

Bovicin HC5, a Lantibiotic Produced by *Streptococcus bovis* HC5, Catalyzes the Efflux of Intracellular Potassium but Not ATP[∇]

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Bovicin HC5, a broad-spectrum lantibiotic produced by *Streptococcus bovis* HC5, catalyzed the efflux of intracellular potassium from *Streptococcus bovis* JB1, a sensitive strain. The level of ATP also decreased, but this decline appeared to be caused by the activity of the F₁F₀ ATPase rather than efflux per se.

Many gram-positive bacteria produce antibacterial peptides that are called bacteriocins (2, 14). Bacteriocins originally were classified as antibiotics (12), but this terminology was abandoned, because the peptides had distinctly different biosynthetic pathways and are ribosomally synthesized (1, 9). Nisin is the best-studied bacteriocin produced by gram-positive bacteria, and it has modified amino acids that are linked by sulfur to make lanthionine rings, hence the term lantibiotic (7). In recent years, concerns over antibiotic resistance have led to the pursuit of alternative antimicrobial substances. Nisin has been used commercially in the food industry for more than 10 years (7, 8, 14), and recent work indicates that it also can be used therapeutically to treat bovine mastitis (6, 25).

Lantibiotics assemble in the cell membranes of sensitive bacteria to form pores that allow the loss of intracellular solutes (4, 5). The ability of nisin to form pores is facilitated by its interaction with lipid II, a common building block for peptidoglycan synthesis (3). The final pore is thought to be composed of eight nisin peptides and four lipid II molecules (3). Previous workers indicated that the nisin pore might be large enough to translocate relatively large intracellular solutes, including ATP (19, 20, 23). This idea has persisted and was reiterated in a recent review: “Nisin was later shown to cause the rapid outflow of small cytoplasmic compounds such as amino acids, ATP, or preaccumulated rubidium, as well as the collapse of vital ion gradients, when administered to gram-positive bacteria” (18).

Direct measurements of ATP efflux are complicated by the fact that ATP in the extracellular volume of bacterial cell suspensions can be as much as 1,000-fold lower than the intracellular concentration. Montville et al. (19) detected ATP in the extracellular space of *Mycobacterium smegmatis* cell suspensions that were treated with nisin A. However, this accumulation accounted for less than 5% of the intracellular ATP, and the incubation period was several hours. The idea that lantibiotics can catalyze ATP efflux is further complicated by another avenue of decreasing intracellular ATP. When the

proton-motive force is dissipated, the membrane-bound F₁F₀ ATPase can hydrolyze ATP in an attempt to reenergized the cell membrane. Because many amino acid transport mechanisms either are proton or sodium symport systems that can act reversibly, membrane deenergization alone could cause a decrease in intracellular ATP.

Streptococcus bovis HC5 produces a lantibiotic (bovicin HC5) that has a broad spectrum of activity (17). Bovicin is a bit smaller than nisin, but it appears to have the same pattern of lanthionine rings as nisin and streptin, a lantibiotic produced by *Streptococcus pyogenes* (24). Because bacteria that can readily become resistant to nisin did not become significantly more resistant to bovicin even after they were repeatedly transferred with sublethal doses, it appeared that bovicin HC5 had useful characteristics (16). The following experiments sought to answer several questions. Would bovicin HC5 cause a decrease in ATP as well as potassium? Would bovicin-treated cells retain their ability to ferment glucose? Could a bovicin HC5-dependent decrease in ATP be explained by the activity of the F₁F₀ ATPase?

Our ability to address these questions was aided by the observation that *S. bovis* JB1 has a facilitated diffusion system for glucose (21). This transport system gives *S. bovis* the ability to catabolize glucose and generate ATP, even if the proton-motive force across the cell membrane has been completely dissipated (22). The use of nitrogen-limited, nongrowing, glucose-energized *S. bovis* JB1 cells had several additional advantages. Because growth was not possible, (i) intracellular potassium and ATP concentrations were easier to estimate, (ii) the ratio of inhibitors to cells did not change, and (iii) the cells only had the constitutive, lower-affinity, proton symport potassium transport system (10).

S. bovis JB1 and HC5 were grown anaerobically in a strongly buffered medium with glucose (22 mM) as an energy source (22). Bovicin HC5 was liberated from *S. bovis* HC5 cells by a procedure involving acidic sodium chloride (pH 2.0, 100 mM) as previously described (17). Antibacterial activity (12,800 activity units [AU] ml⁻¹) was assayed by serially diluting the extract in distilled water (in twofold increments) and placing each dilution (10 μl) on a lawn of *Clostridium sticklandii* SR (10⁸ CFU ml⁻¹). AU (expressed per milliliter) were calculated from the reciprocal of the highest serial dilution showing a visible zone of clearing.

Stationary-phase *S. bovis* JB1 cells (10,000 × g, 15 min,

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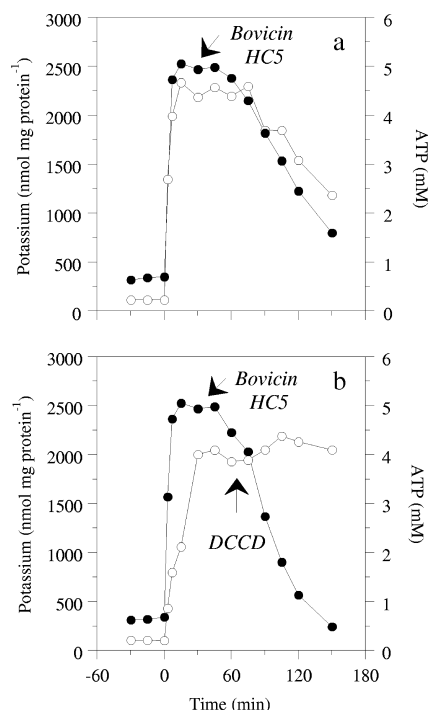


FIG. 1. Ability of nongrowing, glucose-energized *S. bovis* JB1 cells to accumulate and maintain potassium (filled circles) and ATP gradients (open circles). (a) Glucose (22 mM) was added at time zero. Bovicin HC5 (100 AU ml⁻¹) was added at 45 min. (b) The F₁F₀ ATPase inhibitor DCCD was added at 60 min.

22°C) were washed anaerobically in basal medium lacking glucose, ammonia, yeast extract, and Trypticase (pH 6.7). Cell suspensions (160 μg protein ml⁻¹) were energized with glucose (22 mmol ml⁻¹) and treated with tetrachlorosalicylanilide (TCS), *N,N'*-dicyclohexylcarbodiimide (DCCD), and/or partially purified bovicin HC5. The level of intracellular potassium was determined by flame photometry as previously described (17). ATP was assayed using the firefly luciferine luciferase (22). The intracellular ATP concentration was based on the observation that *S. bovis* cells have an intracellular volume/cell protein ratio of 4.6 μl mg⁻¹ (22).

L-Lactic acid was measured by the method of Hohorst (13) using rabbit muscle L-lactate dehydrogenase (EC 1.1.1.27) (Sigma Chemical Co., St. Louis, MO). Glucose was measured by an enzymatic test employing hexokinase (EC 2.7.1.1) (Sigma) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (Sigma) (22). Cell protein was determined by the assay of Lowry et al. (15). All experiments were performed three times. The means, standard deviations, and coefficients of variation were computed. Because the coefficients of variation were less than 10% and the difference among means after treatment was always greater than three standard deviations, statistics are not reported.

The *S. bovis* JB1 washed cell suspensions initially had little ATP or potassium, but both of these parameters increased as soon as glucose was added (Fig. 1a). The cell suspensions did not grow but were able to maintain their potassium gradient and ATP for longer than 150 min. Glucose was never depleted (data not shown). Cell suspensions that were treated with bovicin HC5 lost most of their potassium and depleted more than

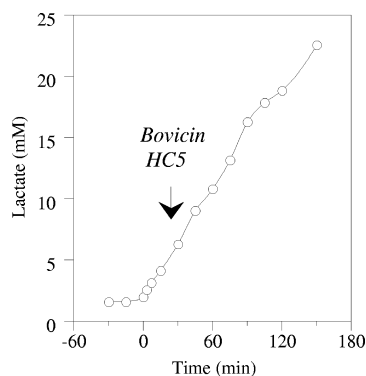


FIG. 2. Lactate production of nongrowing, glucose-energized *S. bovis* JB1 cells. Glucose (22 mM) was added at time zero. Bovicin HC5 (100 AU ml⁻¹) was added at 45 min.

half of their intracellular ATP. However, these same cells retained their ability to ferment glucose and produce lactate for at least 100 min (Fig. 2). The ability of bovicin HC5 to cause a decline in ATP was completely counteracted by DCCD, an inhibitor of the F₁F₀ ATPase (Fig. 1b).

The idea that the effect of bovicin HC5 on intracellular ATP was being mediated via the F₁F₀ ATPase rather than efflux per se was further supported by the effect of the protonophore TCS (Fig. 3). TCS caused an even more rapid decline in ATP than bovicin HC5. This decrease also could be counteracted by DCCD, but the kinetics were different from those observed with bovicin HC5. When the action of bovicin was counteracted by DCCD (Fig. 1b), the level of ATP stayed on a plateau, but eventually it decreased if DCCD was added to TCS-treated cells (Fig. 3). These results indicate that TCS-mediated proton influx was greater than the influx caused by the dosage of bovicin HC5 that we used, and that DCCD was not able to completely inhibit the F₁F₀ ATPase if the influx of protons was great.

The effect of TCS on intracellular potassium is consistent with the observation that the low-affinity potassium transport system of lactic acid bacteria is a reversible potassium sym-

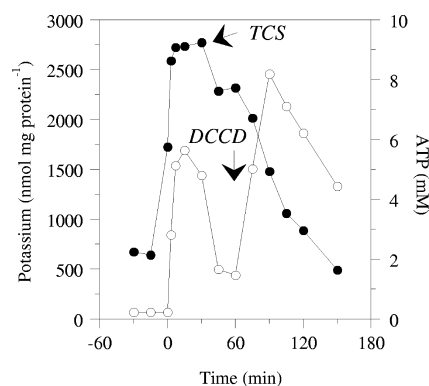


FIG. 3. Ability of nongrowing, glucose-energized *S. bovis* JB1 cells to accumulate and maintain potassium (filled circles) and ATP (open circles). Glucose (22 mM) was added at time zero. The protonophore TCS (10 μM) was added at 45 min. The F₁F₀ ATPase inhibitor DCCD was added at 60 min. *S. bovis* JB1 cells were not exposed to bovicin HC5.

porter (10). Because bovicin-treated *S. bovis* JB1 cells retained their ability to ferment glucose and the decreases in intracellular ATP could be counteracted by DCCD, it is unlikely that bovicin HC5 was catalyzing a significant efflux of ATP. The ability of bovicin HC5 to inhibit sensitive bacteria can be explained by its ability to translocate potassium, and it may be similar to enterocin P, a bacteriocin that forms specific, potassium ion-conducting pores (11).

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