



Physiology and microbial chemistry

The ability of non-bacteriocin producing *Streptococcus bovis* strains to bind and transfer bovicin HC5 to other sensitive bacteria[☆]Bruno M. Xavier^a, James B. Russell^{a,b,*}^a Department of Microbiology, Cornell University, 157A Wing Hall, Ithaca, NY 14853, USA^b Agricultural Research Service, USDA, Ithaca, NY 14853, USA

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ABSTRACT

Streptococcus bovis HC5 produces a broad spectrum lantibiotic (bovicin HC5), but *S. bovis* JB1 does not have antimicrobial activity. Preliminary experiments revealed an anomaly. When *S. bovis* JB1 cells were washed in stationary phase *S. bovis* HC5 cell-free culture supernatant, the *S. bovis* JB1 cells were subsequently able to inhibit hyper-ammonia producing ruminal bacteria (*Clostridium sticklandii*, *Clostridium aminophilum* and *Peptostreptococcus anaerobius*). Other non-bacteriocin producing *S. bovis* strains also had the ability to bind and transfer semi-purified bovicin HC5. Bovicin HC5 that was bound to *S. bovis* JB1 was much more resistant to Pronase E than cell-free bovicin HC5, but it could be inactivated if the incubation period was 24 h. Acidic NaCl treatment (100 mM, pH 2.0) liberates half of the bovicin HC5 from *S. bovis* HC5, but it did not prevent bovicin HC5 from binding to *S. bovis* JB1. Acidic NaCl liberated some bovicin HC5 from *S. bovis* JB1, but the decrease in activity was only 2-fold. Bovicin HC5 is a positively charged peptide, and the ability of *S. bovis* JB1 to bind bovicin HC5 could be inhibited by either calcium or magnesium (100 mM). Acidic NaCl-treated *S. bovis* JB1 cells were unable to accumulate potassium, but they were still able to bind bovicin HC5 and prevent potassium accumulation by untreated *S. bovis* JB1 cells. Based on these results, bovicin HC5 bound to *S. bovis* JB1 cells still acts as a pore-forming lantibiotic.

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1. Introduction

Many Gram-positive bacteria produce small peptides (lantibiotics) that assemble to form pores in cell membranes [1]. Some lantibiotics have a broad spectrum of antibacterial activity, but others are species- or even strain-specific. The specificity of lantibiotics is not entirely clear. Breukink et al. [2] demonstrated that nisin bound lipid II during its incorporation into the cell membrane. However, most Gram-positive bacteria have lipid II and it should be noted that nisin, the most widely used commercial bacteriocin, is a broad rather than narrow spectrum lantibiotic [1].

Previous work indicated that approximately half of the *Streptococcus bovis* strains isolated from the rumen had antibacterial activity [3], and a strain designated as HC5 produced a broad spectrum, positively charged lantibiotic [4]. Because bacteria

known for their ability to become resistant to nisin or other bacteriocins remained sensitive to bovicin HC5, it appeared that bovicin HC5 might be a useful lantibiotic [5]. *S. bovis* JB1 does not produce a lantibiotic, and it has been used as a model organism of bovicin HC5 sensitivity [6–8].

S. bovis HC5 does not liberate significant amounts of cell-free bovicin HC5 until it reaches a stationary phase [9], but competition studies indicated that exponentially growing *S. bovis* HC5 cells could inhibit *S. bovis* JB1 [10]. Cell-associated bovicin HC5 can be liberated from the cell-surface of *S. bovis* HC5 by acidic sodium chloride (pH 2.0, 100 mM), and divalent cations bind to the cell-surface of *S. bovis* JB1 and cause resistance to bovicin HC5 [4,6]. These latter results indicate that cell-surface charge is an important feature for bovicin HC5 sensitivity and its release from *S. bovis* HC5.

Clostridium sticklandii SR, *Clostridium aminophilum* and *Peptostreptococcus anaerobius* are hyper-ammonia bacteria (HAB) that are even more sensitive to bovicin HC5 than *S. bovis* JB1 [4], and they have a different pattern of energy source utilization. HAB do not utilize carbohydrates [11] while *S. bovis* strains use hexoses and do not ferment amino acids [12]. This difference allowed us to examine the ability of *S. bovis* JB1 to bind bovicin HC5 and transfer it to HAB. Subsequent experiments were designed to describe the nature of this transfer.

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2. Materials and methods

2.1. Bacteria, media and growth

S. bovis strains [13] were routinely grown under O₂-free CO₂ at 39 °C in basal medium containing (per liter): 240 mg K₂HPO₄, 240 mg KHPO₄, 520 mg Na₂SO₄, 480 mg NaCl, 100 mg MgSO₄·7H₂O, 64 mg CaCl₂·2H₂O, 600 mg cysteine hydrochloride, 1 g Trypticase (BBL Microbiology Systems, Cockeysville, MD, USA), vitamins and minerals [14]. The medium was adjusted to pH 6.7 with NaOH and autoclaved for 20 min. After the sterile medium had cooled to room temperature, sterile Na₂CO₃ (4 g l⁻¹) was added as a buffer. Cultures were grown in 18 × 150 mm tubes that were sealed with butyl rubber stoppers. Glucose (4 mg ml⁻¹, final concentration) was added to the basal medium after it had been autoclaved. Growth was monitored via changes in the optical density (1 cm cuvette, 600 nm, Gilford 260 spectrophotometer, Oberlin, OH). *C. sticklandii*, *C. aminophilum* and *P. anaerobius* were grown in a similar fashion, except that additional Trypticase (20 mg ml⁻¹) was substituted for glucose.

2.2. Co-incubation experiments

S. bovis cultures were grown in basal medium until they reached the stationary phase (16 h of incubation, 4 mg glucose ml⁻¹, final pH 6.4), and the cells were harvested by centrifugation (4000 × g, 5 °C, 15 min). Cell-free supernatants were removed from the cell pellets. *S. bovis* HC5 supernatant was retained and passed through a 0.45 µm sterile membrane filter (Millipore, Bedford, MA). Non-bacteriocin producing *S. bovis* cells were resuspended in sterile *S. bovis* HC5 supernatant (15 min, 39 °C) and washed with basal medium lacking nitrogen or energy sources. *S. bovis* cells that had been treated with the *S. bovis* HC5 supernatant were re-harvested by centrifugation and washed again in the basal medium lacking nitrogen or energy sources. Bovicin HC5-treated cell pellets were resuspended in basal medium containing Trypticase (20 mg ml⁻¹), and the tubes were inoculated with HAB (1% v/v). After 16 h of incubation (39 °C), the *S. bovis* and HAB cells were harvested by centrifugation, and the cell-free culture supernatant was analyzed for ammonia. In some cases, the non-bacteriocin producing *S. bovis* cells were resuspended in basal medium containing semi-purified bovicin (20 AU ml⁻¹, described below) rather than sterile filtered *S. bovis* HC5 culture supernatant.

2.3. Ammonia production and cell protein

Ammonia production was evaluated using the method of Chaney and Marbach [15]. Six times as much reagent was used to eliminate cysteine inference. *S. bovis* was harvested by centrifugation (10,000 × g, 15 min, 5 °C), and the cell pellets were digested with dilute NaOH (0.2 N, 100 °C, 10 min). Protein content was assayed using the Lowry method [16] using serum albumin as a standard.

2.4. Pronase E treatment

The sterile *S. bovis* HC5 supernatant or non-bacteriocin producing *S. bovis* cells that had been treated with semi-purified bovicin HC5 were treated with Pronase E (4 U ml⁻¹, Sigma Chemical Co., St. Louis, MO) as previously described [17].

2.5. Acidic NaCl and semi-purified bovicin HC5

Stationary phase *S. bovis* cells were treated with acidic NaCl (100 mM NaCl, pH 2.0, 30 min, 39 °C) according to the method of

Yang et al. [18] to produce semi-purified bovicin HC5. Semi-purified bovicin HC5 was obtained by lyophilizing the acidic NaCl extract and resuspending it in sterile distilled water (2 ml, 2500 activity units ml⁻¹). The semi-purified preparation was assayed for antibacterial activity by serially diluting the extract in distilled water (2-fold increments), and placing each dilution (100 µl) in agar wells that had been cut into agar plates inoculated with *C. sticklandii* SR (10⁶ cells ml⁻¹) in an anaerobic glove box (Coy Laboratory Products, Ann Arbor, MI). Activity units (expressed per milliliter) were calculated from the reciprocal of the highest serial dilution showing a visible zone of clearing.

2.6. Potassium accumulation

Stationary phase *S. bovis* JB1 cells were washed and incubated in basal medium lacking Trypticase and yeast extract (39 °C, 60 min). The washed suspensions were then centrifuged (13,000 × g, 5 min) through silicone oil as previously described [4]. The cell pellets were removed with dog nail clippers, digested in 3 N HNO₃ (25 °C, 24 h), and the insoluble cell material was removed by centrifugation (13,000 × g, 1 min). Potassium concentration was determined by flame photometry (Cole-Parmer 2655-00 Digital Flame Analyzer, Cole-Parmer Instruments). *S. bovis* JB1 cells were energized by adding glucose (20 mM) and incubating for 30 min prior to centrifugation through silicone oil. In some cases, untreated *S. bovis* JB1 cells were co-incubated with *S. bovis* JB1 cells that had been treated with acidic NaCl and subsequently allowed to bind semi-purified bovicin HC5.

3. Results

Preliminary experiments indicated that bovicin HC5 was bacteriostatic (but not bactericidal) against *S. bovis* JB1 and as little as 20 AU ml⁻¹ inhibited growth (data not shown). *C. sticklandii* SR, *P. anaerobius* C and *C. aminophilum* F (1% v/v inoculum) grew well in the basal medium that was supplemented with Trypticase (20 mg ml⁻¹), and their deamination activities produced more than 10 mM ammonia after 16 h of incubation (Fig. 1). When a tri-culture of all HAB was used, the ammonia production was approximately 40 mM. If *S. bovis* JB1 cells (160 µg protein ml⁻¹ or approximately 10⁹ viable cells ml⁻¹) were added to the basal medium that was supplemented with Trypticase a little increase in ammonia was detected, and this ammonia could be subtracted to determine the ammonia that was produced by HAB. None of the HAB grew or produced ammonia in the sterile filtered, *S. bovis* HC5 supernatant that was supplemented with Trypticase, and this inhibition was consistent with the ability of *S. bovis* HC5 to liberate cell-free bovicin HC5 after it reaches the stationary phase [9].

HAB were not inhibited by stationary phase *S. bovis* JB1 cells. However, if *S. bovis* JB1 cells were: (1) harvested by centrifugation, (2) resuspended in stationary phase *S. bovis* HC5 cell-free supernatant (30 min, 39 °C), (3) washed with basal medium and (4) added to the basal medium that was supplemented with Trypticase (20 mg ml⁻¹), all three of the HAB and the HAB tri-culture lost much of their ability to grow (<0.1 increased in optical density) and produced little ammonia (Fig. 1). With *C. sticklandii* SR the inhibition was approximately 75%. The ability of non-bacteriocin producing *S. bovis* to bind bovicin HC5 and transfer it to HAB was not restricted to the JB1 strain, and it did not seem to matter if the *S. bovis* had been isolated from the rumen (bovine strains) or the gastrointestinal tract of humans (human strains) (Fig. 2).

The idea that non-bacteriocin producing *S. bovis* strains could bind and transfer bovicin HC5 to HAB was strengthened by the observation that a similar effect could be obtained if semi-purified bovicin HC5 was added to the basal medium, and the inhibition was

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