Production of the Bsa Lantibiotic by Community-Acquired
Staphylococcus aureus Strains

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Lantibiotics are antimicrobial peptides that have been the focus of much attention in recent years with a view to clinical, veterinary, and food applications. Although many lantibiotics are produced by food-grade bacteria or bacteria generally regarded as safe, some lantibiotics are produced by pathogens and, rather than contributing to food safety and/or health, add to the virulence potential of the producing strains. Indeed, genome sequencing has revealed the presence of genes apparently encoding a lantibiotic, designated Bsa (bacteriocin of Staphylococcus aureus), among clinical isolates of S. aureus and those associated with community-acquired methicillin-resistant S. aureus (MRSA) infections in particular. Here, we establish for the first time, through a combination of reverse genetics, mass spectrometry, and mutagenesis, that these genes encode a functional lantibiotic. We also reveal that Bsa is identical to the previously identified bacteriocin staphylococcin Au-26, produced by an S. aureus strain of vaginal origin. Our examination of MRSA isolates that produce the Panton-Valentine leukocidin demonstrates that many community-acquired S. aureus strains, and representatives of ST8 and ST80 in particular, are producers of Bsa. While possession of Bsa immunity genes does not significantly enhance resistance to the related lantibiotic gallidermin, the broad antimicrobial spectrum of Bsa strongly indicates that production of this bacteriocin confers a competitive ecological advantage on community-acquired S. aureus.

Staphylococcus aureus can be a human commensal bacterium, colonizing the skin and mucosal surfaces such as the nares, pharynx, and vagina in approximately 25 to 40% of the population. However, it is also a human pathogen that can cause epidemics of invasive disease. Genome sequencing of S. aureus strains has highlighted that the species is highly clonal, with approximately 78% of the genes being conserved and representing the core genome. The remaining 22% of the genes, which are variable and include those present on genomic islands, pathogenicity islands, prophages, integrated plasmids, and transposons, can in turn be regarded as an accessory genome (for a review, see reference 19) that provides plasmids, and transposons, can in turn be regarded as an accessory genome (for a review, see reference 19) that provides plasmids, and transposons, can in turn be regarded as an accessory genome (for a review, see reference 19) that provides plasmids, and transposons, can in turn be regarded as an accessory genome (for a review, see reference 19) that provides plasmids, and transposons, can in turn be regarded as an accessory genome (for a review, see reference 19) that provides plasmids, and transposons, can in turn be regarded as an accessory genome (for a review, see reference 19) that provides plasmids, and transposons, can in turn be regarded as an accessory genome (for a review, see reference 19) that provides

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revealed the presence of strain FPR3757 (part of the virulent USA300 clonal group) MRSA isolates with a competitive advantage in such environments. Production of an antimicrobial compound may provide CA-MRSA strains, like commensal multidrug-resistant nature, coupled with exposure to antibiotics that are generally active against bacterial species which are closely related to the producing organism, and these antibiotics are thought to have a role in niche competition in many natural environments (41). Lantibiotics have been speculated that the production of an antimicrobial compound may provide CA-MRSA isolates with a competitive advantage in such environments (4, 14). The theory was first suggested when sequencing of strain FPR3757 (part of the virulent USA300 clonal group) revealed the presence of bsa (bacteriocin of S. aureus) genes, which resembled those associated with production of the epidermin subgroup of lantibiotics (2, 60). Lantibiotics are ribosomally produced, posttranslationally modified peptide antibiotics that are generally active against bacterial species which are closely related to the producing organism, and these antimicrobials are thought to have a role in niche competition in many natural environments (41). Lantibiotics have been the focus of much attention in recent years with a view to clinical, veterinary, and food applications (10, 72). Although many lantibiotics are produced by food-grade bacteria or bacteria generally regarded as safe, there have also been a few examples of lantibiotic production by pathogens (11, 46, 69). In this instance, despite the identification of the bsa genes, the production of a lantibiotic by CA-MRSA isolates has remained speculative. Indeed, to date, there has been only one confirmed example of a lantibiotic, i.e., staphylococcin C55 (46), produced by S. aureus and no definitive evidence that CA- (or HA-) MRSA strains produce such compounds. There is, however, some evidence to suggest that staphylococcin Au-26, which is produced by a vaginal isolate of S. aureus and has an inhibitory spectrum encompassing lactobacilli isolated from the endocervix and representative strains of Staphylococcus hominis, Staphylococcus warneri, Streptococcus pyogenes, Streptococcus salivarius, Streptococcus mutans, Lactococcus spp., and oral Neisseria spp., may also be a lantibiotic (63). Here, 17 years after its initial characterization, we have carried out a closer inspection of staphylococcin Au-26 and the associated producer and have established that the staphylococcin Au-26 and Bsa genetic loci are almost identical. Prompted by this finding, we employed a combination of mutagenesis and mass spectrometry (MS) to reveal that these genes are functional in a number of other staphylococci, including a large percentage of CA-MRSA isolates. We suggest that, as a consequence of eliminating competing human microbiota, this lantibiotic contributes strongly to the fitness of these community-associated isolates.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** Bacterial strains, plasmids, and primers used in this study are listed in Tables 1 and 2. S. aureus strains were grown at 37°C in Mueller-Hinton broth/agar (Oxoid) or a blood agar-calcium carbonate mixture (BACa) comprising Columbia blood agar base (Difco, Sparks, MD) supplemented with 5% human blood and 0.1% calcium carbonate (Oxoid). Escherichia coli strains were grown in Luria-Bertani (LB) broth/agar at 37°C with aeration. Micrococcus luteus strains were grown in tryptic soy broth/agar (Merck) or BACa at 37°C with aeration unless otherwise stated. Lactobacillus strains were grown anaerobically on BACa at 37°C. Microbacterium oxydans DPC 6277 was grown aerobically in tryptic soy broth/agar at 30°C. Corynebacterium testudinoris, Staphylococcus epidermidis DPC 6293, and M. luteus DPC 6275 were grown aerobically in Mueller-Hinton broth/agar at 30°C. Psychrobacter sp. strain DPC 6277 was grown aerobically in brain heart infusion broth/agar (Oxoid) at 30°C. Antibiotics were used, where indicated, at the following concentrations: chloramphenicol, 10 μg ml⁻¹ for E. coli and 5 μg ml⁻¹ for S. aureus, and erythromycin, 250 μg ml⁻¹ for E. coli and 5 μg ml⁻¹ for S. aureus. X-Gal (5-bromo-4-chloro-3-indoly-β-D-galactopyranoside) was used at a concentration of 40 μg ml⁻¹.

**Purification and MS analysis.** Staphylococcin Au-26 was purified from cultures of S. aureus strain 26, grown in Columbia agar base-liquid medium (Difco)
agar, and the dishes were incubated overnight at 37°C. The resultant growth was
cus gallinarum
carried out using Hotmaster
bated at 37°C overnight.
BsaBsoeC ...........................
BsaBseqR...........................
BsaA1seqF .........................
Au26LocR..........................
LeuDF ................................
StaphLanBR ......................

Bioassays for antimicrobial activity. Bioactivity was assessed by well diffusion
assays unless otherwise stated. These assays were carried out as described previously (74). Collision-induced dissociation (CID) fragment spectra
were manually interpreted to gain sequence tag information for peptide identi-
fication. The amino acid derivatives dehydroalanine (Dha) and dehydrobutyryline
(Dhb) were considered to represent sites of thioether bridge cleavages under
CID conditions. For colony MS (CMS), bacteria were collected with sterile
plastic loops and mixed with 50 μl of 70% isopropanol adjusted to pH 2 with
HCl. The suspension was subjected to a vortex, the cells were spun down in a
benchtop centrifuge at 14,000 rpm for 2 min, and the supernatant was removed
for analysis. MS was performed with an Axima CFR plus MALDI-TOF mass
spectrometer (Shimadzu Biotech, Manchester, United Kingdom). A 0.5-μl ali-
quot of matrix solution (α-cyano-4-hydroxycinnamic acid (CHCA) at 10 mg ml⁻¹
in 50% acetonitrile–0.1% [vol/vol] trifluoroacetic acid) was placed onto the
target and left for 1 to 2 min before being removed. The residue from the
solution was then air dried, and the resulting sample solution was positioned
onto a precoated sample spot. Matrix solution (0.5 μl) was added to the sample,
and the sample was allowed to air dry and subsequently analyzed in positive-ion
reflectron mode.

Bioassays for antimicrobial activity. Bioactivity was assessed by well diffusion
assays unless otherwise stated. These assays were carried out as described previously (70) or as follows. Molten agar was cooled to 48°C and seeded with the
indicator of choice (~10⁶ cells from a fresh overnight culture per ml). The
incubated medium was dispensed into sterile petri plates in 20-ml volumes,
allowed to solidify, and dried. Wells (4.6 mm in diameter) were bored into the
seeded agar plates, and 50 μl of an antimicrobial-containing sample was placed
into each well before overnight incubation. Defeated antagonism assays were
carried out as described previously (65) or as follows. Aliquots of 10 μl (2 × 10⁶
cells per ml) of overnight cultures of Staphylococcus epidermidis and Staphyloccus
gallinarum were spotted onto petri dishes containing 20 ml of solidified LB
agar, and the dishes were incubated overnight at 37°C. The resultant growth
was killed by UV irradiation, and the plates were flooded with 20 ml molten agar
containing 10⁶ cells of the various CA-MRSA strains per ml. Plates were incub-
ated at 37°C overnight.

General molecular biology techniques. PCR with degenerate primers was
carried out using HotMaster Taq polymerase (Eppendorf, Hamburg, Germany)
in 50-μl reaction volumes containing 41.5 μl PCR-quality water, 5 μl of 10X
buffer, 10 mM deoxynucleoside triphosphate mix (Roche Diagnostics Ltd.,
Lewes, England), 1 μl of each forward and reverse primer (stockers at 0.1
ng μl⁻¹), and 0.5 μl Taq (5 U μl⁻¹). Amplification was carried out with reaction
conditions as follows: initial denaturation at 95°C for 2 min, followed by 30 cycles
of 95°C for 30 s, annealing at 40°C for 30 s, and elongation at 65°C for 1 min, with
final extension at 72°C for 5 min. PCR products were gel extracted with a Qiagen
gel extraction kit, cloned into pGEM-T per the instructions of the manufacturer
(Promega), and sequenced. Amplification of the entire staphylococcal Au-26
locus was carried out using a long-range PCR kit per the instructions of the
manufacturer (Roche Diagnostics, Mannheim, Germany). Plasmid DNA was
isolated from E. coli strains by using the High Pure plasmid isolation kit as
recommended by the manufacturer (Roche Diagnostics, Mannheim, Germany).
Total cell DNA was isolated using the High Pure PCR template preparation kit
according to the recommendations of the manufacturer (Roche Diagnostics,
Mannheim, Germany). The presence of bsa genes was assessed using the fol-
lowing primer pairs: BsaAseqF and BsaBseqR, BsaEseqC and BsaEseqD, and
BsaBseqA and BsaBseqD. E. coli EC10B was used as an intermediate
cloning host for the plasmid pORI280. S. aureus cells were made electropore-
tent by using the protocol outlined by Schenk and Laddaga (58). PCR
was performed according to standard procedures using BioTaq DNA (Bioline), Vent
polymerase (New England Biolabs), and KOD Hot Start DNA polymerase
(Novagen) in a PTC-200 DNA engine (MJ Research). Colony PCR was imple-
mented following lysis of cells in 10% IGEPAL 630 (Sigma-Aldrich) at 94°C for
10 min. Restriction digests and DNA ligations were carried out according to
established procedures using restriction enzymes PstI and EcoRI and T4 DNA
ligase supplied by Roche Diagnostics. DNA sequencing was performed by MWG
Biotech AG.

MLST. Multilocus sequence typing (MLST) of strain 26 was carried out using
previously published protocols (18). Sequencing reactions were carried out using
using the terminator cycle sequencing protocol with Quick Start Dye Terminator
(Buckinghamshire, United Kingdom). Cycle sequencing reactions were carried out
in 10-μl (1/4-strength) reaction volumes containing 0.5 μl of a purified PCR
product, 5 pmol of each primer, 1 μl of halICEO buffer (GeneApp, Hemel
Hempstead, United Kingdom), 2 μl of Dy terminator cycle sequencing Quick Start master
mix (Beckman Coulter), and 6 μl of sterile water. Reaction conditions were as
follows: 40 cycles of 96°C for 20 s and 60°C for 4 min. Reaction products were
analyzed using a CEQ 8800 genetic analysis system, and raw data were analyzed
using Sequencher software (Gene Codes Corporation, MI).

Creation of an RN4220 Bsa⁻ mutant. Two PCR fragments from the DNA
flanking the bsaB gene were generated by using Vent polymerase (New England
Biosciences) with the oligonucleotide pairs BsaBseqA/BsaBseqB and BsaBseqC/
BsaBseqD. The products were mixed in a 1:1 ratio and combined by splicing by
overlap extension (SOE)-PCR using the oligonucleotide pair BsaBseqA/BsaBseq
and transformants were selected on LB agar containing 250 μg ml⁻¹ erythromycin
and 40 μg ml⁻¹ X-Gal. Plasmids were reisolated and introduced by electropor-
tation into an S. aureus NCTC8325-4 derivative, RN4220, containing pVE6007.
RN4220/pVE6007/pORI280 was grown overnight at 30°C, subcultured twice at the nonpermissive temperature of 42°C, plated onto tryptic soy agar
containing 5 μg/ml erythromycin at 42°C, and then subjected to selection for
integration of pORI280 into the RN4220 genome. Plasmid integration was con-
firmed by PCR using primers pORI280R and deltabsafor.

MICs were determined as described by Wiedemann et al. (71). CA-
MRSA strains were grown in Mueller-Hinton broth (Oxoid). Serial twofold
dilutions of the gallidermin peptide in Mueller-Hinton broth were prepared.
Bacteria were added to give a final inoculum density of 10⁸ CFU per ml in a
volume of 0.2 ml. After incubation for 16 h at 37°C, the MIC was read as the
lowest peptide concentration causing inhibition of visible growth.

RESULTS
Identification of staphylococcal Au-26 determinants. Previous
attempts to elucidate the amino acid sequence of staphy-
lococcal Au-26 were hampered by the presence of a modified
residue at position 2 of the peptide, which blocked N-terminal
Edman degradation assays. It was established, however, that
the first residue in this peptide (estimated to have a mass of
2,700 Da) is an isoleucine and that the peptide contains two or
three lantionine residues and thus is likely to be a lantibiotic
(63). Given that isolatecin is also the first residue of the S.
epidermidis-produced lantibiotic epidermin and of two putative epidermin-like peptides potentially encoded within the genome of a number of S. aureus isolates (corresponding to the theoretical Bsa lantibiotic), we speculated that staphylococcin Au-26 may also be an epidermin-like antimicrobial. To assess this possibility, we designed two degenerate primers, one (StaphLanF) designed on the basis of nucleotides conserved within epidermin-encoding genes and a second (StaphLanBR) designed to amplify a region conserved across genes encoding lantibiotic-modifying proteins, i.e., lanB homologues, in a manner analogous to that described previously (31, 73). A PCR product of approximately 450 bp was amplified, and DNA sequencing and BlastX searches against the nonredundant protein sequence database established that this amplicon was almost identical to corresponding regions from the type II vSaβ virulence islands of a number of sequenced genomes of S. aureus strains associated with the putative Bsa lantibiotic (Fig. 1A). Further PCR amplification and partial sequencing were employed to better assess the extent to which this region of the vSaβ island in strain 26 corresponded to the type II versions of the islands from sequenced strains. Amplification with primers LeuDF (designed to amplify a sequence from the leukocidin-encoding gene lukD, located upstream of the Bsa locus in type II vSaβ islands) and StaphLanBR yielded an amplicon 2,043 kb in length, and sequencing revealed that this region displayed 99% identity to bsa gene sequences over the entire length (E value, 0.0) and potentially represented genetic determinants encoding two structural lantibiotic peptides and a partial LanB-encoding gene sequence, which were designated bsaA1Au-26, bsaA2Au-26, and bsaB Au-26, respectively (Fig. 1A). PCR carried out with the primers LeuDF and Au26LocR, designed to amplify a region downstream of the bsa locus (Table 1), yielded a product of >10 kb in length. The length of this product, combined with sequencing of the 3’ ~700 bp of this fragment, confirmed that this was a portion of the S. aureus strain 26 vSaβ island. Given the high level of identity shared between the loci in this strain and other bsa gene-containing strains, sequencing of the remainder of the vSaβ island in S. aureus 26 was deemed unnecessary and a detailed in silico analysis of the sequence information available to date was undertaken.

Detailed in silico analysis of the putative staphylococcin Au-26/Bsa operons. As noted above, the nature of the genes present within the various Bsa-encoding loci indicates that the corresponding strains may produce lantibiotics that are related to the previously characterized lantibiotics epidermin (produced by S. epidermidis DSMZ 3095) (62) and epidermin’ and gallidermin (produced by S. gallinarum DSMZ 4616), which are active against other staphylococci, streptococci, and Propionibacterium acnes (the causative agent of acne) (34), as well as other skin bacteria (59), and have been the subject of a number of studies investigating their potential for clinical application (45) (Fig. 1). In fact, the bsa genes so closely resemble those associated with epidermin production that they have on occasion been incorrectly annotated or referred to as epi (epidermin) genes. bsa genes are located on the type II vSaβ genomic islands in strains MW2 (USA400; multilocus sequence type 1 [ST1]) and FPR3757 and USA300_TCH11516 (USA300; ST8), the laboratory strains NCTC8325-4 and Newman (ST8), and MSSA476 (a CA methicillin-sensitive strain that shares common ancestry with USA400 strains; ST1) (28). While the bsa genes are identical in these strains, related but not identical genes are also present in ET3-1, formerly known as RF122, a representative of a hypervirulent bovine mastitis clone (ST151) (27). In contrast, these genes are absent from the type I forms of the island found in N315 (an ST5 HA-MRSA strain) and Mu50 (an ST5 vancomycin-intermediate S. aureus [VISA] strain) (4, 6, 28) and in JH1 (a VISA strain; GenBank accession no. AAPK01000000) and JH9 (a VISA strain; GenBank accession no. AAPL00000000), both single-locus variants of ST5 (64), and are also absent from the type III island found in MRSA252 (an ST36 HA-MRSA strain). Thus, among genome-sequenced S. aureus strains, ST1, ST8, and ST151 strains possess the island and all ST5 and ST36 strains lack it. Notably, a fragment of one of the Bsa-associated genes, bsaG, corresponding to the 92 C-terminally located residues of the intact type II protein, is present in type I vSaβ islands, establishing that the bsa genes were lost from type I islands rather than acquired by type II islands (3). The chromosomal location of the bsa genes is not atypical in the context of epidermin-like determinants in that the gallidermin determinants are also situated on the chromosome of S. gallinarum (61); however, the location does contrast with that of the plasmid-associated epidermin genes (62).

The predicted prepropeptide and propeptide domains of staphylococcin Au-26 elements BsaA1 Au-26, and BsaA2 Au-26 share 83% identity (39 of 47 amino acids are identical) and 77% identity, respectively. The most notable difference between the two prepropeptides arises from a leucine start codon (TTG) in the gene encoding BsaA1Au-26. This is also the only difference between the BsaA1Au-26 and BsaA1 prepropeptides. UUG start codons have been reported to lead to reduced levels of expression in Gram-positive bacteria (1, 75). The predicted BsaA1Au-26 propeptide shares 86, 64, and 68% identity with the ET3-1 BsaA1 (BsaA1ET3-1), epidermin, and gallidermin propeptides, respectively, while the deduced 22-amino-acid sequence of the BsaA2 Au-26 propeptide is identical to that of the BsaA2 propeptide and shares 81, 77, and 77% identity with those of the BsaA1ET3-1, gallidermin, and epidermin propeptides, respectively. Alignment of the propeptide forms of these, and other, epidermin-like peptides (Fig. 1B) reveals that while 9 of the 10 C-terminally located amino acids are conserved in all such peptides (excluding mutacin I), the same is true for only 4 of the 12 N-terminally located residues.

Interestingly, BsaA1, BsaA1 Au-26, and BsaA1ET3-1 do not possess the conserved CTPGC stretch of residues in the unmodified peptide, thought to be essential for binding of peptides of this nature to their target, i.e., the precursor of cell wall peptidoglycan, lipid II (29). All three contain an alternative stretch corresponding to CSFGC in the unmodified peptide. It should also be noted that the presence of two lantibiotic structural gene homologues is unusual in that epidermin and gallidermin producers possess only one such gene. Should these peptides be produced and undergo ring formation in a manner equivalent to that observed for epidermin and gallidermin, we predict the masses to be 2,281 Da for BsaA1 and 2,091 Da for BsaA2 (assuming all hydroxy-amino acids not involved in bridge formation are completely dehydrated). It should also be noted that while the annotation of the genome of FPR3757 (a USA300 CA-MRSA strain) (14) did not include a bsaA1 gene, we can confirm that it is indeed present.
FIG. 1. *In silico* analysis of *bsa* and homologous genes. (A) Visual representation of epidermin and Bsa (BsaAu-26. Bsa, strain COL) Bsa [BsaCOL], and BsaET3-1 loci. Numbers represent the percentages of identity to the corresponding *bsa* gene (except for *bsaA1*, *bsaX*, and *bsaA2*, which are compared in greater detail in panels B and C). Homologous genes share a color (white indicates the absence of homologues). Dashed lines indicate unsequenced regions within the Bsa Au-26 operon. Three products (i, ii, and iii) amplified from within this region in *S. aureus* strain 26 are indicated. The locus tags indicated correspond to those for *lukE*. Bsa* represents the identical *bsa* genes in strains MW2 (accession no. NC_003923), FPR3757 (accession no. NC_007793), USA300 TCH1516 (accession no. NC_010079), NCTC8325-4 (accession no. NC_007795), Newman (accession no. NC-009641), and MSSA476 (accession no. NC_002953). Accession numbers for the COL and ET3-1 genomes are NC_002951 and NC_007622, respectively. (B) Alignment of the predicted amino acid sequences of the Bsa and BsaET3-1 structural propeptides with those of other epidermin-like and related peptides. (C) Alignment of the predicted amino acid sequences of BsaX and BsaXET3-1 [BsaX(RF122)]. The sole difference is indicated by an arrow. (D) Predicted BsaA2 structure (modified residues are shaded in black).
Similarly, although annotation of the *S. aureus* USA300_TCH1516 genome suggests the existence of a hypothetical protein (USA300HOU_1816) encoded upstream of *bsa*A2, our inspection of this region has revealed the existence of a *bsa*A1 gene. Interestingly, in strain COL (a HA-MRSA strain which is related to NCTC8325-4 and is classified as ST250, i.e., an ST8-like sequence type [6, 23]), there is a divergently transcribed open reading frame (ORF; SACOL1879) located upstream of *bsa*A2, which is predicted to encode an IS1181 transposase (12). This finding indicates the ongoing evolution of the locus mediated by the transposition of mobile DNA. In this strain, *bsa*A1 is interrupted but the *lukDE* genes are present (Fig. 1A).

Of the other proteins potentially encoded within the genomic island, BsaB, BsaC, and BsaD correspond to those required for posttranslational modification of epidermin-like peptides, BsaP is predicted to be involved in leader cleavage/transport (2, 22, 36, 60), and BsaEFG are likely to serve as immunity proteins to protect the producing strain from that which it produces (49, 51). Our analysis reveals that the *bsa* genes in MW2, FPR3757, USA300_TCH1516, NCTC8325-4, Newman, and MSSA476 (which are 100% identical across these strains) are 88 to 97% identical to their ET3-1 equivalents (Fig. 1A). The level of homology to the corresponding epidermin genes is lower (40 to 72% identity) (Fig. 1A), and there are no *bsa* equivalents of *epiH*, *epiT*, and *epiQ*. During sequencing of ET3-1, an additional ORF located in this region, *sab*1684, was annotated (27). This ORF, which has been designated *bsa*X, is located between *bsa*A1<sub>ET3-1</sub> and *bsa*A2<sub>ET3-1</sub> but has the opposite orientation. Our analysis has revealed that, although not always annotated, such an ORF exists between *bsa*A1 and *bsa*A2 in the other genome-sequenced *S. aureus* strains and that the predicted 63-amino-acid products *BsaX*<sub>ET3-1</sub> and *BsaX* differ by only one residue (Fig. 1C). *bsa*X and *bsaX*<sub>ET3-1</sub> do not display a significant degree of homology to any other ORFs and are not present in epidermin- or gallidermin-producing strains, and the absence of an obvious ribosomal binding site means that it is not yet clear if these ORFs correspond to genes.

*bsa* and *bsa*<sub>Au-26</sub> encode a lantibiotic. A number of approaches were taken to definitively confirm that the *bsa* and *bsa*<sub>Au-26</sub> loci encode an active lantibiotic (Bsa/staphyloccin Au-26). The first strategy involved purification and investigation of the active staphyloccin Au-26 peptide. Cation-exchange chromatography analysis of ammonium sulfate-precipitated fractions from the broth supernatant of *S. aureus* strain 26 revealed a titer of 64 activity units/ml against *M. luteus* NCIMB9278 (i), *S. epidermidis* DPC 6293 (ii), *Psychrobacter* sp. strain DPC 6277 (iii), *M. luteus* NCIMB9278 (iv), *M. luteus* DPC 6275 (v), and *M. oxydans* DPC 6277 (vi). (B) Results from agar well diffusion assays with cell-free supernatant from *S. aureus* ET3-1 using *M. luteus* NCIMB9278 as an indicator. (C) Results from deferred antagonism assays assessing the activities of *S. aureus* RN4220 and RN4220::pOri280*bsaB* against *M. luteus* NCIMB9278.

[Fig. 2. Results from agar-based antimicrobial assays with representative *Bsa*<sup>+</sup> strains. (A) Results from agar well diffusion assays highlighting the activities of partially purified Bsa produced by *S. aureus* NCTC8325 against *C. testudinoris* DPC 6273 (i), *S. epidermidis* DPC 6293 (ii), *Psychrobacter* sp. strain DPC 6277 (iii), *M. luteus* NCIMB9278 (iv), *M. luteus* DPC 6275 (v), and *M. oxydans* DPC 6277 (vi).]
mutant derivative of NCTC8325-4 (35). This process involved the use of the pVE6007/pOri280 plasmid pair that was originally developed to generate mutants of Lactococcus lactis (9, 38) and has more recently also been applied to mutagenesis in other Gram-positive bacteria (43). The polar disruption of *bsaB* was targeted, as plasmid integration at this point would be expected to prevent lantibiotic biosynthesis. Thus, a 600-bp *bsaB* fragment was amplified and cloned into the RepA vector pOri280, and the resultant construct was introduced into an RN4220 strain containing the temperature-sensitive RepA vector pVE6007. Following temperature upshift and loss of pVE6007, pORI280 integrants were identified by antibiotic selection and checked by PCR. The resultant mutant was designated RN4220::pORI280*bsaB*. The antimicrobial activities of the parental and mutant strains were assessed by deferred antagonism agar diffusion assays using *M. luteus* NCIMB9278. From these assays, it was apparent that disruption of the Bsa operon resulted in a strain that was unable to produce an antimicrobial (Fig. 2C).

**Bsa status of PVL* CA-MRSA isolates.** The CA-MRSA strains that have spread most rapidly belong to one of five clonal groups and are associated with specific STs in each case, i.e., ST1 (corresponding to the USA400 clonal group) (48, 66), ST8 (USA300 and USA500) (7, 15, 21, 25, 33), ST59 (USA1000) (30, 68), ST80 (Europe) (66), and ST30 (Pacific) (15, 48). Although not absolute (57), an association between the clinical spectrum of infections caused by CA-MRSA and the presence of Panton-Valentine leukocidin (PVL) genes, which code for the production of cytolysins that cause tissue necrosis and leukocyte destruction, has been noted (24). When *bsa* genes were first identified, it was suggested that possession of these genes might represent an alternative distinctive feature of CA-MRSA isolates. However, as is evident from analyses of genome-sequenced strains (as described above) and comparative genomics studies, an exact correlation does not exist (8). Nonetheless, the production of the Bsa lantibiotic by CA-MRSA strains may be significant given the virulent nature of many of these isolates, in particular the PVL-positive (PVL*) forms due to their need to be more competitive than their HA-MRSA counterparts with respect to flourishing on the skin surface. We therefore set about investigating the distribution of *bsa* genes among a selection of 21 HA, health care-associated (HCA), and CA PVL* isolates. PCR-based analysis established that eight of the isolates, M02/0088 (ST80), M04/0101 (ST80), M04/0266 (ST8), M05/0028 (ST8), M05/0060 (ST8), M05/0199 (ST8), M05/0259 (ST8), and M05/0267 (ST8), possessed the *bsa* genes (Table 3). Thus, both ST80 strains and all except one of seven ST8 strains tested possessed the genes (ML224 being the exceptional strain), whereas all strains of the ST5, ST22, ST30, and ST154 genotypes lacked the genes. Notably, during the present study it was determined that strain 26 is also a representative of the ST8 lineage and has a methicillin-sensitive phenotype. With respect to the MRSA isolates, it was also evident that only 1 (M02/0088) of 5 HA/HCA strains (20%) but 7 of 16 CA strains (44%) possessed the Bsa operon (Table 3).

Deferred antagonism antimicrobial assays, using NCIMB9278 as an indicator, were conducted with the 21 PVL* strains. The results demonstrated a close correlation between the presence of the Bsa operon and antimicrobial activity, in that all except one of the eight *bsa* strains inhibited the indicator whereas none of the *bsa*-negative strains exhibited antimicrobial activity (Table 3). Perhaps significantly, the only *bsa* strain that did not exhibit antimicrobial activity was M02/0088, i.e., the sole HCA *bsa* strain. Thus, from an antimicrobial activity perspective, none of the HA/HCA strains exhibited activity whereas 44% of CA strains did. Finally, CMS analysis of all 21 strains was carried out. Here, a perfect correlation between antimicrobial activity and the presence of a peak corresponding to BsaA2, but not BsaA1, was observed (Table 3). The results demonstrated a close correlation between the presence of the Bsa operon and antimicrobial activity, in that all except one of the eight *bsa* strains inhibited the indicator whereas none of the *bsa*-negative strains exhibited antimicrobial activity (Table 3). Perhaps significantly, the only *bsa* strain that did not exhibit antimicrobial activity was M02/0088, i.e., the sole HCA *bsa* strain. Thus, from an antimicrobial activity perspective, none of the HA/HCA strains exhibited activity whereas 44% of CA strains did. Finally, CMS analysis of all 21 strains was carried out. Here, a perfect correlation between antimicrobial activity and the presence of a peak corresponding to BsaA2, but not BsaA1, was observed (Table 3).

**Investigation of the susceptibilities of Bsa-producing CA-MRSA strains to epidermin-like lantibiotics.** In addition to the inhibition of competing microorganisms, another potential advantage associated with the presence of the Bsa operon may theoretically be the provision of immunity against other epidermin-like lantibiotics either produced by members of the human (or animal) microbiota or introduced in the form of a clinical therapeutic. All lantibiotic producers possess self-protective immunity mechanisms. One of the advantages associated with the application of lantibiotics for clinical use is that these immunity mechanisms are usually quite specific, which makes resistance, as a consequence of possessing homologous immunity genes, rare (for a review, see reference 17). Initially, deferred antagonism assays were carried out to investigate if Bsa-producing MRSA strains were more resistant than strains without Bsa to the activity of the related lantibiotics epidermin and gallidermin. Although the epidermin producer failed to inhibit the target strains, with the exception of strain 26, re-

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**TABLE 3. Bsa statuses (genotypic and phenotypic) of PVL* MRSA strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>MLST</th>
<th>Acquisition category</th>
<th><em>bsa</em> status</th>
<th>Antimicrobial activity</th>
<th>Result from CMS analysis</th>
<th>Gallidermin MIC (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1394 ST30</td>
<td>CA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.75</td>
</tr>
<tr>
<td>E1401 ST22</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>-</td>
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<tr>
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<td>-</td>
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</tr>
<tr>
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<td>-</td>
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<tr>
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<td>-</td>
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</tr>
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<tr>
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<td>+</td>
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<tr>
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<tr>
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<td>+</td>
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</tr>
<tr>
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<td>+</td>
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<tr>
<td>M05/0267 ST8</td>
<td>CA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1.8</td>
</tr>
</tbody>
</table>

*Acquisition category as reported by Rossney et al. (57). Determined using the primer pairs *BsaAlseqF*/*BsaBseqR*, *BsaEFsoeC*/*BsaEFsoeD*, and *BsaBseqA*/*BsaBseqB.* Activity was assessed using *M. luteus* NCIMB9278 as an indicator.

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**Note:** The average MIC (± the standard deviation) for strains with a *bsa*-negative status is 1.85 (±1.24) μM, and the average MIC for strains with a *bsa*-positive status is 2.39 (±0.90) μM. Statistical analysis revealed these values not to be significantly different ($F_{1,9} = 0.727; P > 0.05$ by one-way analysis of variance).
FIG. 3. CMS analysis of *S. aureus* isolates. Gray box, data for *S. aureus* strain 26 and NCTC8325-4; white box, data for CA-MRSA strains. Masses (expressed in daltons) corresponding to the predicted mass of BsaA2 are indicated by a larger font.
Regardless of their bsa status (data not shown), the gallidermin producer effectively inhibited growth to various extents (Fig. 4). To more accurately assess gallidermin sensitivity, MIC determination assays were carried out with purified gallidermin (Table 3). Here again, sensitivity levels did vary within both the bsa+ and bsa-negative groups. However, when the bsa+ strains were examined in combination, it was apparent that they did not possess statistically enhanced resistance to gallidermin ($F_{1,19} = 0.727; P > 0.05$ by one-way analysis of variance). It would thus seem that BsaEFG does not provide protection against gallidermin and that this lantibiotic could be employed as an anti-CA-MRSA chemotherapeutic option. By extension, the lack of immunity against gallidermin suggests that no protection would be provided against other, more distantly related lantibiotics either.

**DISCUSSION**

Lantibiotics are antimicrobial peptides, many of which have potent and broad-ranging antimicrobial activities. In response to the ever-increasing emergence of antibiotic-resistant bacteria, many researchers have investigated the application of these compounds in clinical settings, with some very positive results (for a review, see Piper et al. [52]). One of the areas that has received most attention is the application of lantibiotics as anti-*S. aureus* agents, with nisin, lacticin 3147, Pep5, mersacidin, gallidermin, epidermin, and others having potential in this regard. However, the production of lantibiotics is not a feature associated solely with nonpathogenic Gram-positive bacteria. Although the focus of much less attention, a number of pathogens have also been shown to produce these compounds. It may be that production of various mutacins by *S. mutans*, streptin and streptococcins by *S. pyogenes*, and staphylococcin C55 by *S. aureus*, etc., could provide these microorganisms with a competitive advantage when colonizing/infecting a human host. In the most extreme case, that of cytolysin produced by enterococci, the lantibiotic is itself a cytolytic virulence factor (11).

From the findings of the present study, it is evident that the bsa genes initially identified during the course of *S. aureus* genome-sequencing projects do indeed encode a novel epidermin-like lantibiotic. The individual genes closely resemble those within the corresponding Epi and Gdm operons, but there are key differences in that no BsaH or BsaT homologues exist and, thus, export of the Bsa lantibiotic must progress in a manner that differs from that for epidermin and gallidermin. The presence of two structural peptide-encoding genes is particularly noteworthy. Although a number of two-peptide lantibiotics which are active by virtue of the combined activities of two quite different lanthionine-containing peptides exist (37), the benefits of possessing multiple highly homologous structural peptide-encoding genes is not evident. Despite this, a number of lantibiotic producers, especially among strains producing the type AII (lacticin-481-like) lantibiotics (e.g., ruminococcin A [40], mutacin K8 [56], streptococcin A-M49 [32], streptococcin A-FF22 [42], and macedocin [50]), carry multiple copies of homologous structural genes. This phenomenon has previously also been associated with the other epidermin-like peptides mutacin I (53), mutacin III (54), and mutacin B-Ny266 (5). In the case of mutacin B-Ny266, there is only 57.4% amino acid identity between the two putative structural gene products and there is no evidence for the transcription of the second structural gene. For mutacin I and mutacin III, inser- tional inactivation of the first structural gene abolished anti microbial activity while inactivation of the second did not and, thus, the role of the latter gene has remained undetermined. Similarly, the significance of the presence of bsaA1 or bsaA1$_{Au-26}$ remains unclear. Although our failure to detect a peptide with a mass corresponding to that of BsaA1 indicates that in this instance BsaA2 may be the sole significant peptide, further analysis will be required to confirm this conclusion unequivocally. The fact that BsaA1 was not detected in these analyses indicates that the peptide is not produced in a fully modified form, is present in very low abundance, or is not produced at all. Although the UUG start codon may have an impact on the production of BsaA1$_{Au-26}$, it does not explain the apparent lack of production of BsaA1 by strains other than strain 26.

The production of a lantibiotic by clinical MRSA strains is potentially of great significance. Our investigations focused on *pvl*+ MRSA strains responsible for HA, HCA, and CA infections. From these studies, it was apparent that there was a particular association between strains with an ST8 or ST80 genotype and the presence of the *bsa* genes; it was also evident that although 44% of strains associated with CA infections possessed these genes, exhibited antimicrobial activity, and
produced a BsaA2 peptide, none of the HA/HCA strains produced the antimicrobial. While a larger collection of strains will need to be assessed to determine definitively whether this finding represents a general association between Bsa production and CA strains in general or reflects a more specific finding, it is noteworthy that the lantibiotic is particularly interesting in light of the extreme coding genes, the high proportion of ST8 strains that produce the lantibiotic is particularly interesting in light of the extreme success of the USA300 (ST8) CA strain. It was also noteworthy that the sole bsaA7 strain that did not produce the Bsa lantibiotic, M02/0088, was, despite being an ST8 strain, isolated from a patient with an HCA infection. While the basis for nonproduction remains undetermined, we have established that the disruption of bsaA1 through transposase insertion, akin to that observed in COL (ST8), has not occurred (K. M. Daly, unpublished data). It will be interesting to investigate M02/0088 in greater depth to determine if the absence of Bsa production is a reflection of its adaptation to the health care environment where, due to the presence of antibiotics, competition from other bacteria is greatly reduced. Although Bsa production by S. aureus COL has yet to be assessed, it may be that the presence of the IS1/181 transposase also has an impact on the ability of this HA-MRSA isolate to produce a lantibiotic.

As the genes required for production of staphylococcin Au-26/Bsa are relatively widespread in staphylococci and it has now been established that these genes do indeed encode a functioning lantibiotic, it seems logical to assume that the producing strains must derive some associated benefit. Given that strain 26 was originally recovered from an endocervical environment, one could hypothesize that Au-26 plays a role in staphylococcal toxic shock syndrome. However, strain 26 was not associated with this disease and does not express toxic shock syndrome toxin 1 (63). In addition, an in silico survey of the prevalence of the tst gene reveals that only strain ET3-1 carries both the lantibiotic-encoding genes and tst. Thus, Bsa is unlikely to play a significant role in invasive S. aureus disease in the vagina. Instead, we propose that Bsa is important for the survival of staphylococci in the vagina, where antagonistic activity against competing commensal bacteria, including lactobacilli, would be beneficial (indeed, although perhaps a coincidence, the HCA bsa-positive strain, M02/0088, was originally isolated from an episiotomy wound specimen). Similarly, skin-associated CA-MRSA strains face competition from other members of the skin microbiota, including coagulase-negative staphylococci, Micrococcus spp., Corynebacterium and Streptococcus spp. (P. acnes, P. avidum, and P. granulosum), Acinetobacter, and Streptococcus spp., as well as common transient species such as E. coli, Bacillus species, and Pseudomonas aeruginosa, while bovine mastitis-causing S. aureus strains, such as ET3-1, encounter an equally complex microbial challenge in order to establish an infection in the community setting, i.e., environments where exposure to antibiotics does not eliminate/reduce the competing microbiota. While production of an antimicrobial is of obvious advantage to a strain competing against a complex microbial consortium, the presence of the associated immunity proteins provides a less obvious, but potentially important, advantage. These immunity proteins may be present in lantibiotic producers themselves, although more recently, the phenomenon of immune mimicry (i.e., the provision of immunity as a consequence of possessing immunity gene homologues not associated with lantibiotic production) has also been reported (16). Given that the bsaE, bsaF, and bsaG genes are most closely related to the genes responsible for epidermin and gallidermin immunity, it was deemed most likely that cross-protection, if observed, would be against these lantibiotics. As the epidermin producer did not possess sufficient antimicrobial activity to inhibit any of the targets other than S. aureus strain 26, the possibility of cross-protection from gallidermin became the focus of greatest attention. Gallidermin has been shown in a number of studies to possess activity against skin-associated microorganisms, including P. acne (34), and is viewed as an antimicrobial with commercial potential. From our investigations, it was notable that despite homology between the BsaEFG and GdmEFG proteins, the presence of the Bsa operon did not confer a significantly higher level of resistance on the associated strains. Thus, while additional investigations using isogenic mutants containing nonpolar deletions of immunity genes or using indicators carrying heterologously expressed immunity determinants are required, it would seem that the presence of these genes does not preclude the use of gallidermin (or, presumably, more distantly related lantibiotics) as an antimicrobial to target/prevent CA-MRSA infection.

In conclusion, we have established that many S. aureus isolates from the community, including disease-causing antibiotic-resistant forms, produce an antimicrobial agent that enhances their ability to inhibit competing bacteria while potentially reducing their sensitivity to similar such compounds. However, now that the significance of the Bsa operon has been uncovered, attempts can be made to reduce the competitiveness of bsaA7 strains by developing means by which production can be switched off or using other lantibiotic-producing members of the skin microbiota to control their expansion.

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References


