Physiology and microbial chemistry

The ability of non-bacteriocin producing Streptococcus bovis strains to bind and transfer bovicin HC5 to other sensitive bacteria

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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.
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 KH2PO4, 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 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 cystine 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 1010 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...
dependent on the cell density (Fig. 3). If the density of *S. bovis* JB1 cells was less than 32 mg protein ml⁻¹, little inhibition could be demonstrated, but there was a linear decrease in ammonia production as the *S. bovis* JB1 cell density was increased to 160 mg protein ml⁻¹. *S. bovis* JB1 cells that had bound bovicin HC5 were as inhibitory as *S. bovis* HC5 cells. Once again, no inhibition by *S. bovis* JB1 cells was observed unless the cells had been allowed to bind bovicin HC5. When the concentration of the semi-purified bovicin HC5 was varied from 0 to 10 AU ml⁻¹, the *S. bovis* JB1 cells (160 mg ml⁻¹) had nearly as much capacity to inhibit *C. sticklandii* SR as cell-free bovicin HC5 (Fig. 4).

Recent work indicated that the cell-associated activity of *S. bovis* HC5 cells was more resistant to Pronase E (a commercial mixture of peptidases and proteinases) than cell-free bovicin HC5 [17], and a similar effect was observed for bovicin HC5 that had been allowed to bind to *S. bovis* JB1 cells. The cell-free supernatant of *S. bovis* HC5 that was treated with Pronase E for 2 h lost most of its ability to inhibit the ammonia production of *C. sticklandii* SR, but bovicin HC5 that was bound to *S. bovis* JB1 cells resisted a similar treatment (data not shown). The resistance of *S. bovis* JB1 cell-associated bovicin HC5 to degradation by Pronase E was, however, not complete. If the incubation time with Pronase E was increased to 24 h, even the *S. bovis* JB1 cell-associated bovicin HC5 lost most of its activity (data not shown).

Acidic NaCl (100 mM, pH 2.0) is used to remove bacteriocins from lactic acid bacteria [18]. However, *S. bovis* JB1 cells that were treated with acidic NaCl still bound bovicin HC5 and transferred it to *C. sticklandii* SR (Fig. 5). *S. bovis* JB1 cells that were first treated with bovicin HC5 and then subjected to acidic NaCl were only 2-fold less potent than cells that had not been treated with acidic NaCl. However, if large amounts of divalent cations (100 mM magnesium or calcium) were added, the *S. bovis* JB1 cells completely lost their ability to bind bovicin HC5 and transfer it to HAB (data not shown).

Stationary phase *S. bovis* JB1 cells that were washed and incubated in the basal medium lacking nitrogen sources or glucose had an intracellular potassium concentration of only 700 nmol mg protein⁻¹ min⁻¹, and those treated with acidic NaCl had even less potassium (Fig. 6). When the treated and untreated
cells were mixed together (160 mg protein \textsuperscript{-1} ml\textsuperscript{-1}, each) the value was approximately 350 nmol mg protein \textsuperscript{-1} min\textsuperscript{-1}. When glucose (20 mM) was added, the untreated cells accumulated potassium and had approximately 2400 nmol mg protein \textsuperscript{-1} min\textsuperscript{-1} at either pH 6.7 or 5.5. The bovicin and acidic NaCl-treated cells did not accumulate potassium at either pH, but they were able to prevent the potassium accumulation of untreated cells at pH 5.5. The mixture of treated and untreated cells was still able to accumulate potassium at pH 6.7.

### 4. Discussion

Rumen is a very complex ecosystem that is inhabited by a variety of bacteria, protozoa and fungi, and molecular techniques indicate that the taxonomy is continuously changing [19]. A variety of Gram-positive ruminal bacteria produce bacteriocins [20], and microbiologists have typically focused on cell-free bacteriocin activity because it can diffuse through agar and create zones of clearing that are observed visually [1]. The role of bacteriocins in ruminal ecology has been stymied by the observation that it is difficult if not impossible to detect cell-free activity using standard methods of agar diffusion [unpublished results]. However, previous work showed that mixed rumen bacteria inhibited from cattle fed a grain-based ration that were harvested by centrifugation inhibited a tri-culture of HAB and decreased the initial rate of ammonia production by 50% [21]. Because bacteria taken from a cow fed timothy hay were unable to inhibit the HAB tri-culture, it appeared that cows fed hay have fewer bacteriocin producing bacteria. This idea was consistent with the observation that cows fed hay have a 2-fold greater specific rate of ammonia production than cows fed mostly grain [21,22].

Many bacteriocin producing, lactic acid bacteria have cell-associated activity that can be released by treatment with acidic NaCl [18], and this method was used to prepare semi-purified bovicin HCS [4]. Competition experiments between \textit{S. bovis} JB1 indicated that bovicin HCS production allowed JB1 to dominate the co-cultures even though HCS had a slower growth rate than JB1 and cell-free bovicin HCS could not yet be detected [10]. These results support the idea that cell-free bovicin HCS is only detected in stationary phase cultures [9], and the cell-associated activity seems to be more important [17]. The cell-associated
bovicin HC5 activity was more resistant to Pronase E, and this characteristic could be important in the rumen because many ruminal microorganisms produce proteinases [20] and peptidases [23].

The observation that non-bacteriocin producing S. bovis strains could bind bovicin HC5, transfer it to HAB and decrease the growth and ammonia production of HAB, is yet another reason why bacteriocin activity may be difficult to detect in cell-free ruminal fluid (see above). Comparisons of semi-purified bovicin HC5 alone and with the amount of bovicin HC5 that S. bovis JB1 was able to bind indicated that S. bovis JB1 bound large amounts of bovicin HC5, and bovicin HC5-treated S. bovis JB1 cells were approximately as potent as S. bovis HC5 cells. Bovicin HC5 that was bound to S. bovis JB1 was more resistant to Pronase E than cell-free bovicin HCS, and this observation supports the idea that this type of binding also confers increased stability. Further work will be needed to determine the mechanism of bovicin HC5 transfer from S. bovis JB1 to HAB, but it should be noted that agglutination per se was not readily observed.

As previously noted, acidic NaCl extracts large amounts of bacteriocins from lactic bacteria [18], but previous work indicated that it was only able to liberate approximately half of the bovicin HC5 from S. bovis HC5 cells [17]. Our observation that acidic NaCl-treated S. bovis JB1 cells retained large amounts of bovicin HC5 indicates that S. bovis might have two different methods of binding. Acidic NaCl-resistant binding might be common to all S. bovis. Whereas, the acidic NaCl-susceptible binding might be the one more closely related to the production and secretion of bovicin HC5 by S. bovis HC5.

A recent review by Hasper et al. [24] noted that the lantibiotic, nisin, has an “alternative bactericidal mechanism of action.” This alternative activity is mediated at the level of lipid II and cell wall synthesis rather than pore formation and the loss of intracellular solutes. Our previous work indicated that acidic NaCl-treated S. bovis HC5 cells were unable to accumulate potassium, and the activity that remained on the cells after acidic NaCl treatment prevented potassium accumulation by S. bovis JB1 [17]. These results indicated that the cell-associated activity was still acting as a pore-forming lantibiotic and not just as an inhibitor of cell wall synthesis. Our current work indicated that acidic NaCl-treated S. bovis JB1 cells had approximately the same ability to bind bovicin HC5 as untreated S. bovis JB1 cells. The acidic NaCl-treated S. bovis JB1 cells were also able to transfer bovicin HC5 to untreated S. bovis JB1 cells and prevent potassium accumulation at pH 5.5 but not 6.7. This latter result is consistent with the observation that bovicin HC5 is much more active at an acidic pH [7].

Further work will be needed to define more precisely the binding of bovicin HC5 to S. bovis JB1 and other non-bacteriocin producing strains. However, it should be noted that lantibiotics first pass through the extracellular glycocalyx that typically carries a net negative charge. Most lantibiotics [11], including bovicin HC5 [4] are positively charged peptides, and the glycocalyx has been implicated in at least some forms of bacteriocin resistance [25]. Previous work indicated that S. bovis JB1 cells that were incubated and washed with large amounts of calcium or magnesium became bovicin HC5-resistant, and this resistance was correlated with an increased ability of the cells to bind Congo red, an anionic dye [8]. Because our current work indicated that high concentrations of calcium or magnesium blocked the ability of S. bovis JB1 cells to bind bovicin HC5 and transfer it to C. sticklandii SR, it appears that the binding is electrostatic.

The observation that bovicin HC5 either directly or indirectly via non-bacteriocin producing bacteria such as S. bovis JB1 can inhibit HAB has practical significance. For many years it was assumed that carbohydrate fermenting ruminal bacteria were responsible for the wasteful degradation of amino acids in the rumen, but these bacteria are in most cases resistant to monensin, the most commonly used feed additive in American rations. HAB have much greater deamination activities, can produce as much as 90% of the ruminal ammonia [21] and are sensitive to both monensin [11] and bovicin HC5 [4]. Because monensin has been banned by the European Union, bovicin HC5 may offer an alternative way of decreasing excess ruminal ammonia [26]. Very recently published work likewise indicates that the cell-associated bacteriocin of S. bovis HC5 is more potent and less likely to be degraded by peptidases than the cell-free form [17].

References