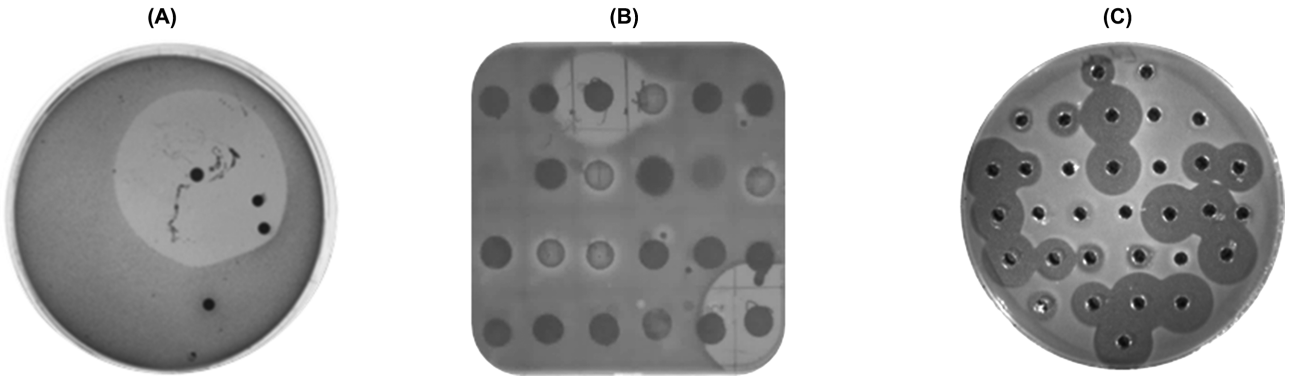


Figure 1. (A) Overlay of an original plate containing dilution of a toe swab with *L. delbrueckii* ssp. *bulgaricus* LMG 6901. **(B)** Spot assay of 10 μL of inoculum from colonies of varying morphologies isolated from swab dilution plates, overlaid with *L. delbrueckii* ssp. *bulgaricus* LMG 6901. **(C)** Well diffusion assay of antimicrobial-producing skin isolates in agar seeded with *L. delbrueckii* ssp. *bulgaricus* LMG 6901 to confirm that the positive result in spot assay was due to antimicrobial substance secreted into CFS.

antimicrobial-producing strains. To confirm species, degenerate primers targeting the *dnaJ* gene (Shah *et al.* 2007) forward primer, 5′-GCCAAAAGAGACTATTATGA-3′, and reverse primer, 5′-ATTGYTTACCYGTTTGTGTACC-3′, were used for the PCR reactions, following conditions described by Shah *et al.* (2007). Quantification of the extracted DNA and PCR products was conducted using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, 168

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Third Avenue Waltham, MA USA 02451). Sequencing was conducted by Genewiz (Hope End, Takeley, Essex, CM22 6TA, United Kingdom) and analyses of the sequencing data were performed utilising Lasergene 8 software (DNAStar Inc., Madison, WI). Basic local alignment search tool (BLAST) on the National Centre for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov) was used to compare the sequencing data of the isolates to existing genomic data.

Heat stability and protease sensitivity

To establish the heat stability of bacteriocins produced, CFS were incubated for 10 min at a range of temperatures, 37, 60, 70, 80, 90, 100°C, and for 15 min at 121°C before performing a WDA in 1.5% MRS agar seeded with 0.25% *L. delbrueckii* ssp. *bulgaricus* LMG 6901 as previously described; see Fig. S2A (Supporting Information).

To investigate whether the antimicrobials produced were proteinaceous in nature, the CFS of all antimicrobial-producing isolates were incubated with Proteinase K (Sigma) to a final concentration of 20 mg/mL at 37°C for 1 hr and tested for antimicrobial activity by a WDA using *L. delbrueckii* ssp. *bulgaricus* LMG 6901 as the target organism as described above. The corresponding CFS, mixed with an equivalent volume of water, was used as a control; see Fig. S2B and S2C (Supporting Information).

Cross-immunity

To determine the relatedness of the bacteriocins produced by the strains, cross-immunity assays were performed as follows: CFS of all producing strains were tested against each other by conducting WDA assays as previously described using each producing strain as a producer and also as a target strain. Each producing strain was inoculated individually (at 0.25%) into BHI agar, which was allowed to solidify and 50 μL of the pH-neutralised CFS of each strain was added to wells of all plates; see Fig. 2.

Spectrum of inhibition

A spectrum of inhibition was completed on pH-adjusted CFS of all antimicrobial-producing isolates using 28 indicator strains by a WDA as described previously. All indicator strains were diluted to the same optical density, ($OD_{600\,nm}$ 0.8), using a Biochrom Libra S2 Colorimeter (Biochrom Ltd., Cambourne Business Park, Cambridge, United Kingdom) before seeding the agar. Indicator strains employed to detect antimicrobial activity and their optimal growth conditions are listed in Table 1. Zone size was measured as follows: area of zone πr^2 − area of well πr^2 in millimetres.

Pulsed Field Gel Electrophoresis

All isolates identified as *Staphylococcus* species were fingerprinted using Pulsed Field Gel Electrophoresis (PFGE) as described by Bannerman *et al.* (1995) with minor modifications. Cultures were inoculated at 0.02% and grown in a shaking incubator overnight in 5 mL BHI broth at 37°C. The overnight culture (0.7 mL) was centrifuged for 2 min at 4500 × *g* in an Eppendorf centrifuge. The supernatant was discarded, and the cell pellets were washed in 1 mL of sterile TE buffer (0.1 M Tris Cl, 0.15 M NaCl, 0.1 M EDTA, pH7.5) and recentrifuged. Washed cells were resuspended in 0.3 mL sterile EC buffer (6 mM Tris-HCl, 1 M NaCl, 0.1 M EDTA, 1% Sarkosyl). A solution of lysostaphin (1 mg/mL) was prepared in 20 mM sodium acetate (pH 4.5) and 2 μL was added to the cell suspension and then vortexed. A 2% Sea Plaque agarose (Bio-Rad Laboratories Ltd., Watford, Hertfordshire, United Kingdom) solution was prepared in EC buffer and 300 μL added to 300 μL lysostaphin-cell suspension was mixed gently to avoid the formation of bubbles and then quickly pipetted into PFGE plug molds. The agar plugs were allowed to solidify at room temperature for 10 min. When solidified, the plugs were incubated in 3 mL EC buffer overnight at 37°C. After overnight incubation, the EC buffer was removed and replaced with 3 mL TE buffer (10 mM Tris Cl, 1 mM EDTA) and incubated for 1 hr at 55°C without shaking. Prior to electrophoresis, the plugs were cut into small slices of 2 × 5 mm and transferred into Eppendorf tubes containing 125 μL of 20U *Sma*1 restriction enzyme (New England Biolabs, 75–77 Knowl Piece, Wilbury Way, Hitchin, Herts, United Kingdom) in buffer. Plugs were incubated overnight with shaking (300 rpm) at 25°C. Following digestion, trimmed slices of plug were loaded onto the PFGE comb, fixed with 1% PFGE-grade agarose (Bio-Rad Laboratories) in 0.5X Tris-borate EDTA (TBE) buffer. Lambda PFGE marker (New England Biolabs) was loaded onto the gel as described above and used as a reference ladder. The gel was run in 0.5X TBE buffer using a CHEF-DR II®PFGE apparatus using the following gel running parameters: initial pulse 5 s, final pulse 40 s, voltage 200 V or 6 V/cm for 20 hr at 12–14°C. The gel was stained for 2 hr with ethidium bromide (0.5 μg/mL), destained for 1 hr in distilled water and photographed using a GelDoc-It Imager (Ultra-Violet Products Ltd., Cambridge, United Kingdom).

Activity units

For the 13 different antimicrobial-producing strains identified from PFGE, bacteriocin activity units (AU) were calculated as described by Nilsen, Nes and Holo (2002) based on the WDA. Briefly, 50 μL of CFS was diluted twofold and dilutions were dispensed into wells of plates seeded with indicators listed in Table 4. AU were calculated by multiplying the reciprocal of the lowest dilution of the CFS, which produced zones of inhibition by 20 in order to obtain AU/mL; see Table 4. This assay was performed in duplicate.

MALDI TOF mass spectrometry

Colonies from the 13 antimicrobial-producing skin isolates were mixed with 50 μL propan-2-ol 0.1% TFA, vortexed three times and centrifuged at 16 000 × *g* for 30 s. MALDI TOF mass spectrometry was performed on the CFS using an Axima TOF^2 MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK). An aliquot (0.5 μL) of matrix solution (α-cyano 4-hydroxy cinnamic acid, 10 mg mL^{-1} in acetonitrile-0.1% (v/v) trifluoroacetic acid) was deposited onto the target and left for 10 s before being removed. The residual solution was allowed to air-dry and 0.5 μL of the sample solution was deposited onto the pre-coated sample spot; 0.5 μL of matrix solution was added to the deposited sample and allowed to air-dry. The sample was then analysed in the positive-ion linear mode and peptide masses were compared to the bacteriocin database 'Bactibase' to identify putative bacteriocins.

RESULTS AND DISCUSSION

The human skin microbiome is home to trillions of bacteria but is predominately colonised by members of the genus *Staphylococcus* (Otto 2010). The skin is the defensive barrier that separates our internal organs from the external world. It acts as a first line of defence against pathogens, and the layers of skin help prevent harmful substances from accessing the body (Sanford and Gallo 2013; Zeeuwen *et al.* 2013). Significant therapeutic potential has

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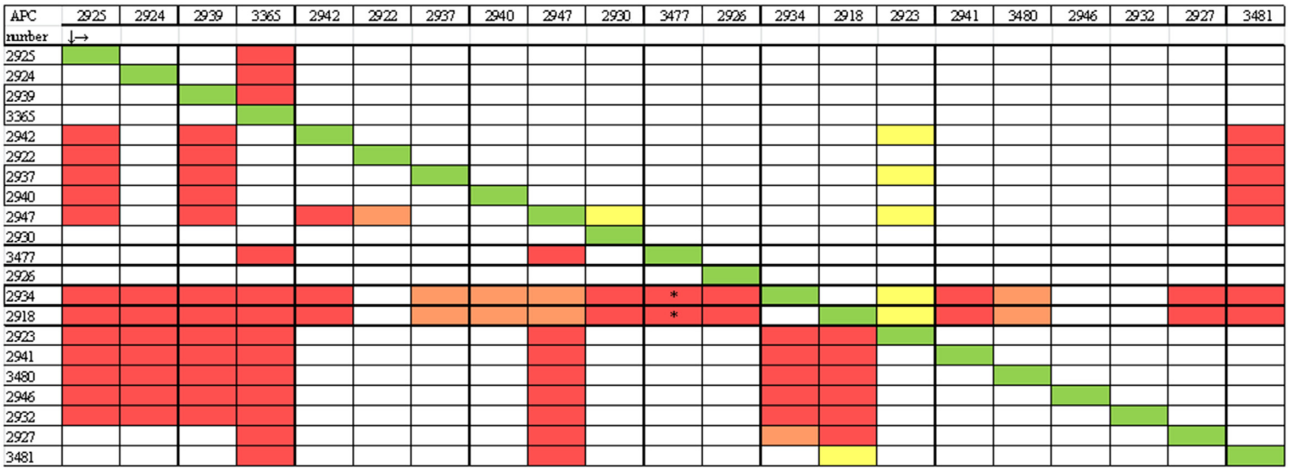


Figure 2. Cross-immunity assays using CFS from all the antimicrobial-producing strains tested against themselves and each other, with the list of antimicrobial-producing isolates down the left-hand side and the indicator strains across the top of the table. The diagonal green line reveals that the antimicrobial producer is immune to its own antimicrobial, as expected. The red box represents inhibition of the indicator strain indicating that these strains might be different or sensitive to each other. (orange) = small/ medium hazy zones. (yellow) = very small zones but sensitive. White boxes represent no inhibition, revealing target strains not inhibited by the producing strain, indicating they are immune (related) to the bacteriocins produced by other producers. * = initially sensitive but bactostatic activity observed.

been identified for the staphylococcal genus, including the production of class 1a lantibiotics such as epidermin, nukacin, gallidermin and aureocin (Schnell et al., 1989, 1992; Sashihara *et al.* 2000; Netz *et al.* 2002), as well as a novel antibiotic lugdenin (Zipperer *et al.* 2016). Consequently, a culture-based approach was adopted in this study to isolate antimicrobial-producing bacteria with a particular focus on those that exhibit inhibitory activity against bacteria involved in the pathogenesis of skin infections. These include MRSA, *S. epidermidis, Cutibacterium acnes* and a number of mastitic pathogens, such as *Streptococcus uberis*, *Streptococcus bovis, Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *S. aureus*.

Detection and isolation of antimicrobial-producing skin isolates

Twenty volunteers of mixed age, gender and race were recruited to provide swabs from seven body locations. The body sites were chosen based on the varied bacterial compositions of different skin areas (Grice and Segre 2011). Superficial swabs were employed in this study because the swabbing method was less invasive and was reported to yield a largely similar microbiota profile to that associated with skin scraping of epidermis or punch biopsy of the full thickness of epidermis and dermis when next-generation sequencing methods were used (Grice *et al.* 2008). Over 90,000 colonies were initially screened for antimicrobial activity; these were isolated from 7 sites from the 20 individuals (140 sites) using the agar overlay technique on the original isolation plates. Samples were serially diluted and spread-plated onto the surface of BHI and MSA agar plates and all plates that contained visible growth were counted as potential antimicrobial producers and overlaid with *L. delbrueckii* ssp. *bulgaricus* or MRSA/*L. innocua*, respectively. At this stage of the study, the genetic relationship between the colonies was not determined. Spot bioassays on selected individual colonies and original plate overlays identified 101 possible antimicrobial-producing isolates (see the supplementary information for more details). To confirm that an antimicrobial substance was secreted into the growth medium, neutralised CFS was tested against *L. delbrueckii* ssp. *bulgaricus* LMG 6901 by WDA and this reduced the number of possible bacteriocin producers to 25, giving an observed frequency of 0.03% from the 90 000 colonies screened in this study. Due to small zone sizes, four of the antimicrobial producers were discarded. *Lactobacillus delbrueckii* ssp. *bulgaricus* LMG 6901 was selected as the primary indicator for all assays because of its insensitivity to acids, thus reducing the frequency of false positives (Casey *et al.* 2004). While it is now accepted that many bacteria in natural ecosystems have the genetic potential to produce bacteriocin-like inhibitors, their detection *in vitro* is largely dependent on the screening methods undertaken. The low frequency observed in this study may contribute to the fact that only *L.* delbrueckii ssp. *bulgaricus* was used as the primary indicator bacterium.

Identification of antimicrobial-producing skin isolates

16S rRNA sequencing of the 25 antimicrobial-producing isolates revealed four *Bacillus* species—*Bacillus licheniformis* (2), *Bacillus endophyticus* and *Bacillus safensis*—but the remainder were coagulase-negative *Staphylococcus* species. However, additional analysis by amplification of the *dnaJ* gene was required to further differentiate the *Staphylococcus* species. Five *Staphylococcus* species were identified, including *S. capitis* (9), *S. hominis* (4), *S. epidermidis* (1), *S. simulans* (1) and *S. warneri* (6) (Table 2). *Staphylococcus capitis*, a frequent coloniser of human skin, was the most prevalent species isolated (43%) followed by *S. warneri* (29%), which has been reported to be a less-frequent skin coloniser (Otto 2010)). Twelve strains (~50%) of antimicrobial-producing staphylococci were isolated from the toe web-space body area. The four antimicrobials which were omitted from the study, as they were considered poor producers, were *Bacillus* species.

Characterisation of antimicrobial-producing skin isolates

A characteristic of bacteriocins is that they are relatively heat stable and are susceptible to degradation by proteolytic enzymes (Jack, Tagg and Ray 1995). Antimicrobial activity in the CFS from all strains was eliminated following Proteinase K treatment, indicating that the antimicrobials were proteinaceous in nature

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Table 2. Identification of antimicrobial-producing strains isolated from skin.

Strain Identity	Strain number	Subject no.	Body area	PFGE lane no.
Staphylococcus hominis	APC 2925	SM019	toe web-space	1$
Staphylococcus hominis	APC 2924	SM012	toe web-space	2
Staphylococcus hominis	APC 2939	SM019	gluteal crease	3$
Staphylococcus hominis	APC 3365	SM020	gluteal crease	4
Staphylococcus warneri	APC 2942	SM009	axillary vault	5*
Staphylococcus warneri	APC 2922	SM001	toe web-space	6
Staphylococcus warneri	APC 2937	SM006	gluteal crease	7*
Staphylococcus warneri	APC 2947	SM007	toe web-space	8
Staphylococcus warneri	APC 2940	SM007	gluteal crease	9*
Staphylococcus warneri	APC 2930	SM015	toe web-space	10
Staphylococcus epidermidis	APC 3477	SM015	toe web-space	11
Staphylococcus simulans	APC 2926	SM002	toe web-space	12
Staphylococcus capitis	APC 2934	SM003	inguinal crease	13
Staphylococcus capitis	APC 2918	SM001	alar crease	14
Staphylococcus capitis	APC 2923	SM007	toe web-space	15
Staphylococcus capitis	APC 2941	SM010	umbilicus	16
Staphylococcus capitis	APC 3480	SM009	umbilicus	17
Staphylococcus capitis	APC 2946	SM008	toe web-space	18
Staphylococcus capitis	APC 2932	SM008	toe web-space	19
Staphylococcus capitis	APC 2927	SM015	toe web-space	20π
Staphylococcus capitis	APC 3481	SM015	toe web-space	21π

$, *, π These symbols reflect identical PFGE patterns from Figure 3.

(Fig. S2, Supporting Information). In addition, all antimicrobials were stable up to 100°C for 10 min and showed only a 50% reduction in activity after autoclaving at 121°C for 15 min (Fig. S2, Supporting Information).

One of the features of bacteriocin production is that the genome of producers must harbour immunity gene(s), which protect producers from the antimicrobial effects of the bacteriocin they produce (Cotter, Hill and Ross 2005; Yang *et al.* 2014). Cross-immunity assays were performed to determine whether the producing strains were sensitive to the antimicrobial activity of the other producers (Fig. 2). To determine the genetic relatedness of the 21 staphylococci isolated, the strains were fingerprinted using PFGE and similarities were categorised on the basis of the procedure described by Tenover *et al.* (1995). Strains of the same species are closely related, in some cases only differing from each other by a single DNA band, for example *S. hominis* strains APC 2925, APC 2924 and *S. capitis* strains APC 2934 and APC 2918 (Fig. 3). However, the PFGE analysis revealed 13 genetically different antimicrobial-producing staphylococci—three *S. hominis*, four *S. warneri*, one *S. epidermidis*, one *S. simulans* and four *S. capitis*.

Combining data from cross-immunity assays and PFGE analysis revealed that antimicrobials produced by skin isolates of the same species and of the same or similar pulsotype were immune to each other, suggesting they are producing the same bacteriocin and are possibly the same strain. For example, two *S. hominis* strains APC 2925 and APC 2939, isolated from different body areas (toe web-space and gluteal crease) of the same subject, yielded the same pulsotype (lanes 1 and 3 on PFGE gel) and had the same immunity pattern as another *S. hominis* strain, APC 2924, from a different subject whose pulsotype differed by just one *Sma*1 digestion DNA band (lane 2 on PFGE gel). This was also the case for two *S. capitis* strains from two different subjects (APC 2934 and APC 2918, lanes 13 and 14 of PFGE gel). This shows that some different strains that are closely related can produce the same bacteriocin.

PFGE profiles demonstrated that the same pulsotype can be shared across a number of individuals, with the same *S. capitis* pulsotype isolated from four different subjects (Fig. 3, lanes 15–19) and three subjects sharing the same *S. warneri* pulsotype: APC 2942, APC 2937 and APC 2940 (Fig. 3, lanes 5, 7 and 9). Interestingly, we also showed that the same subject can carry more than one strain of the same species; *S. warneri* APC 2947 and APC 2940 were isolated from the subject 7. These strains were isolated from different locations on the body and exhibited different pulsotypes—(Fig. 3, lanes 8 and 9). *S. warneri* APC 2947 had a very different pulsotype to other *S. warneri* strains isolated in this study, and while it was immune to the bacteriocin produced by others of the same species, it had a different immunity pattern in that it was inhibited by *S. capitis* skin isolates unlike other *S. warneri* (Figs 2 and 3, lane 8).

Bacteriocins such as thuricin CD and nisin have either a narrow or broad spectrum of activity, respectively (Rea *et al.* 2010; Shin *et al.* 2016), and both of these characteristics may be beneficial to the producing strain depending on the niche inhabited by the bacterium. In a crowded environment like the skin, bacteriocin production might help producing strains establish themselves in a particular skin environment. For example, the production of narrow spectrum bacteriocins may confer a competitive advantage to strains, allowing them to inhibit closely related species that would be competing for similar nutrients on the skin (Dobson *et al.* 2012). Table 3 shows the spectrum of inhibition of the bacteriocins produced against a panel of bacteria including skin pathogens such as *C. acnes*, *S. epidermidis*, *Corynebacterium xerosis* (*C. xerosis*) and MRSA. The majority of *S. warneri* exhibited the broadest spectrum of activity, inhibiting nine of the indicator strains, while the inhibitory activity of three strains (APC 2930, APC 3477 and APC 2926) was shown to exhibit a narrow inhibition spectrum—inhibiting only the primary indicator *L. delbrueckii* ssp. *bulgaricus* LMG 6901.

While the majority of our commensal or resident skin microbiota are beneficial to skin health, occasionally the microbes that inhabit our skin can develop into opportunistic pathogens, for

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Table 3. Spectrum of inhibition of bacteriocin-producing skin isolates against indicator strains subjected to well diffusion assays. +<50 mm^2; ++50–150mm^2; +++150–249mm^2; ++++ 250–349mm^2; 350–449mm^2; ++++++>450 (calculated as area of zone πr^2 – area of well πr^2 in millimetres).

		S. hominis				*S. warneri*						*S. epidermidis*	*S. simulans*	*S. capitis*								
		Producer strains																				
		2925	2924	2939	3365	2942	2922	2937	2947	2940	2930	3477	2926	2934	2918	2923	2941	3480	2946	2932	2927	3481
Corynebacteria xerosis	DPC5629	++	++	++	–	+++	+++	+++	+++	+++	–	–	–	–	–	–	–	–	–	–	–	–
Cutibacterium acnes	LMG 16711	–	–	–	++	–	–	–	–	–	–	–	–	++	++	++	++	++	++	++	–	–
Lactobacillus bulgaricus	LMG6901	++	++	++	++++	++++++	++++++	+++++	++++++	++++++	+++	+++++	+	++	++	+++++	++++++	++++++	++++++	++++++	+++++	+++++
Lactobacillus fementum	APC 2582	++	++	++	+	+++	++	++	++	++	–	–	–	++	++	+	+	+	+	+	+	+
Listeria monocytogenes	DPC 6893	++	++	++	–	–	–	–	–		–	–	–	–	–	–	–	–	–	–	–	–
MRSA	DPC5645	–	–	–	–	–	–	–	–	–	–	–	–	++	++	–	–	–	–	–	–	–
Staphylococcus aureus	ATCC 25923	–	–	–	–	–	–	–	–	–	–	–	–	++	++	–	–	–	–	–	–	–
Staphylococcus aureus	DPC 7016	–	–	–	++	++	++	++	++	–	–	–	–	++	++	–	–	–	–	–	–	–
Staphylococcus epidemidis	DPC 5990	–	–	–	–	–	–	–	–	–	–	–	–	+++++	+++++	–	–	–	–	–	–	–
Staphylococcus simulans	APC 3482	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++	++	++	++	++	++	++
Streptococcus agalactiae	APC 1055	++	++	++	–	+++	+++	+++	+++	+++		–	–	–	–	+	+	+	+	+	–	–
Streptococcus agalactiae	ATCC 13813	++	++	++	++	+++	+++	+++	+++	+++	–	–	–	–	–	–	–	–	–	–	–	–
Streptococcus bovis	DPC 6491	++	++	++	+	++	++	++	++	++	–	–	–	–	–	+++	+++	+++	+++	+++	–	–
Streptococcus dysgalactiae	APC 3484	++	++	++	++	++	++	++	++	++	–	–	–	–	–	–	–	–	–	–	–	–
Streptococcus tiberis	DPC 5344	–	–	–	–	+++	+++	+++	+++	++	–	–	–		–	+++	+++	+++	+++	+++	++	++

Other indicator strains that the skin antimicrobial producers were tested against can be seen on Table 1.

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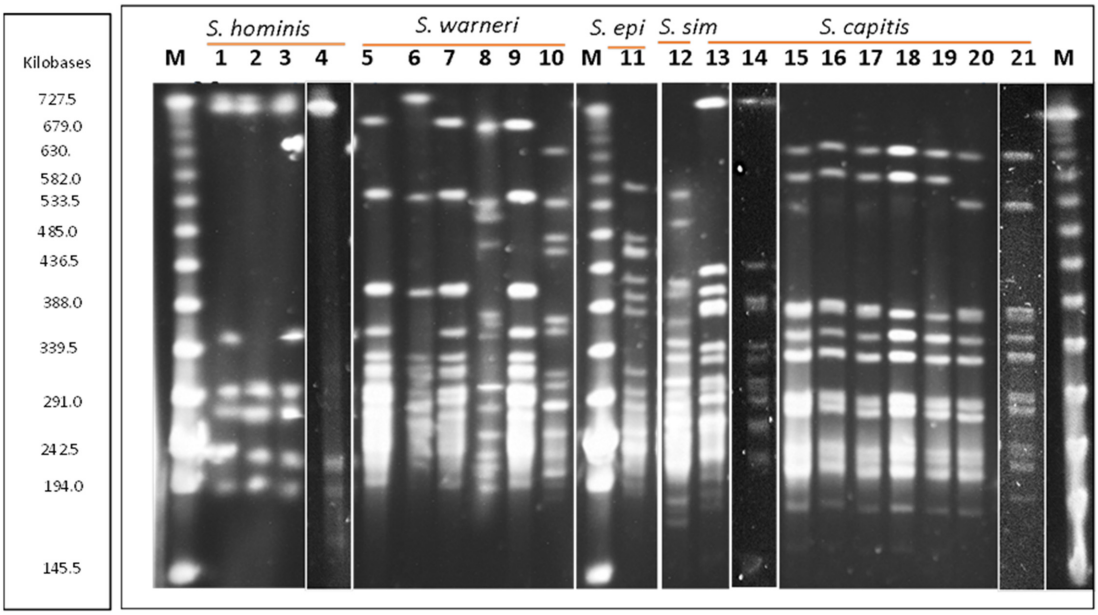


Figure 3. PFGE macrodigestion patterns of staphylococcal skin isolates following genomic digestion with *Sma*1. Lanes 1–4 = *Staphylococcus hominis*: APC 2925, 2924, 2939 and 3365; lanes 5–10 = *Staphylococcus warneri*: APC 2942, 2922, 2937, 2947, 2940 and 2930; lane 11 = *Staphylococcus epidermidis*: APC 3477; lane 12 = *Staphylococcus simulans* APC 2926; lanes 13–21 = *Staphylococcus capitis* APC 2934, 2918, 2923, 2941, 3480, 2946, 2932, 2927 and 3481. Lanes 4, 14 and 21 were taken from other PFGE gels with the same lambda marker (M) and input into this gel. epi = epidermidis; sim = simulans. Artefact in lane 3 below the first band is not a *Sma*1 DNA band.

example *S. lugdunensis* and *S. capitis* (Böcher *et al.* 2009; Cameron *et al.* 2015). Even the most abundant species of *Staphylococcus* on human skin, *S. epidermidis,* can become pathogenic if the conditions are suitable (Cogen, Nizet and Gallo 2008). Disruptions to the microbial composition of the skin are responsible for skin conditions such as psoriasis, atopic dermatitis, impetigo and acne, and sometimes these imbalances can also prevent healing of chronic wounds (Grice 2014). Two *S. capitis* strains (APC 2934 and APC 2918) isolated in this study exhibited inhibitory activity of 160 AU/mL against *S. epidermidis* (DPC 5990) (Tables 3 and 4).

S. aureus in particular is known for developing antibiotic resistance (Chambers and Deleo 2010). MRSA was first identified in 1960 and since then has become one of the most prevalent antibiotic resistant pathogens worldwide and is characterised by swollen red lesions containing pus following infection of skin wounds; it also causes many other diseases, including pneumatic diseases that can result in mortality in the elderly. A study investigating incidence of MRSA in *S. aureus* isolates in the Prince of Songkhla Hospital, Thailand found that 60.9% of the *S. aureus* isolates were methicillin resistant (Indrawattana *et al.* 2013), while a study on skin and soft tissue infections carried out in medical centres in Europe revealed that the most prevalent pathogen was MRSA (22.5%) (Johnson 2011). MRSA is first treated with glycopeptide antibiotics such as vancomycin, (Santajit and Indrawattana 2016). However, the development of novel antimicrobial therapies for methicillin-resistant and vancomycin-resistant *S. aureus* are included in the second highest priority group in a list of priority pathogens published by the WHO in a recent report describing antimicrobial resistance as a 'global emergency' (http://www.who.int/antimicrobial-resistance/en/). Alternative interventions to alleviate the problems posed by MRSA and other antimicrobial resistant pathogens are urgently required. *S. capitis* strains APC 2934 and APC 2918 with very similar pulsotypes (Fig. 3, lanes 13 and 14) were isolated in this study from two different subjects, from different body areas, namely the inguinal crease and alar crease, and were immune to each other's antimicrobial activity (Fig. 2). These strains exhibited inhibitory activities, 80 AU/mL and 40 AU/mL respectively, against a strain of the skin pathogen MRSA (DPC 5645) (Tables 3 and 4).

Many adolescents and adults will experience the effects of acne vulgaricus at some point in their lives (Webster 2002). Patients suffering from mild acne are often prescribed topical antibiotics or antiseptics at the beginning of treatment, such as clindamycin, erythromycin, benzoyl peroxide, topical retinoid or a combination of these products. However, if these are ineffective, or if the acne is classified as moderate to severe, then patients are often prescribed systemic treatments including oral antibiotics including tetracyclines, erythromycin, trimethoprim–sulfamethoxazole hormone therapy and retinoid-isotreretinoin, often for long periods (Rathi 2011). According to *TIME Health*, acne patients stay on antibiotics for four times the recommended time period. Eight skin isolates in this study displayed inhibitory activity of 40 AU/mL against *C. acnes*, which is a leading cause of acne vulgaricus (see Tables 3 and 4). Seven of these isolates were *S. capitis*; however, there were three distinct pulsotypes. *S. hominis* APC 3365 was also active against *C. acnes*.

C. xerosis is a Gram-positive aerobic bacterium, and a commensal of the human skin. *C. xerosis* can cause bacteremia, skin infections, endocarditis and pneumonia in immune-compromised patients (Pessanha *et al.* 2003). Three *S. hominis* and five *S. warneri* demonstrated inhibitory activities of 40 AU/mL and 80 AU/mL against *C. xerosis* (Tables 3 and 4), and against other *Corynebacterium* species (results not shown). PFGE analysis revealed five distinct pulsotypes (two *S. hominis* and three *S. warneri* strains (Fig. 3)). In addition to highlighting the therapeutic potential of coagulase-negative staphylococci on human skin by inhibiting skin pathogens, we also observed the ability of skin commensals to inhibit other pathogens such as *Listeria monocytogenes* (Table 3, APC 2924, APC 2925, APC 2939-2 distinct strains from PFGE, Fig. 3) and *Streptococcus* species

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Table 4. Activity units per mL (AU/mL) from well diffusion assays of the 13 different bacteriocin-producing skin isolates against indicator strains. (*S. hominis* APC 2925, 2924, 3365; *S. warneri* APC 2922, 2937, 2947, 2930; *S. epidermidis* APC 3477; *S. simulans* APC 2926; *S. capitis* APC 2934, 2918, 2923, 2927).

		Bacteriocin producing strains												
Indicator strains		2925	2924	3365	2922	2937	2947	2930	3477	2926	2934	2918	2923	2927
Corynebacterium xerosis	DPC 5629	40	40	–	80	80	80	–	–	–	–	–	–	–
Cutibacterium acnes	LMG 16711	–	–	40	–	–	–	–	–	–	40	40	40	–
Lactobacillus bulgaricus	LMG 6901	80	80	320	640	640	640	160	320	40	80	80	1280	640
Lactobacillus fermentum	APC 2582	40	40	20	80	80	80	–	–	–	20	20	40	20
Listeria monocytogenes	DPC 6893	40	40	–	–	–	–	–	–	–	–	–	–	–
MRSA	DPC 5645	–	–	–	–	–	–	–	–	–	80	40	–	–
Staphylococcus aureus	ATCC 25923	–	–	–	–	–	–	–	–	–	40	40	–	–
Staphylococcus aureus	DPC 7016	–	–	–	80	20	40	–	–	–	40	40	–	–
Staphylococcus epidermidis	DPC 5990	–	–	–	–	–	–	–	–	–	160	160	–	–
Staphylococcus simulans	APC 3482	–	–	–	–	–	–	–	–	–	–	–	40	40
Streptococcus agalactiae	APC 1055	20	20	20	320	80	80	–	–	–	–	–	20	–
Streptococcus agalactiae	ATCC 13813	20	20	–	80	80	80	–	–	–	–	–	–	–
Streptococcus bovis	DPC 6491	40	40	20	40	40	80	–	–	–	–	–	320	–
Streptococcus dysgalactiae	APC 3484	40	40	–	80	40	40	–	–	–	–	–	–	–
Streptococcus uberis	DPC 5344	–	–	–	80	40	40	–	–	–	–	–	40	20

involved in bovine mastitis, such as *St. uberis, St. agalactiae* and *St. dysgalactiae* (Table 3). While traditionally *S. aureus* was the most common cause of mastitis in dairy cows, more recently *St. uberis* has been shown to be increasingly associated with infection (Kromker, *et al.* 2014). In this study, three *Staphylococcus* species, *S. hominis, S. warneri* and *S. capitis* inhibited a range of *Streptococcus* species (*St. uberis, St. bovis, St. agalactiae* and *St. dysgalactiae*), which have been implicated in bovine mastitis (Table 3) with AU ranging from 20 to 320 AU/mL (Table 4). *Streptococcus uberis* is of particular interest as it has been increasingly shown to be one of the causative agents of bovine mastitis in the UK. This shows that the antimicrobial-producing human skin isolates may also have applications in veterinary medicine for the treatment of mastitis, given their ability to inhibit both *Staphylococcus* and *Streptococcus* species, and could aid in the search for non-antibiotic solutions for mastitis treatment to reduce antibiotics in the food chain. Mastitis in humans is also a major health issue (Michie, Lockie and Lynn 2003). *Staphylococcus epidermidis* and streptococci are among the most prevalent causative agents of this infection, found in 87.6% and 68.6% of mastitic human breast milk samples, respectively (Marín *et al.* 2017). Bacteriocin producers from human skin, identified in this study, exhibited inhibitory activity against *Staphylococcal* and *Streptococcal* species, thus highlighting their potential as live biotherapeutics for the treatment of human mastitis.

Colony mass spectrometry can be used to identify antimicrobial peptides if they can be matched to known peptide masses in the Bactibase database. However, in this case the peptide masses detected did not match any known bacteriocins, suggesting these isolates are potentially producing novel bacteriocins. The Maldi-TOF MS profiles of the 13 antimicrobial-producing staphylococci are shown in Fig. S3, Supporting Information.

CONCLUSION

There is a need to identify new antimicrobial strains with the potential to outcompete pathogens in the skin environment. The skin is a protective defence, shielding us from the external environment (Yamasaki and Gallo 2008). This study has highlighted that the skin microbiota is home to many bacteriocin-producing strains, which may indicate that the skin microbiota is an important tool in our fight against antimicrobial resistance. Indeed, this screening has resulted in the isolation of a set of 13 novel bacteriocin-producing human skin isolates with potential to restore imbalances of skin microbiota as well as to inhibit skin pathogens such as MRSA and *C. acnes*. More importantly, these strains may prove useful as probiotics for topical skin applications to provide colonization resistance by replacement of skin pathogens and particularly MRSA. Further characterisation studies will be carried out on these bacteriocin-producing skin isolates.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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Conflicts of interest. None declared.

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