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Ubericin A, a Class IIa Bacteriocin Produced by *Streptococcus uberis*▽†

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***Streptococcus uberis*, a causal agent of bovine mastitis, produces ubericin A, a 5.3-kDa class IIa (pediocin-like) bacteriocin, which was purified and characterized. The *uba* locus comprises two overlapping genes: *ubaA* (ubericin A precursor peptide) and *ubaI* (putative immunity protein). Ubericin A is the first streptococcal class IIa bacteriocin to be characterized.**

Streptococcus uberis is the main environmental causative agent of bovine mastitis in New Zealand and a prolific producer of proteinaceous antibacterial substances (bacteriocins) targeting other strains of *S. uberis* and/or other mastitis-associated species, such as *Streptococcus agalactiae* and *Enterococcus faecalis* (2, 11, 18–21). Due to their diversity, the bacteriocins elaborated by *S. uberis* can also be exploited by being incorporated into formulations designed as a preventive treatment against bovine mastitis, a strategy similar to that achieved with the lantibiotics nisin and lacticin 3147 (3, 15). It is therefore important, both practically and conceptually, to elucidate the complete *S. uberis* antimicrobial repertoire.

To date, two *S. uberis* bacteriocins (nisin U and uberolysin) have been biochemically characterized and their genetic loci identified (20, 21). However, during screening of *S. uberis* strains for novel inhibitory activities using an agar-based deferred-antagonism method, we found that *S. uberis* strain E produces a heat-stable antibacterial activity with an inhibitory spectrum that was distinguishable from that of nisin U or uberolysin by the absence of activity against *Micrococcus luteus* and certain streptococcal species, such as *Streptococcus pyogenes*, *Streptococcus salivarius*, and *Streptococcus agalactiae* (Table 1). Moreover, this new inhibitory agent was particularly potent against *Listeria* spp. and also inhibited *S. uberis* 42 (Table 1), a strain known to synthesize (and to be immune to) both nisin U and uberolysin (20, 21). The aim of the present study was to determine the biochemical and genetic characteristics of the inhibitory agent produced by *S. uberis* strain E.

(Parts of this work were presented at the 7th ASM Conference on Streptococcal Genetics, Saint Malo, France [22].)

The bacteriocin elaborated by *S. uberis* E was purified from 2 liters of supernatant recovered from an 18-h Todd-Hewitt broth culture as follows. First, protein (crude bacteriocin preparation) was precipitated with ammonium sulfate (80% saturation at 4°C), harvested by centrifugation (15,000 × *g*, 30 min, 4°C), redissolved in 400 ml buffer A (20 mM 2-morpholinoethanesulfonic acid, pH 5.8), and applied to a 5-ml HiTrap CM Fast Flow (GE Healthcare Life Sciences, Little Chalfont, United Kingdom) cation-exchange column (equilibrated with buffer A). The column was then developed with a linear gradient of 0 to 0.5 M NaCl (in buffer A) at a flow rate of 5 ml/min. Biologically active fractions (using *Listeria grayi* ATCC 19120 as the indicator strain) were lyophilized, redissolved in 600 μl of 10% (vol/vol) aqueous acetonitrile containing 0.1% (vol/vol) trifluoroacetic acid, and applied (200-μl aliquots) to a 50- by 2-mm (5-μm-pore-size) Gemini C_{18} reversed-phase high-performance liquid chromatography column (Phenomenex Inc., Torrance, CA) at a flow rate of 0.4 ml/min. The column was developed at a flow rate of 0.4 ml/min over 30 min in a linear gradient of 10 to 50% (vol/vol) aqueous acetonitrile (containing 0.1% [vol/vol] trifluoroacetic acid).

An active fraction corresponding to a single absorbance peak containing the purified bacteriocin was further characterized by matrix-assisted laser desorption ionization–time of flight mass spectrometry and N-terminal amino acid sequencing (Protein Microchemistry Facility, Department of Biochemistry, University of Otago). Mass spectrometry conducted using a Finnigan LaserMAT 2000 (Thermo BioAnalysis) mass analyzer yielded a single peak with an average mass of 5,270.5 Da (Fig. 1A). Automated Edman degradation using a Procise Model 492 pulsed liquid/gas-phase microsequencer (Applied Biosystems, Foster City, CA) disclosed the following N-terminal amino acid sequence: KTVNYGNGLYXNQKKXWVNWSETATTIVNNSIMNGLTGGN, where the unidentified residue, X, could represent cysteine. Homology searches using the BLASTP algorithm (http://www.ncbi.nlm.nih.gov/BLAST) revealed that the inhibitory agent produced by *S. uberis* E, now designated ubericin A, is most similar (64% identity, 78% similarity) to leucocin C, a class IIa bacteriocin produced by *Leuconostoc mesenteroides* (GenBank accession no. P81053). The class IIa (or pediocin-like) bacteriocins are a large group of antibacterial peptides produced by lactic acid bacteria typified by their potent antilisteria activity, the presence of a highly conserved pentapeptide motif (YGNG[V/L]) or "pediocin box," and at least one disulfide bond that is essential for biological activity (4, 6). Indeed, ubericin A possesses all these characteristics, including the YGNGL motif (underlined above) and an essential disulfide bond (Fig. 1B), thus validating ubericin A as a new member of the pediocin-like family of gram-positive peptide bacteriocins.

The inhibitory spectrum of purified ubericin A accounts for the activity produced by *S. uberis* strain E in deferred-antagonism assays (Table 1). It is noteworthy that growth of small numbers of colonies within the zones of inhibition was ob-

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TABLE 1. Inhibitory spectra of *Streptococcus uberis* strain E and purified ubericin A

Indicator bacterium (n[a])	No. of strains inhibited by deferred antagonism[b]	Sensitivity to purified ubericin A peptide[c]
Micrococcus luteus (1)	0	−
Streptococcus uberis 42 (1)	1	+
Streptococcus uberis 0140J (1)	0	−
Listeria monocytogenes (7)	7[d]	++++
Listeria monocytogenes L45ER (1)[e]	0	−
Listeria grayi ATCC 19120 (1)	1[d]	++++
Listeria grayi 19120ER (1)[e]	0	−
Listeria seeligeri (2)	2	++++
Listeria ivanovii (2)	2	++++
Other *Listeria* spp. (3)[f]	3	++++
Enterococcus faecalis ATCC 19433 (1)	1[d]	++
Enterococcus faecalis 19433ER (1)[e]	0	−
Other *Enterococcus. faecalis* (5)	5[d]	+
Streptococcus bovis strain 83 (1)	1[d]	++
Streptococcus bovis 83ER (1)[e]	0	−
Enterococcus hirae (1)	1	++++
Lactococcus lactis (1)	1	++
Streptococcus pyogenes (5)	0	−
Streptococcus salivarius (5)	0	−
Streptococcus dysgalactiae (1)	0	−
Streptococcus agalactiae (2)	0	−
Streptococcus gordonii (1)	0	−
Streptococcus mitis (2)	0	−
Streptococcus mutans (1)	0	−
Staphylococcus aureus (1)	0	−

[a] *n*, no. of strains tested.
[b] Deferred antagonism assays were carried out (with *S. uberis* E as the producer strain) according to the method of Tagg and Bannister (17) except that CABCa medium (Columbia agar base [Becton Dickinson, Sparks, MD] plus 0.1% [wt/vol] calcium carbonate) was used.
[c] Aliquots (10 μl) of ubericin A (partially purified using cation-exchange chromatography) were deposited on the surface of CABCa medium and allowed to dry. Each indicator strain was then applied using a sterile cotton swab charged from diluted (1:100) 18-h Todd-Hewitt broth cultures. *L. grayi* ATCC 19120 was used as the positive control in all assays. Diameter of inhibitory zones: −, no inhibition; +, 6 to 10 mm; ++, 11 to 15 mm; +++, 16 to 20 mm; ++++, >20 mm.
[d] Growth of resistant colonies (see the text) was observed within the zone of inhibition.
[e] Selected ubericin A-resistant derivative isolated from deferred-antagonism assays.
[f] Comprises one strain each of *Listeria innocua*, *Listeria murrayi*, and *Listeria welshimeri*.

served with strains of *Listeria monocytogenes*, *Listeria grayi*, *E. faecalis*, and *Streptococcus bovis*, especially when assayed by the deferred-antagonism method (Table 1). Upon subculture and retesting of a selection of these colonies, they were found to be completely resistant to the purified peptide (Table 1). Whereas the development of resistance to ubericin A in *Listeria* spp. and *E. faecalis* is consistent with previous findings for other class IIa bacteriocins (7, 8), our discovery of ubericin A resistance in *S. bovis* was unexpected. In *L. monocytogenes* and *E. faecalis*, development of resistance to class IIa peptides is attributed to the loss of a functional mannose-specific phosphotransferase system (EII_t^{Man}) presumed to be the bacteriocin receptor (6–8). It would therefore be of interest to ascertain if a similar resistance mechanism applies for *S. bovis*.

The genetic locus responsible for ubericin A production (designated *uba*) was identified by PCR-based methods including "vectorette" PCR (16). The erythromycin resistance gene *ermAM* (1) served as the "vectorette" component. A complete list of oligonucleotide primers and their sequences is listed in Table S1 in the supplemental material. The *uba* locus contains two genes, *ubaA* and *ubaI*, which overlap by one base pair, i.e., the last nucleotide of the *ubaA* stop codon is the first nucleotide of the *ubaI* start codon. *ubaA* encodes the 70-amino-acid ubericin A prepeptide, which comprises the mature ubericin A propeptide (49 amino acids) preceded by a 21-amino-acid secretion signal peptide containing a "double-glycine" (GG) motif, a distinctive characteristic of many peptide bacteriocins produced by gram-positive bacteria (5, 14). The deduced amino acid sequence of ubericin A not only has a calculated molecular mass (5,271.5 Da) consistent with that obtained by mass spectrometry but also confirms the residues (Cys^{11} and Cys^{16}) that form the essential disulfide bond (Fig. 2). *ubaI* specifies UbaI, a 99-amino-acid polypeptide displaying 52% identity (71% similarity) to MunC, the protein which confers immunity to the class IIa peptide mundticin ERL35 (GenBank accession no. AAQ95743), indicating that the probable function of UbaI is to protect the ubericin A producer strain from the lethal effects of its own bacteriocin.

The *S. uberis* genome sequence reference strain 0140J does exhibit antilisteria activity but differs from strain E in not inhibiting enterococci or *S. bovis* (19; G. A. Burtenshaw, unpublished results), leading us to speculate that 0140J may produce a variant of ubericin A having a somewhat different inhibitory spectrum. However, when a BLAST search of the *S. uberis* 0140J genome sequence (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/s_uberis) was conducted using the *uba* locus as the query sequence, we found that *ubaA* is completely absent (Fig. 2), indicating that strain 0140J produces another as-yet-unidentified antilisteria agent. On the other hand, strain 0140J does contain a *ubaI* homologue, which encodes a polypeptide with four amino acid differences ($Thr^1 \rightarrow Ser$, $Ala^2 \rightarrow Tyr$, $Glu^{16} \rightarrow Gly$, and $Phe^{67} \rightarrow Leu$) from UbaI in strain E. These substitutions, however, do not appear to compromise the function of the protein, since the growth of strain 0140J is not inhibited upon exposure to purified ubericin A (Table 1).

The majority of class IIa bacteriocins, like the ubericin A prepeptide, contain secretion leader sequences with GG motifs (4, 6). These peptides are usually secreted (with concomitant proteolytic cleavage of the signal peptide) via dedicated export complexes usually composed of an ATP-binding cassette (ABC) transporter and an accessory protein (5, 6). The ABC transporter component characteristically comprises three domains: (i) a N-terminal peptidase, (ii) a membrane-spanning permease, and (iii) a C-terminal ATPase (5). Since most of the genetic loci encoding class IIa bacteriocins described to date are organized such that the transport-associated genes are located adjacent to the bacteriocin structural genes (4, 6), we anticipated that the ubericin A export locus would reside close to *ubaAI*. However, the gene immediately downstream of *ubaAI* is *fgp*, which encodes formamidopyrimidine glycosylase, a conserved "housekeeping" protein (Fig. 2). Moreover, additional bioinformatic analyses and sequencing of the DNA regions downstream of *ubaAI* revealed a genetic complement and organization identical to those of strain 0140J (Fig. 2).

In order to identify the ubericin A-associated ABC transporter in *S. uberis* strain E, we used a pair of degenerate PCR primers that bind to DNA segments (ca. 1.8 kb apart) encoding the conserved N-terminal peptidase and C-terminal ATPase

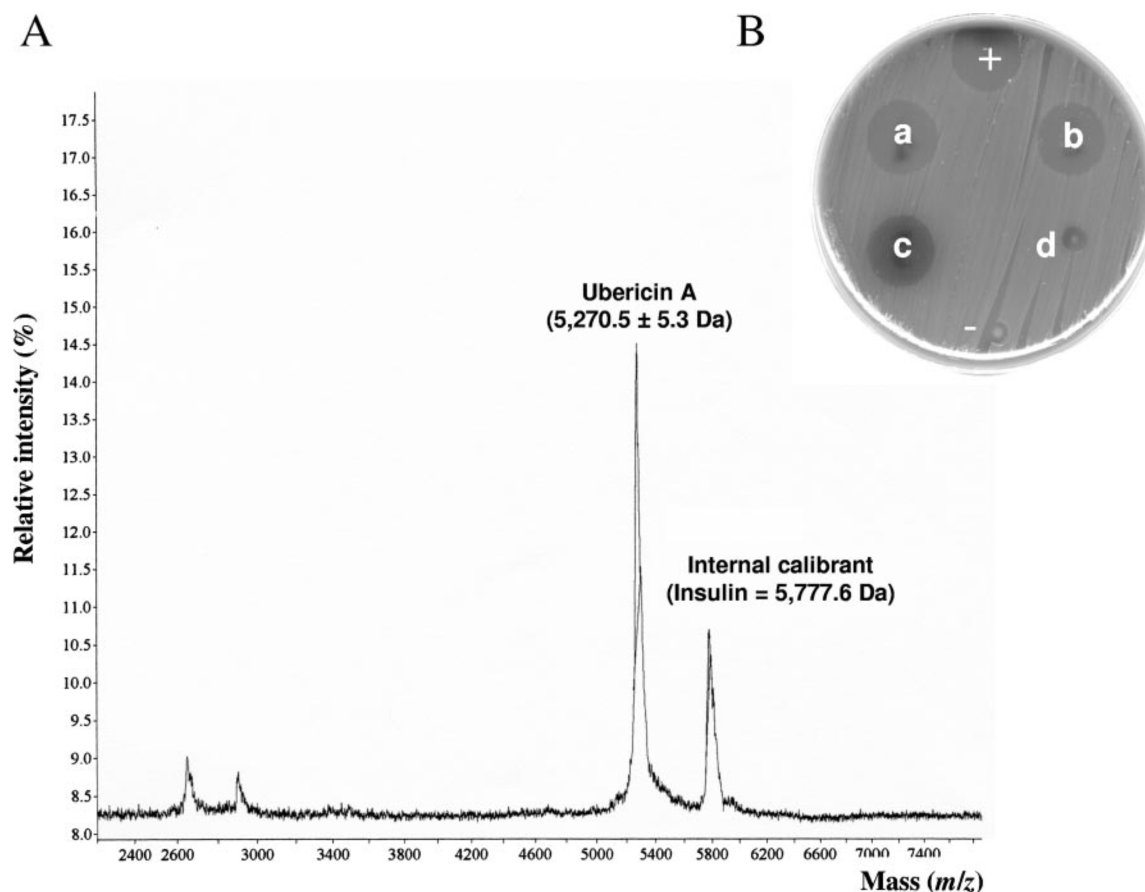

FIG. 1. (A) Matrix-assisted laser desorption ionization–time of flight mass spectrometric analysis of purified ubericin A. Only the primary ubericin A (5,270.5 Da) and internal mass standard (insulin; 5,777.6 Da) peaks are labeled. (B) Ubericin A contains an essential disulfide bond. Purified ubericin A (ca. 40 pmol) was subjected to the reductive alkylation protocol of Jack et al. (10). +, untreated ubericin A (positive control); −, reagent-only control; a, ubericin A plus reaction buffer; b, ubericin A plus 4-vinylpyridine; c, ubericin A plus 2-mercaptoethanol; d, ubericin A plus 2-mercaptoethanol plus 4-vinylpyridine. The indicator strain is *L. monocytogenes* L45.

domains. Amplicons of the expected size were cloned into pBluescript II (Stratagene), and five clones were sequenced, all of which corresponded to an internal segment of an open reading frame (ORF), ORF1 (Fig. 2), in strain 0140J, which encodes a 717-amino-acid polypeptide displaying considerable homology (at least 50% identity) to ABC transporters known to secrete peptide bacteriocins. Interestingly, ORF1 is the only gene specifying a three-domain ABC transporter in the strain 0140J genome, and no apparent accessory protein-encoding counterpart could be detected (Fig. 2). Furthermore, two ORFs (ORF2 and ORF3) located immediately downstream of ORF1 could potentially encode a putative two-component signal transduction system (Fig. 2). Sequencing of additional PCR amplicons (obtained with genomic DNA of strain E as a template) and subsequent PCR analyses revealed not only perfect conservation of ORF1 to ORF3 in strain E but also that the intergenic spacing between ORF1 and *ubaAI* (after subtracting 0.2 kb, the size of *ubaA*) was comparable to that found in strain 0140J (Fig. 2). Since the biosynthesis of some class IIa bacteriocins, e.g., carnobacteriocin A (12), is induced via two-component signal transduction systems, it is tempting to speculate that ORF2 and ORF3 might be involved in ubericin A biogenesis. Future functional genomic studies will focus on elucidating the roles, in both strains E and 0140J, of all three ORFs in bacteriocin production.

In conclusion, we have characterized ubericin A, which to the best of our knowledge is the first class IIa bacteriocin to be characterized from a member of the genus *Streptococcus*. Ubericin A bears the distinctive hallmarks of other members of this bacteriocin class, including potent antilisteria activity, the "pediocin box," and an essential disulfide bond. In addition, *ubaA* and *ubaI* are organized in an overlapping fashion, a genetic configuration not previously reported for any class IIa bacteriocin. An overlapping bacteriocin gene system has so far been described only for the locus encoding sakacin Q, an unmodified nonpediocin bacteriocin produced by *Lactobacillus sakei*, in which case the expression of *sppQ* (bacteriocin) and *spiQ* (immunity) depends on translational coupling (13). Whether translational coupling plays a role in expression of the *uba* locus remains to be determined. Finally, this work has further highlighted the diversity of the *S. uberis* bacteriocin repertoire, which now includes members of three (i.e., class I [lantibiotics], class II [unmodified peptides], and class IV [cyclic peptides]) of the four known classes (9) of bacteriocins pro-

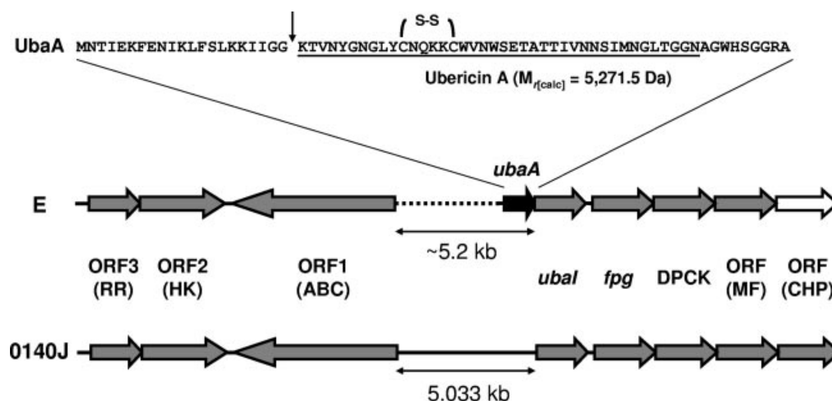

FIG. 2. Genetic organization of the ubericin A (*uba*) locus in *S. uberis* E and comparison with the corresponding region of the *S. uberis* genome sequence reference strain 0140J. For simplicity, the ORFs are not drawn to scale. The incompletely sequenced DNA region and gene (CHP) in strain E are indicated by the dotted line and unfilled arrow, respectively. The deduced amino acid sequence of the ubericin A prepeptide (UbaA) is shown with the residues obtained by N-terminal sequencing underlined. The inverted arrow highlights the cleavage site of the signal peptide, and the disulfide bond (—S—S—) formed between Cys^{11} and Cys^{16} is also indicated. ORF1, putative ABC transporter; ORF2(HK) and ORF3(RR), putative histidine kinase and response regulator, respectively; *fpg*, formamidopyrimidine glycosylase; DPCK, dephosphocoenzyme A kinase; ORF(MF), putative membrane fusion protein; ORF(CHP), conserved hypothetical protein of unknown function.

duced by gram-positive bacteria. From a practical perspective, any antimastitis formulation incorporating *S. uberis* bacteriocins will likely be a "bacteriocin cocktail" containing at least ubericin A, nisin U, and uberolysin.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this article have been deposited in GenBank under accession numbers EF203953 (*ubaAI* locus) and EF203954 (putative bacteriocin-associated ABC transporter).

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