Bovicin HC5, a bacteriocin from *Streptococcus bovis* HC5

Hilário C. Mantovani,¹ Haijing Hu,² Randy W. Worobo² and James B. Russell^{1,3}

Author for correspondence: James B. Russell. Tel: +1 607 255 4508. Fax: +1 607 255 3904.
e-mail: jbr8@cornell.edu

^{1,2} Section of Microbiology¹ and Department of Food Science and Technology (NYSAES)², Cornell University, Ithaca, NY 14853, USA

³ Agricultural Research Service, USDA, Ithaca, NY 14853, USA

Previous work indicated that *Streptococcus bovis* HC5 had significant antibacterial activity, and even nisin-resistant *S. bovis* JB1 cells could be strongly inhibited. *S. bovis* HC5 inhibited a variety of Gram-positive bacteria and the spectrum of activity was similar to monensin, a commonly used feed additive. The crude extracts (ammonium sulfate precipitation) were inactivated by Pronase E and trypsin, but the activity was resistant to heat, proteinase K and α -chymotrypsin. Most of the antibacterial activity was cell associated, but it could be liberated by acidic NaCl (100 mM, pH 2.0) without significant cell lysis. When glycolysing *S. bovis* JB1 cells were treated with either crude or acidic NaCl extracts, intracellular potassium declined and this result indicated the antibacterial activity was mediated by a pore-forming peptide. The peptide could be purified by HPLC and matrix-assisted laser desorption ionization time-of-flight analysis indicated that it had a molecular mass of approximately 2440 Da. The terminal amino acid sequence was VGXRYASXPGXSWKYVXF. The unnamed amino acid residues (designated by X) had approximately the same position as dehydroalanines found in some lantibiotics, but samples that were reduced and alkylated prior to Edman degradation did not have cysteine residues. The only other bacteriocin that had significant similarity was the lantibiotic precursor of *Streptococcus pyogenes* SF370, but the identity was only 55%. Based on these results, the bacteriocin of *S. bovis* HC5 appears to be novel and the authors now designate it as bovicin HC5.

Keywords: rumen, lactic acid bacteria, purification, N-terminal amino acids

INTRODUCTION

Beef cattle in the United States are routinely fed antibiotics to alter ruminal fermentation, and this alteration is mediated by a decrease in Gram-positive species that produce large amounts of hydrogen, a precursor of methane, and ammonia, a wasteful end product of protein degradation (Russell & Strobel, 1989). Monensin, the most common feed additive, is not used by physicians and does not seem to increase antibiotic resistance, but the widespread use of antibiotics in animal feed has been criticized (Russell & Rychlik, 2001). Some Gram-positive ruminal bacteria produce bacteriocins that inhibit other related species

(Kalmokoff *et al.*, 1996) and *in vitro* experiments indicated that the bacteriocins nisin and monensin had similar effects on ruminal fermentation (Callaway *et al.*, 1997).

Nisin is too expensive to be used as a feed additive and experiments with the ruminal bacterium *Streptococcus bovis* indicated that resistance developed quickly (Mantovani & Russell, 2001). Whitford *et al.* (2001) screened several ruminal streptococci for their ability to produce bacteriocins, and they purified and sequenced a peptide from *Streptococcus gallolyticus* LRC0255 (bovicin 255). Bovicin 255 inhibited some freshly isolated strains of *S. bovis*, but many isolates were not inhibited and adaptation greatly decreased its potential activity (Mantovani *et al.*, 2001). Because nisin-resistant *S. bovis* JB1 could not be inhibited by bovicin 255, it appeared that there was a common mechanism of resistance.

Abbreviations: MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; TFA, trifluoroacetic acid.

Ruminal isolations yielded a lactic-acid-producing bacterium (HC5) that was identified by 16S rDNA to be *S. bovis* (Mantovani *et al.*, 2001). *S. bovis* HC5 had a broader antibacterial spectrum than *S. gallolyticus* LRC0255 and freshly isolated strains of *S. bovis* did not adapt to *S. bovis* HC5 (Mantovani *et al.*, 2001). The antibacterial activity of *S. bovis* HC5 appeared to be a bacteriocin, but further work was needed to demonstrate that it was indeed a pore-forming peptide. The following experiments describe the purification and characterization of the *S. bovis* HC5 bacteriocin.

METHODS

Cells and growth. Nisin-sensitive and -resistant *S. bovis* JB1 (Mantovani & Russell, 2001) and *S. bovis* HC5 were previously described (Mantovani *et al.*, 2001). The bacteria were grown anaerobically in a basal medium (39 °C) that contained (per litre): 22 mmol glucose, 1.7 mmol K_2HPO_4 , 2.1 mmol KH_2PO_4 , 3.6 mmol $(NH_4)_2SO_4$, 8.3 mmol NaCl, 0.75 mmol $MgSO_4 \cdot 7H_2O$, 0.43 mmol $CaCl_2 \cdot 2H_2O$, 2.8 mmol cysteine hydrochloride, 38 mmol Na_2CO_3 , Casamino acids (5 mg ml⁻¹; Difco), Trypticase (a pancreatic digest of casein, 10 mg ml⁻¹; BBL Microbiology Systems), 5 g yeast extract, vitamins and minerals (Cotta & Russell, 1982). The basal medium was prepared under O₂-free CO₂ and was dispensed into tubes (18 × 150 mm) that were sealed with butyl rubber stoppers. To release the bacteriocin from the cells, Tween 80 (1 µl ml⁻¹) was sometimes added to the basal medium. Basal medium was adjusted to pH 6.7 with NaOH and glucose fermentation never caused >0.2 unit decrease in pH. Growth was monitored via changes in optical density (1 cm cuvette, 600 nm, Gilford 260 spectrophotometer) and the ratio of cell protein to optical density was 160 µg protein ml⁻¹ per optical density unit.

Spectrum of activity. *S. bovis* HC5 was spotted onto basal medium plates and these plates were incubated anaerobically for 24 h at 39 °C. Molten agar (basal medium, 4 mg glucose ml⁻¹) inoculated with target bacteria (approx. 10⁶ viable cells ml⁻¹) was poured over agar plates that already had a *S. bovis* HC5 colony (deferred antagonism assay). The agar overlays were incubated anaerobically for 4 h at 25 °C (a temperature that does not allow growth of most ruminal bacteria). The agar overlays were then incubated anaerobically at 39 °C for 48 h and zones of clearing were measured.

Crude extracts of the *S. bovis* HC5 bacteriocin. Stationary-phase *S. bovis* HC5 cells were harvested by centrifugation (20 min, 8200 g, 4 °C). The cell-free supernatant was treated (4 °C, 1 h) in stepwise fashion with increasing amounts of $(NH_4)_2SO_4$, and precipitated materials were harvested by centrifugation (30 min, 8200 g, 4 °C). The 40–60% $(NH_4)_2SO_4$ fraction was resuspended in K_2HPO_4 buffer (6.6 ml, 100 mM, pH 6.0) and dialysed (3500 M_r cut-off; Pierce Chemicals) against potassium phosphate buffer. Crude extracts were in some cases treated with peptidases and proteinases (4 U Pronase E, 11.3 U trypsin, 12 U proteinase K, 41 U α-chymotrypsin ml⁻¹) and activity was assessed by adding these extracts to agar wells that had been cut into agar plates inoculated with *S. bovis* JB1 (10⁵ cells ml⁻¹). The agar plates were incubated anaerobically at 39 °C and bacteriocin activity was assessed from the size of the zone of clearing.

Intracellular potassium. Stationary phase *S. bovis* JB1 cells (10 ml, approximately 160 µg protein ml⁻¹) were harvested by centrifugation (4000 g, 15 min, 22 °C), washed anaerobically

in basal medium lacking ammonia, and resuspended in 10 ml of the same medium. The washed cell suspensions were energized with glucose (22 mmol ml⁻¹) and some suspensions were treated with either nisin (1 µM) or partially purified *S. bovis* HC5 bacteriocin (amount equivalent to a culture density of 160 µg protein ml⁻¹). Samples (1 ml) were centrifuged (13000 g, 5 min, 22 °C) through 0.3 ml silicon oil (1:1 ratio, Dow-Corning 550 and 556). The microcentrifuge tubes were frozen (–20 °C) and the bottom of the tubes containing the cell pellets were removed with a pair of dog nail clippers. Cell pellets were digested (22 °C, 24 h, 3 M HNO₃) and insoluble cell debris was removed (13000 g, 5 min, 22 °C). Potassium was determined with a flame photometer (model 2655-00 digital flame analyser; Cole-Parmer Instruments).

Purification of *S. bovis* HC5 bacteriocin. Stationary-phase *S. bovis* cultures were harvested by centrifugation and the cells were washed in sodium phosphate buffer (5 mM, pH 6.7). The cell pellets were resuspended in acidic sodium chloride (100 mM, pH 2.0, 2 h, 4 °C). The cell suspensions were recentrifuged to remove cells and the cell-free supernatant was lyophilized. The lyophilized material was resuspended in sterile water. The bacteriocin extract was then applied to an SP Sepharose column (1.0 × 10 cm; Amersham Pharmacia) washed with 3 vols water followed by 0.2 M and 0.4 M NaCl. Multiple 100 µl injections of the active fractions were applied to a Discover BIO wide pore C-18 column [4.6 × 150 mm, 5 µm Supelco, 1 ml 0.1% trifluoroacetic acid (TFA) in water min⁻¹, 2% acetonitrile gradient min⁻¹, 22 °C]. Antibacterial activity of the eluted fractions was assayed with *Bacillus subtilis* ATCC 6537, an aerobic bacterium that was also sensitive. The final purification of the active peptide was completed by reinjecting the active fractions onto a Discovery RP amide C16 column using ethanol as a carrier solvent (1% min⁻¹, 4.6 mm × 25 cm, 5 µm Supelco). The active fractions were then collected and lyophilized.

The purified peptide was separated by Tris/Tricine SDS-PAGE (16.5% acrylamide) (Ausubel *et al.*, 1997). One half of the gel was washed in water (10 min), and fixed with glutaraldehyde (5%, 1 h; Sigma), prior to staining with Coomassie brilliant blue R-250 (0.025%, 1 h; Sigma). Gels were destained overnight in acetic acid (10%, v/v). The other half of the gel was fixed (10% acetate/20% 2-propanol, v/v, 30 min) and washed with MilliQ-H₂O (1 h). The gel was then covered with moist Kimwipes (Kimberly Clark), overlaid with soft agar (10 ml) containing 10⁶ cells *B. subtilis* ATCC 6537 and incubated overnight at 37 °C.

Determination of N-terminal amino acid sequence of bovicin HC5. Purified bacteriocin from *S. bovis* HC5 was subjected to Edman degradation analysis on a PE/ABD Procise 494 cLC Protein Sequencing System (Harvard Microchemistry Facility, Cambridge, MA).

Mass spectrometry of bovicin HC5. The *S. bovis* HC5 bacteriocin obtained by HPLC purification was added to a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) sample plate and the sample was supplemented with 1 ml of an α-cyano-4-hydroxy-trans-cinnamic acid solution (10 mg in 50% acetonitrile and 0.3% TFA). The mixture was mixed and allowed to dry at room temperature prior to mass spectrometry. The mass spectrometry data were acquired on a Voyager DE-STR MALDI-TOF MS system (Perspective Biosystems) with delayed extraction in the reflectron mode.

Other analysis. Cell protein was determined by the Lowry method, using serum albumin as a standard.

RESULTS

Spectrum of activity

When *S. bovis* HC5 was grown anaerobically on basal medium agar plates and overlaid with molten agar containing *S. bovis* JB1 cultures, a distinct zone of clearing was observed (Table 1). *S. bovis* HC5 also inhibited *S. bovis* 33317 and 15351, a variety of other Gram-positive bacteria and even Gram-negative *Prevotella* strains. None of the target bacteria were inhibited when *S. bovis* JB1 (a non-bacteriocin-producing strain) was employed. *S. bovis* HC5 did not inhibit *S. bovis* 2703, *Escherichia coli* K-12, *Acidaminococcus fermentans* ATCC 25085, *Selenomonas ruminantium* HD4 and D or *Megasphaera elsdenii* MEAW106. These latter two bacteria are closely related to Gram-positive species, but they have outer membranes (Stackebrandt *et al.*, 1985).

Crude extracts and potassium depletion

The *S. bovis* HC5 bacteriocin could be precipitated by ammonium sulfate precipitation (40–60 % fraction) and dialysis. The bacteriocin extract retained activity if it was autoclaved (120 °C, 20 min) or treated with 12 U proteinase K ml⁻¹ and 41 U α -chymotrypsin ml⁻¹, but it was inactivated by 4 U Pronase E ml⁻¹ and 11.3 U

Fig. 1. The effect of proteinases, peptidases and heat (121 °C, 20 min) on the activity of partially purified bacteriocin that was added to agar wells. The agar was inoculated with approximately 10^5 *S. bovis* JB1 cells and incubated at 39 °C for 24 h.

Table 1. Spectrum of activity of partially purified *S. bovis* HC5 bacteriocin

Bacterial strain	Zone of inhibition*
<i>Streptococcus bovis</i> JB1	++
<i>Streptococcus bovis</i> 33317	++
<i>Streptococcus bovis</i> 15351	+++
<i>Streptococcus bovis</i> 2703	—
<i>Streptococcus gallolyticus</i> LRC0255	+++
<i>Streptococcus equinus</i> 9812	++
<i>Ruminococcus albus</i> B199	+++
<i>Enterococcus faecalis</i> CG110	++
<i>Enterococcus faecalis</i> NCK 337	++
<i>Clostridium sticklandii</i> SR	++++
<i>Clostridium aminophilum</i> F	+++
<i>Peptostreptococcus anaerobius</i> C	++++
<i>Butyrivibrio fibrisolvens</i> 49	+++
<i>Butyrivibrio fibrisolvens</i> JL5	+++
<i>Bacillus subtilis</i> ATCC 6537	++++
<i>Lactobacillus plantarum</i> LII a I	+++
<i>Lactococcus lactis</i> C2	++++
<i>Listeria monocytogenes</i> 104035	+++
<i>Prevotella bryantii</i> B ₁ 4	+
<i>Prevotella brevis</i> GA-33	+
<i>Selenomonas ruminantium</i> HD4	—
<i>Selenomonas ruminantium</i> D	—
<i>Escherichia coli</i> K-12	—
<i>Acidaminococcus fermentans</i> ATCC 25085	—
<i>Megasphaera elsdenii</i> MEAW106	—

* No inhibition (—) to strong inhibition (>15 mm, ++++).

Fig. 2. The effect of *S. bovis* HC5 bacteriocin extracts on the intracellular potassium levels of *S. bovis* JB1 cells that were energized with glucose (22 mM). Cells were treated at time zero (arrow) with extracts that were obtained from culture supernatant ammonium sulfate precipitation (●) or cell pellets that were treated with acidic NaCl (■). The cells were also treated with 1 μ M nisin (▲). Untreated controls (○) are also shown.

trypsin ml⁻¹ (Fig. 1). Bacteriocin extracts that were bubbled with O₂ and returned to the anaerobic glove box remained active (data not shown). When the extract was subjected to Tris/Tricine PAGE, a variety of peptides were observed (data not shown).

When stationary-phase *S. bovis* JB1 cells were washed and incubated in a basal medium containing glucose

Fig. 3. A high pressure liquid chromatogram of purified bovicin HC5. Extracts obtained from cells by acidic NaCl (100 mM, pH 2.0) and collected from an SP Sepharose column were applied to a C-18 column (4.6 × 150 mm, Supelco, 1 ml 0.1% TFA in water min⁻¹, 2% acetonitrile gradient min⁻¹, 22 °C).

(22 mM) but lacking nitrogen sources, the cells did not grow, but the intracellular potassium was >2800 nmol (mg protein)⁻¹ (Fig. 2). When 1 µM nisin was added, intracellular potassium declined rapidly and virtually no potassium could be detected after 10 min. If bacteriocin extract was added, potassium also declined, but the rate of this decrease was noticeably slower than that observed with nisin. Potassium efflux was not observed if the extract was treated with Pronase E (data not shown).

Purification of the *S. bovis* HC5 bacteriocin

The crude bacteriocin extract had contaminating peptides from the basal medium and it appeared that much of the activity was not being recovered. If Tween was deleted from the basal medium, little activity was detected in the culture supernatant. Cells that were harvested by centrifugation and treated with acidic NaCl (100 mM, pH 2.0) did not lyse, but they released bacteriocin. The activity (based on the original number of cells) was at least 10-fold greater than that in ammonium sulfate extracts and the rate of potassium efflux from glycolysing *S. bovis* JB1 cells was faster (Fig. 2).

When the acidic NaCl extract was applied to an SP Sepharose column, the antibacterial activity could be eluted by 0.4 M NaCl. HPLC (C18 column) indicated that this fraction had a variety of peaks, but only one peak had activity. Rechromatography of the active fraction (RP amide, C16 column) yielded a single purified peptide that had activity (Fig. 3). Tris/Tricine PAGE supported the idea that bacteriocin had indeed

Fig. 4. Tris/Tricine PAGE of nisin (A) and purified bovicin HC5 (B). The gel was fixed with glutaraldehyde and stained with Coomassie brilliant R 250 blue. Molecular mass standards are shown at the left. Lane C shows an unstained PAGE that was overlaid with soft agar containing *Bacillus subtilis* ATCC 6537.

Fig. 5. A MALDI-TOF MS chromatogram of the purified bovicin HC5. Results indicated that the purified bacteriocin had a molecular mass of approximately 2440 Da.

been purified (Fig. 4). When the purified bacteriocin was lyophilized and subjected to Edman degradation, the N-terminal amino acid sequence could be determined (VGXRYASXPGXSWKYVXF), but there were four residues (indicated by an X) that did not correspond to any of the 20 amino acids commonly found in proteins. Mass spectrometry based on MALDI-TOF indicated that the purified bacteriocin had a molecular mass of approximately 2440 Da (Fig. 5).

DISCUSSION

Early work (Iverson & Mills, 1976) indicated that some strains of *S. bovis* produced bacteriocins and Whitford *et al.* (2001) recently reported that *S. gallolyticus*

Bovicin HC5	V	G	X	R	Y	A	S	X	P	G	X	S	W	K	Y	V	X	F
<i>S. pyogenes</i> lantibiotic	V	G	S	R	Y	L	C	T	P	G	S	C	W	K	L	V	C	F

Fig. 6. Alignment of the N-terminal amino acid sequence of bovicin HC5 and the lantibiotic precursor of *S. pyogenes* SF370. Identical residues are shown on a black background and conserved substitutions are shown on a grey background.

LRC0255 (a bacterium previously classified as *S. bovis*) inhibited a variety of streptococci. However, recent work indicated that sterile-filtered *S. gallolyticus* LRC0255 culture supernatants could not inhibit nisin-resistant *S. bovis* JB1 cultures (Mantovani *et al.*, 2001). Because nisin-resistant cells were bovicin-resistant, and this phenotype develops quickly, it is unlikely that either bovicin 255 or nisin would have a marked impact on *S. bovis* ecology *in vivo*. However, previous work indicated that *S. bovis* HC5 could inhibit both nisin-sensitive and nisin-resistant *S. bovis* JB1 cultures and a variety of freshly isolated *S. bovis* strains, and adaptation could not be demonstrated (Mantovani *et al.*, 2001). Based on these previous results, it appeared that the *S. bovis* HC5 bacteriocin had potential as an agent to manipulate ruminal ecology and fermentation.

Some bacteriocins are highly specific and can only inhibit closely related strains (Jack *et al.*, 1995), but *S. bovis* HC5 was able to inhibit a variety of Gram-positive ruminal bacteria as well as Gram-positive species from other habitats (Table 1). *Sel. ruminantium* and *M. elsdenii* are closely related to Gram-positive bacteria, but these species have outer membranes (Stackebrandt *et al.*, 1985) and were resistant to the antibacterial activity of *S. bovis* HC5. *S. bovis* HC5 did not inhibit *E. coli* or *A. fermentans*, but a small zone of clearing was observed with *Prevotella bryantii*, a Gram-negative ruminal bacterium that has been used as a model of monensin resistance (Callaway & Russell, 2000). Based on these results, it appeared that *S. bovis* HC5 has approximately the same spectrum of activity as monensin, a commonly used antibiotic in cattle rations (Russell & Strobel, 1989).

The idea that the antibacterial activity of *S. bovis* HC5 was a bacteriocin was supported by the observation that it could be precipitated by ammonium sulfate and inactivated by Pronase E, a mixture of proteinases and peptidases. The crude extracts were resistant to α -chymotrypsin, proteinase K and heat, and these properties could be advantageous for commercial applications. Many bacteriocins are peptides that insert into cell membranes to form pores, but some bacteriocins are thought to inhibit peptidoglycan synthesis and DNA replication (Jack *et al.*, 1995; Sablon *et al.*, 2000). Because the crude extract catalysed potassium efflux from *S. bovis* JB1, it appeared to contain a pore-forming peptide.

Bacteriocins are frequently cell associated and detergents are often added to the culture media to promote bacteriocin release (Parente & Ricciardi, 1999; Nel *et al.*, 2001). The bacteriocin activity of *S. bovis* HC5 cell-

free supernatant was greatly enhanced by Tween 80. Bacteriocin activity could be precipitated from the cell-free supernatant by ammonium sulfate, but it had an abundance of contaminating peptides. Some of these peptides could be removed by dialysis, but even this latter treatment did not remove all of the contamination.

Yang *et al.* (1992) noted that bacteriocins of some lactic acid bacteria could be dislodged from the cell surface by acidic NaCl, and this treatment liberated *S. bovis* HC5 bacteriocin without causing detectable cell lysis. Because the cells could be washed prior to the acidic NaCl treatment, contaminating peptides from the basal medium were largely eliminated. HPLC indicated that the acidic NaCl extracts had some inactive peptides, but the active peptide could be purified and concentrated by rechromatography. The active peptide could not be silver stained, and other workers have noted a similar phenomenon (Carolissen-Mackay *et al.*, 1997; Pattnaik *et al.*, 2001; Villani *et al.*, 1995). However, it reacted with Coomassie stain after the gel had been fixed with glutaraldehyde (Fig. 4).

Edman degradation analysis indicated that the N-terminal amino acid sequence had 4 amino acid residues that did not correspond to any of the 20 amino acids commonly found in proteins. Lantibiotics have rings that are created from the condensation of cysteine and dehydro-amino acids (e.g. dehydroalanine) (DeVos *et al.*, 1995; Guder *et al.*, 2000). The unnamed amino acids had approximately the same position as the N-terminal dehydroalanines of nisin, subtilin and epidermin, but samples that were reduced and alkylated prior to Edman degradation did not have cysteine residues. Because cysteine residues were not detected under these conditions, it appeared that the unnamed residues were modified or other unusual amino acids.

A BLAST search of GenBank sequences indicated that our N-terminal amino acid sequence was unique. The only other bacteriocin that had significant similarity was the lantibiotic precursor of *S. pyogenes* SF370 (Ferreti *et al.*, 2001), but the identity was only 55% (Fig. 6). Based on these results, the bacteriocin of *S. bovis* HC5 appears to be novel and we now designate it as bovicin HC5. Further work will be needed to locate and sequence the bovicin HC5 gene.

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Proprietary or brand names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product, and exclusion of others that may be suitable.

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