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A Bacteriocin of *Actinobacillus actinomycetemcomitans*

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An inhibitory factor from *Actinobacillus actinomycetemcomitans* Y4 was isolated, and its properties indicated that it was a bacteriocin (actinobacillicin). The bacteriocin was active against *Streptococcus sanguis* strains, *Streptococcus uberis* (FDC1), and *Actinomyces viscosus* T14 as well as other strains of *A. actinomycetemcomitans*, but not against other crevicular bacteria, including other streptococci and actinomycetes. The activity of this bacteriocin was inhibited by pronase, trypsin, and heat (45 min at 56°C) but not by DNase, RNase, phospholipase, exposure to UV light, or low pH (1.0 to 6.5). Although actinobacillicin markedly inhibited glycolysis in *S. sanguis*, the primary mechanism of its bactericidal action appears to be alterations in cell permeability, with the resultant leakage of RNA, DNA, and other essential intracellular macromolecules. These findings provide an ecologic explanation for the reciprocal growth relationship between *A. actinomycetemcomitans* and *S. sanguis*/*Actinomyces viscosus* observed in localized juvenile periodontitis.

The cytotoxic potential of *Actinobacillus actinomycetemcomitans* for eucaryotic cells is well known. The leukotoxin (1), the factors inhibiting the growth of gingival fibroblasts (23) and endothelial (26) and epithelial cells (11), and the potent toxicity of the endotoxin (12) all provide compelling evidence that *A. actinomycetemcomitans* is remarkably toxic to other cells. Many investigators have speculated that these products are responsible, in part, for the ability of this organism to produce rapid peridontal breakdown in localized juvenile periodontitis (LJP) (28).

One might reasonably ask whether this toxic potential extends to procaryotic cells and, in particular, to the microflora of the gingival crevice, where *A. actinomycetemcomitans* colonizes during episodes of periodontal disease. Virtually nothing has been reported about this aspect of the ecology of *A. actinomycetemcomitans* apart from two preliminary reports (B. F. Hammond, R. H. Stevens, P. Bonner, and S. E. Lillard, J. Dent. Res. 63A, abstr. 830, p. 263, 1984; B.F. Hammond, R. H. Stevens, and S. E. Lillard, J. Dent. Res. 64A, abstr. 726, p. 255, 1985). Yet the clinical observations about the clear reductions of supragingival plaque in LJP are so well known as to be included in textbook descriptions of LJP as a disease entity (14). Mention is often made of the excellent oral hygiene status of LJP patients (14), indicating minimal amounts of supragingival plaque. Another study showed differences in subgingival plaque in LJP associated with *A. actinomycetemcomitans* and in other disease states (13).

One of the specific ecologic relationships of *A. actinomycetemcomitans* that has been noted repeatedly in clinical studies but never explained fully is its reciprocal relationship with *Streptococcus sanguis* and *Actinomyces viscosus* (16, 29). Both of these gram-positive bacteria fail to colonize crevicular areas after the establishment of *A. actinomycetemcomitans* but when the actinobacillus is eliminated or greatly reduced by tetracycline therapy, there is subsequent recolonization of these areas by *S. sanguis* and *Actinomyces viscosus*. Hillman and Socransky (10) described the reverse phenomenon of *A. actinomycetemcomitans* being inhibited by *S. sanguis* and *Actinomyces viscosus*, concluding that certain plaque-associated bacteria prevent infection by putative pathogens such as this actinobacillus. However, this observation does not answer the question of why *S. sanguis* and *Actinomyces viscosus* lose out in competition with *A. actinomycetemcomitans* (16). It is known, for example, that *A. actinomycetemcomitans* in some instances may account for as much as 70% of the crevicular flora in LJP lesions. Although the data presented here do not rigorously exclude an adherence-related explanation, it is unlikely that adherence plays a major role, since the inhibited organisms are already there.

We propose the explanation that *A. actinomycetemcomitans* produces in vivo an extracellular factor, a bacteriocin initially designated actinobacillus inhibitory factor, that is directly toxic to these two major plaque formers (*S. sanguis* and *Actinomyces viscosus*) in minute quantities. Evidence is also presented that this bacteriocin produces alterations in the cell permeability of target bacteria, with resultant leakage of RNA, DNA, and other essential intracellular macromolecules and cofactors.

MATERIALS AND METHODS

Microorganisms. Laboratory strains of *A. actinomycetemcomitans* and other oral bacteria listed in Table 1 were obtained from our laboratory collection and from the Forsyth Dental Center (FDC) collection. Cells were grown and harvested as described previously (9), and identification of new *A. actinomycetemcomitans* isolates was done by the procedures of Hammond and Stevens (9) and Slots (21). Other oral bacteria were grown as described by Tanner et al. (27).

Assays for inhibitory activity of the actinobacillus bacteriocin. (i) Inhibition of growth on agar plates. Plates of tryptic soy agar (Difco) were inoculated with 0.1 ml of an 18-h tryptic soy broth culture of *S. sanguis* ATCC 10556 and spread over the surface of the plate with a sterile cotton swab. Wells (4 mm diameter) were then cut, and samples of the actinobacillus sonic extract (or other more purified preparations with suspected bacteriocin activity) were placed into the wells. The sonic extracts were prepared as described previously (23). Plates were placed at 4°C for 6 h to allow diffusion of the inhibitory factor into the agar and then placed in the incubator for 18 to 24 h at 37°C. Clear zones surrounding the wells were measured as an indicator of bacteriocin activity. Similar results were obtained by the

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TABLE 1. Sensitivity of various oral bacteria to the Y4 bacteriocin

Organism	Strain	Sensitivity
Actinobacillus actinomycetemcomitans	Y4	−
	627	+
	511	+
	29523	+
	KB1	+
	29522	+
	N27	+
	652	+
	651	+
	29524	−
	650	−
	2112	−
Bacteroides gingivalis	381	−
Bacteroides intermedius	Lab strain B1	−
Bacteroides melaninogenicus	Lab strain BM	−
Capnocytophaga gingivalis	FDC 27	−
Capnocytophaga ochracea	FDC 25	−
Capnocytophaga sputigena	FDC 4	−
Eikenella corrodens	FDC 10M	−
Fusobacterium nucleatum	FDC 364	−
Neisseria sicca	Lab strain	−
Veillonella alkalescens	ATCC 17745	−
Wolinella recta	FDC	−
Actinomyces viscosus	T14V	+
Actinomyces israelii		−
Actinomyces naeslundii	ATCC 12104	−
Lactobacillus casei	L324M	−
Rothia dentocariosa	ATCC 14191	−
Streptococcus mutans	6715	−
Streptococcus salivarius	18BH	−
Streptococcus sanguis	ATCC 10556	+
	G9B	+
	ATCC 10557	+
	M5	+
Streptococcus uberis	FDC #1	+

Hillman et al. (10) modification of the overlay technique of Fredericq. In certain cases 18-h cultures of *Actinomyces viscosus* T14V were the indicator organisms, and these organisms were grown as described previously (4). In other cases the organisms listed in Table 1 were used as indicator organisms. In an effort to determine the location of the bacteriocin in growing cultures, two samples of supernatant fluid from 72-h cultures of *A. actinomycetemcomitans* (strains Y4 and 627) were examined. Extracellular vesicle preparations obtained from the clarified 100,000 × *g* fraction of a Y4 broth culture (19) were examined by the well-plate technique (10 μg/ml per well). The second and more direct demonstration of the extracellular location of the bacteriocin was determined by using a 10,000 × *g* supernatant of a 72-h culture of *A. actinomycetemcomitans* 627 which was concentrated 200 times by lyophilization. A drop of this material was added to wells in a plate as described above. Control wells with dialyzed and undialyzed concentrates of the same broth (uninoculated) as well as the 627 sonic extract (10 μg/ml) were run simultaneously.

(ii) Inhibition of glycolysis. To obtain a more quantitative determination of bacteriocin activity, we took advantage of the homofermentation of glucose by this strain of *S. sanguis* and reasoned that this property should provide a quantitative measure of cell metabolism. Washed cells of an 18-h culture of *S. sanguis* were adjusted to a turbidity of 100 on the Klett Summerson photoelectric colorimeter (660 filter) and added to a reaction mixture containing glucose (100 μg/ml) and sodium phosphate-buffered saline (0.015 M) at pH 7.4. The final volume of the reaction mixture was 2.0 ml. The stoppered reaction mixture was incubated in a 37°C waterbath for 1 h, and samples were removed from duplicate tubes at various intervals. Residual glucose was measured by the colorimetric procedure of Somogyi (22) and lactic acid by the method of Barker and Summerson (2). In every instance inhibition was directly proportional to the amount of bacteriocin added, either as crude sonic extracts or partially purified preparations (see Fig. 4).

Partial purification of bacteriocin. Sonic extracts (22) of washed cells were centrifuged at 100,000 × *g* for 60 min, and the lyophilized supernatant was reconstituted to 2 ml with 0.1 M phosphate-buffered saline, pH 7.4, before application to a column of Sephadex G-100 (100 by 2.0 cm) previously equilibrated with the same buffer. Column fractions were monitored for bacteriocin activity by the well-plate technique (see above), and all fractions showing bacteriocin activity were pooled and dialyzed against distilled H_2O and lyophilized for future testing.

Effect of bacteriocin on permeability of *S. sanguis*. Saline-washed 18-h cells of *S. sanguis* ATCC 10556 were exposed to crude sonic extracts of *A. actinomycetemcomitans* (strain Y4 or 627) or partially purified preparations of the bacteriocin (see below) for periods of up to 1 h. The reaction mixtures contained Tris hydrochloride buffer, pH 7.8, or Tris buffer containing 0.5 M sucrose, *S. sanguis* cells standardized to a turbidity of 100 Klett units (660 filter), and approximately 10 to 50 μg (protein) of the partially purified material or 750 μg (protein) of the crude sonic extract. Controls included (i) heat-inactivated (56°C for 1 h) sonic extract or partially purified preparation and (ii) *S. sanguis* cells without the actinobacillus extracts or partially purified bacteriocin preparation. Samples were removed at 15-min intervals, and after centrifugation the supernatants were analyzed for RNA (20) and DNA (5). The centrifuged *S. sanguis* cells remaining at the end of the incubation period were examined by phase contrast microscopy for protoplast formation, ghosts, and intact cells with a Leitz phase contrast microscope (oil immersion objective).

RESULTS

Production and general properties of the bacteriocin. *A. actinomycetemcomitans* Y4 was the first strain to be examined for its ability to inhibit the growth of *S. sanguis*. Crude extracts of strain Y4 were clearly inhibitory to the growth of *S. sanguis* ATCC 10556 on agar plates (Fig. 1). Essentially identical results were observed when lawns of *Actinomyces viscosus* or *S. uberis* were used instead of *S. sanguis*. A number of other strains of this actinobacillus were also sensitive to the inhibitory action of the Y4 extract, whereas other indigenous oral bacteria, including representatives of the genera *Bacteroides*, *Capnocytophaga*, *Fusobacteria*, *Wolinella*, *Lactobacillus*, *Eikenella*, *Veillonella*, *Rothia*, and *Neisseria* were not (Table 1). It was also interesting that except for *S. uberis* and the three other strains of *S. sanguis*, other streptococci (*S. mutans* and *S. salivarius*) and other *Actinomyces* (*A. naeslundii* and *A. israelii*) were also not inhibited.

Washed sensitive cells of either *Actinomyces viscosus* or *S. sanguis* were killed by exposure to crude sonic extracts of strain Y4, and the percent inhibition was dose dependent (Fig. 2). In contrast, the negative control, *S. mutans* ATCC 6715, showed no inhibition at any concentration of Y4 extract. The inhibitory activity was lost after exposure to heat for 30 min at 56°C but retained after 10 min at 56°C and

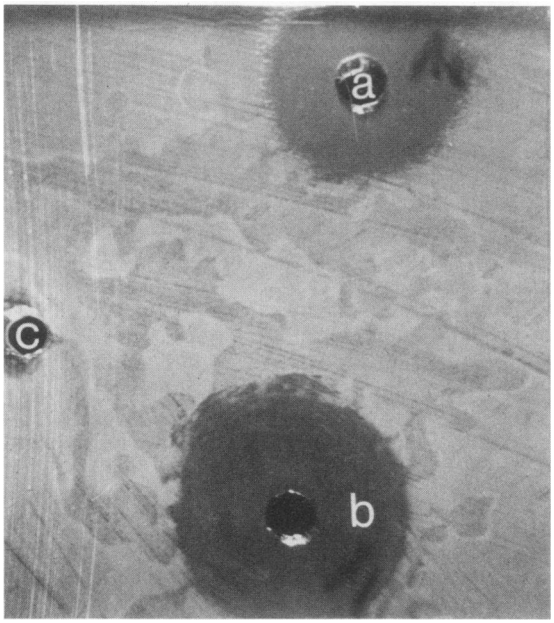

FIG. 1. Growth inhibition of *S. sanguis* ATCC 10556 lawn by crude sonic extracts of *A. actinomycetemcomitans* Y4. (a) 100 μg of protein per ml; (b) 250 μg of protein per ml; (c) heat-inactivated (56°C for 30 min). All wells contained approximately 0.5 ml of sonic extract.

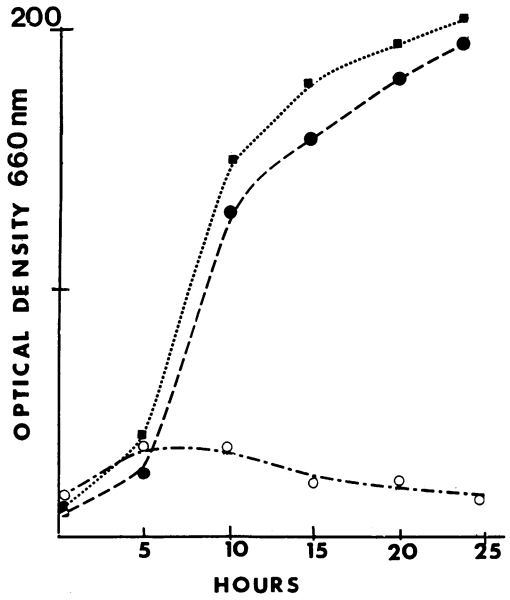

FIG. 3. Effect of *A. actinomycetemcomitans* Y4 crude sonic extract (250 μg of protein per ml) on the growth of *S. sanguis* ATCC 10556 in tryptic soy broth measured turbidometrically over 24 h (Klett Summerson photoelectric colorimeter). Symbols: ●, *S. sanguis* alone (control); ■, *S. sanguis* with heat-inactivated sonic extract; ○, *S. sanguis* with Y4 sonic extract.

lost after exposure to proteolytic enzymes (pronase or trypsin at either 0.1 or 1%). Controls of the enzymes alone at either 0.1 or 1% did not inhibit growth of the indicator organisms in the bacteriocin plate assay. The activity was not affected by exposure to UV light (253.7 nm for 20 min at a distance of 12 in. [ca. 30 cm], phospholipase C and D (1 mg/ml), and DNase and RNase (Sigma) at 1 mg/ml and at low pH (1.0 to 6.5); the activity was not lost after extended dialysis.

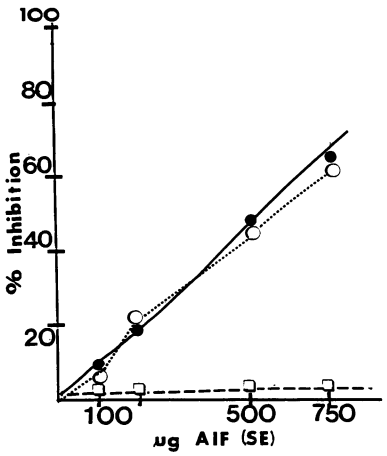

FIG. 2. Dose-activity relationship of *A. actinomycetemcomitans* Y4 crude sonic extract and inhibition of growth of *S. sanguis* ATCC 10556 (●), *Actinomyces viscosus* T14V (○), and *S. mutans* ATCC 6715 (□). Washed cells (18-h culture) were exposed to various concentrations of crude sonic extract for 30 min, after which dilutions of the exposed cells were plated on brain-heart infusion broth (Difco), and the percent inhibition was determined by comparison with identical dilutions of control (nonexposed) cells. Heat-inactivated sonic extracts had no effect on the growth of any of the target cell populations. AIF, Actinobacillus inhibition factor; SE, sonic extract.

When inhibition was checked with growing cells in liquid medium, a clearcut difference in the growth of *S. sanguis* cells was observed (Fig. 3). Minimal growth occurred in the presence of 100 μg of the crude extract containing the bacteriocin per ml, whereas the controls showed luxuriant growth in the absence of the bacteriocin or with the heat-inactivated preparation.

The bacteriocin had an extracellular location as well. Both the untreated extracellular vesicle preparation of strain Y4 suspended in saline (10 μg/ml) (19) and a concentrated broth supernatant of strain 627 (72-h culture) showed zones of inhibition (ca. 10 cm) in lawns of *S. sanguis* ATCC 10556 in the well-plate assay. The control wells containing uninoculated broth supernatant concentrates were negative.

Mechanisms of action: inhibition of glycolysis. In an effort to determine the effect of the actinobacillus bacteriocin on the metabolism of *S. sanguis* target cells, differences in glycolytic rate were checked in the presence and absence of a partially purified preparation of the bacteriocin. The lyophilized pooled fractions from a Sephadex G-100 column were indeed active in inhibiting both the growth and glycolytic rate of *S. sanguis*. The inhibition of glycolysis in the presence of the G-100 pooled fractions (15 μg of protein per ml) was obvious after 30 min (Fig. 4). At 60 min there was roughly a 60% inhibition of glycolysis compared with control cells (only glucose substrate present or substrate coupled with heat-inactivated G-100 fractions). When these experiments on the glycolytic rate were repeated with crude sonic extracts (75 μg of protein per ml) of *A. actinomycetemcomitans*, essentially similar results were obtained. Moreover, the amount of crude material needed was so little that inhibition of glycolysis became the standard assay procedure for bacteriocin activity used in conjunction with the agar plate-well technique. The correlation has been complete and

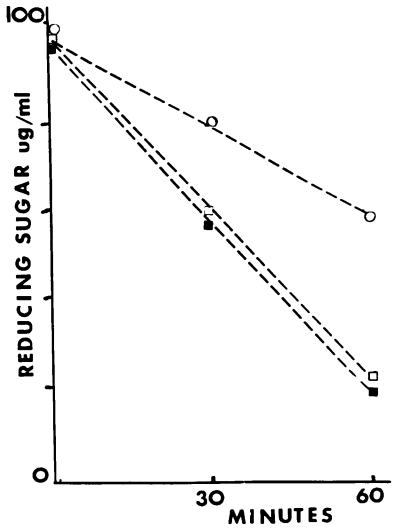

FIG. 4. Glycolysis inhibition of *S. sanguis* ATCC 10556 by *A. actinomycetemcomitans* Y4 sonic extract. The reaction mixture contained glucose, phosphate-buffered saline, washed 18-h cells of *S. sanguis* and strain Y4 sonic extract (75 μg of protein per ml; Sephadex G-100 column fraction). The final volume of the reaction mixture was 2.0 ml. Symbols: ○, cells with Y4 sonic extract; □, cells with heat-inactivated Y4 sonic extract; ■, control *S. sanguis* cells in buffer and glucose.

consistent: whenever bacteriocin activity was detected by growth inhibition on agar plates, the same preparation inhibited glycolysis in *S. sanguis*.

Effect of bacteriocin on permeability of *S. sanguis*. A number of preliminary experiments showed that exposure of resting *S. sanguis* cells to the actinobacillus bacteriocin resulted in a continuous increase in soluble materials, with an absorption maximum of 260 to 280 nm. To test the possibility that this bacteriocin disrupted membrane function, experiments were designed to monitor the possible release of intracellular macromolecules and determine whether bacteriocin activity would induce protoplast formation in an osmotically protected environment.

The release of RNA from washed *S. sanguis* cells after exposure to the bacteriocin is shown in Fig. 5. In the presence of regular Tris hydrochloride buffer alone (0.01 M), there was continuous release of RNA over the 60-min period, whereas in an osmotically protected environment (addition of 0.5 M sucrose to the Tris buffer) the release of both macromolecules was decreased by 50% or greater. The four controls for both experiments (*S. sanguis* cells alone under both osmotic environments and *S. sanguis* plus heat-inactivated bacteriocin under both osmotic environments) were all negative, showing no detectable release of RNA. When identical experiments were performed to check for DNA release, essentially similar results were obtained. These data indicate that in the absence of osmotic protection (0.5 M sucrose), *S. sanguis* cells exposed to the bacteriocin release intracellular DNA and RNA.

Phase contrast micrographs of control cells and osmotically protected cells showed clearly that protoplasts were formed when the bacteriocin was present. The protoplasts were the classic wall-less forms, much less dense than control cells and with a much less regular outline. Unprotected cells in the presence of the bacteriocin were extremely difficult to visualize unless the reaction mixture was centrifuged and the sediment examined. Most of the sedimented material resembled debris, and although there were some intact cells, the morphological differences between them and the osmotically protected cells were quite obvious. The overall impression was that the bacteriocin had markedly changed the surface architecture of these streptococcal cells and that other metabolic changes were subsequently triggered by this alteration(s) (leakage of intracellular macromolecules, reduced glycolytic rate).

DISCUSSION

The *Actinobacillus* inhibitory factor is a protein that satisfies most of the major criteria used to define a bacteriocin. Accordingly, this factor might be more appropriately designated an actinobacillicin, in keeping with the convention in bacteriocin nomenclature of adding the suffix -cin to the genus or species name of the producing organism. It is a nondialyzable protein with a bactericidal mode of action; it has a relatively narrow spectrum of activity centered around the homologous producer species (25), although it is active against two other taxonomically remote species, *Actinomyces viscosus* and *S. sanguis* and *S. uberis*. In its sensitivity to various chemical (proteolytic enzymes) and physical agents (56°C for 30 min), its pattern is not unlike that of a large number of other bacteriocins, and in its activity against the two taxonomically remote organisms described here it bears a marked similarity to most other bacteriocins of oral origin (8). It is distinct from the actinobacillus bacteriophage in its physical characteristics (24), spectrum of activity, sensitivity to trypsin, and relative insensitivity to UV light. Similarly, it is distinct from other factors produced by this organism that are cytotoxic for eucaryotic cells. Because it has no effect on leukocyte viability, a smaller molecular size, and comparative insensitivity to heat, it is distinct from the leukotoxin. Also, since the factor is also functionally distinct from the fibroblast cytotoxin (23) in having no effect on fibroblast proliferation (unpublished data) and because of its much higher molecular weight, it is also separate from the fibroblast inhibitory factor (R. H. Stevens, C. Gatewood, and B. F. Hammond, J. Dent. Res. 63, abstr. 1202, p. 263, 1984). The genetic basis of its production has not yet been identified, and the mechanism of attachment to specific cell receptors is unknown.

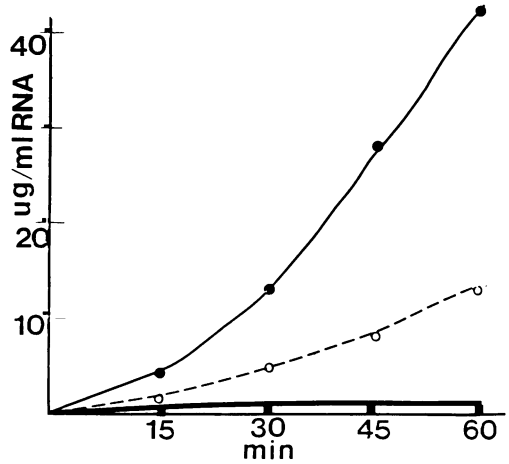

FIG. 5. RNA released from *S. sanguis* ATCC 10556 in the presence of Y4 sonic extract and Tris buffer (●), in the presence of Y4 sonic extract and 0.5 M sucrose (○), and controls (■), which included heat-inactivated Y4 sonic extract and no Y4 sonic extract in Tris buffer or 0.5 M sucrose.

Many of the characteristics of this bacteriocin are consistent with an ecological function. It is active against resting and growing cells of two specific species of oral bacteria, *S. sanguis* and *Actinomyces viscosus*, which are known to have a reciprocal relationship in vivo with the producer bacterium, *A. actinomycetemcomitans*. As indicated elsewhere, one of the salient ecological features of human periodontal disease associated with this organism is the absence of these two target organisms at the disease site. The bactericidal nature of this factor could provide an easy explanation for this clinical observation. Moreover, the bacteriocin is active within a wide range of pHs; its activity is enhanced by normal human serum; it is active under aerobic and anaerobic conditions; and it is extracellular. A preliminary study has recently shown that the bacteriocin is active in the presence of saliva or crevicular fluid and can be demonstrated directly in human subgingival plaque by serologic methods (B. F. Hammond, S. E. Lillard, and R. H. Stevens, J. Dent. Res. 65, abstr. 484, p. 223, 1986).

The bactericidal mechanisms of action appears to be related to its effect on the cell surface. Presumably, there is some muralytic activity associated with this biological protein, resulting in the formation of osmotically fragile protoplasts that leak essential macromolecules such as RNA, DNA, and proteins. Thus, actinobacillicin may have a mode of action similar to colicin M (3) and pesticin (6, 7). The inhibition of glycolysis by *S. sanguis* cells is probably an indirect effect of the leakage of metabolic enzymes, cofactors, and related metabolic machinery. Thus, glycolysis (measured as the increase in lactic acid) is not appreciably affected in cells exposed to lethal amounts of bacteriocin as long as the cells are osmotically protected. The formation of protoplasts that show very little leakage of RNA, DNA, and protein while protected osmotically also points to the fact that the bacteriocin does not appear to damage the cell membrane directly.

It is not clear what ecological advantage to the human host would derive from the in vivo activity of this bacteriocin, because *S. sanguis* is often considered an indicator of periodontal health, i.e., *S. sanguis* may prevent colonization or recolonization of some periodontopathic organisms such as *A. actinomycetemcomitans*. It could also be argued that one of the strategies for successful replacement therapy for or prevention of periodontal disease would be to maintain populations of *S. sanguis* and eliminate any ecological threat (e.g., bacteriocins) to its maintenance. In any event, one of the characteristic features of ecology is that balancing mechanisms must exist in all ecosystems to maintain a natural equilibrium. Since a large majority of human oral *A. actinomycetemcomitans* isolates possess the bacteriocin trait (Hammond, unpublished data), it is reasonable to assume that it has some advantage in nature—possibly as some kind of ecological control mechanism. Studies are in progress to determine a more precise role for this bacteriocin in human oral ecology.

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