INFECTION AND IMMUNITY, Mar. 1987, p. 692–697
0019-9567/87/030692-06$02.00/0
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Vol. 55, No. 3

Purification and Biochemical Properties of a Bacteriocin from *Actinobacillus actinomycetemcomitans*

R. H. STEVENS,* S. E. LILLARD, AND B. F. HAMMOND

Department of Microbiology, School of Dental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Received 28 August 1986/Accepted 21 November 1986

Extracts of certain strains of *Actinobacillus actinomycetemcomitans* are inhibitory to strains of *Streptococcus sanguis* such as *S. sanguis* ATCC 10556. The isolation of a protein from an *A. actinomycetemcomitans* sonic extract which copurified with the inhibitory activity was accomplished by preparative isoelectric focusing, Sephadex G-100 gel filtration chromatography, and preparative polyacrylamide gel electrophoresis (PAGE). The resulting isolated protein, which focused at a pH of 6.1 to 6.3, appeared as a single band in anionic nondissociating PAGE analysis. This protein could be dissociated into two subunits with molecular weights of 50,000 and 70,000, which were resolvable by PAGE analysis. A 1,758-fold increase in specific activity was seen in the purified inhibitory protein compared with the crude sonic extract starting material. The properties of the inhibitory activity in the *A. actinomycetemcomitans* extract are characteristic of a bacteriocin. Accordingly, we propose the name actinobacillicin for the inhibitory protein.

The characteristic alterations in the dental plaque microflora which are seen in the various forms of periodontal disease have been the subject of numerous studies (20–24, 29, 30, 34, 39). The results of these studies indicated that one or more bacterial species which are either absent or present in low numbers in plaque at periodontally healthy sites may appear and increase to significant numbers at periodontally diseased sites. Conversely, other species which are routinely found to compose a high proportion of the microflora in plaque taken from healthy sites may decrease in number or even disappear in plaque taken from sites of periodontal disease. Nowhere is there a better example of these dynamics in plaque microbial populations than in the case of juvenile periodontitis (JP).

Numerous studies have indicated that a high proportion (90 to 100%) of JP patients harbor *Actinobacillus actinomycetemcomitans* (4, 18, 32, 41), although more recently some conflicting data have been reported by other investigators (25, 38). In patients harboring *A. actinomycetemcomitans*, the organism has been reported to account for a significant (3 to 15%) proportion of the microbial flora (17, 18, 22–24). In comparison, *A. actinomycetemcomitans* is found relatively infrequently in plaque from periodontally healthy individuals and, when detected, accounts for a small proportion of the total microbial flora (23, 32, 33, 41). At the same diseased sites where the emergence of one microorganism (*A. actinomycetemcomitans*) is seen, there appears to be a concomitant diminution in the numbers of other microorganisms, such as *Streptococcus sanguis*, which normally accounts for a significant proportion of the plaque flora at periodontally healthy sites (22, 23, 40). Population shifts such as these are not merely coincidental, but frequently the result of interactions between the different microbial populations competing within the same ecological niche (2). With this in mind, we explored the possibility that the observed reciprocal relationship between *A. actinomycetemcomitans* and certain species in the normal plaque population might be the result of an antibiotic-mediated antagonism (5; B. F. Hammond, R. H. Stevens, P. Bonner and S. E. Lillard, J. Dent. Res. 63, abstr. 830, p. 263, 1984). In a companion paper (6) we report the existence of a bacteriocinlike actinobacillus inhibitory factor (AIF) in extracts of strains of *A. actinomycetemcomitans*, which is bacteriocidal for strains of *S. sanguis* and *Actinomyces viscosus*. The present study is concerned with the isolation and physicochemical characterization of the active substance responsible for the observed effects.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *A. actinomycetemcomitans* 511 was originally isolated from a JP patient at the Forsyth Dental Center and kindly provided by S. Socransky. This strain was routinely grown at 37°C to the early stationary phase in a modified fluid thioglycolate broth (0.75% dextrose, 0.5% yeast extract, 1.5% trypticase peptone, 0.25% NaCl, 0.075% L-cysteine, 0.05% sodium thioglycolate, 0.4% $NaHCO_3$). The indicator strain, *S. sanguis* ATCC 10556, was grown at 37°C in BHI broth.

A sonic extract (SE) was prepared from washed strain 511 cells as described previously (36). The SE was subjected to ultracentrifugation at 100,000 × *g* for 1 h, and the resulting supernatant (SE 100,000 × *g* supernatant) was collected, dialyzed against distilled water at 4°C, and lyophilized.

Detection of AIF activity. The AIF activity was detected by one of the two methods described previously (6). Briefly, the first method consisted of placing the sample to be tested in wells formed in BHI agar plates which had previously been coated with a broth culture of the indicator organism (*S. sanguis*). After 5 to 6 h at 4°C to allow diffusion from the wells, the plates were shifted up to 37°C overnight and then examined for zones of inhibition in the lawn of the indicator cells. Alternatively, washed cells of the same indicator strain were added to a reaction mixture (2 ml) containing the test material and glucose (100 μg/ml of 0.015 M sodium phosphate-buffered saline, pH 7.4). The mixture was then incubated at 37°C for 1 h, and the residual glucose was measured by the Somogyi procedure (35) and lactic acid production was assayed by the method of Barker and Summerson (1). A decrease in the glucose utilization or lactic acid production in challenged compared with control cells was considered a measure of inhibitory activity.

AIF isolation. (i) IEF. The preparative isoelectric focusing (IEF) procedure used was a modification of the method

* Corresponding author.

described by Winter et al. (LKB Application Note 198 [revised], LKB Produkter A.B., Bromma, Sweden). Portions (200 mg, dry weight) of the lyophilized SE 100,000 × *g* supernatant were resuspended in 6 to 10 ml of 0.5% glycine. In some cases, 10% (wt/vol) dodecyl trimethyl ammonium bromide (DTAB) was added to a final concentration of 0.1%. The addition of the cationic detergent (DTAB) was made as a result of our previous observations that proteins in lyophilized bacterial extracts often exist as aggregated polymers (unpublished data). The detergent was used to disaggregate the protein polymers prior to application of the SE 100,000 × *g* supernatant to the preparative IEF gel. It was found that DTAB had the additional virtue of precipitating the contaminating nucleic acid from the extract while leaving the proteins in solution (unpublished observations). The sample, including the precipitate which formed after addition of the DTAB, was transferred to a dialysis membrane and dialyzed against 0.5% glycine overnight at 4°C. The DTAB was subsequently eliminated from the SE 100,000 × *g* supernatant since (i) much of the detergent was precipitated with the nucleic acid and removed by centrifugation (see below), (ii) any unprecipitated detergent was dialyzed out of the SE preparation prior to IEF, and (iii) any DTAB remaining in the SE 100,000 × *g* supernatant following dialysis was electrophoresed towards the cathode end of the IEF gel whereas the strain 511 proteins (as well as the AIF activity) focused towards the anodal end of the gel. After dialysis, any precipitate in the SE 100,000 × *g* supernatant preparation was removed by centrifugation (12,000 × *g* for 10 min) and the sample was concentrated down to a volume of 3 ml with a Minicon B concentration cell (Amicon Corp., Danvers, Mass.). The concentrated sample was applied to a partially dried Ultrodex (LKB Inc., Gaithersburg, Md.) flat-bed IEF gel containing 5% pH 5 to 8 carrier ampholyte (Pharmacia, Inc., Piscataway, N.J.). Focusing was carried out for 14 to 18 h at 8°C with a constant power of 8 W, a maximum current of 18 mA, and a maximum potential of 1,400 V. At the conclusion of IEF a transfer blot of the gel surface was made, which was subsequently fixed (10% trichloroacetic acid, three washes), stained (0.2% Coomassie Brilliant Blue R-250 in destaining solution), and destained (destaining solution: 100 ml of methanol, 100 ml of acetic acid, 500 ml of distilled water) to detect the focused protein bands in the gel. After blotting, the gel was sectioned, and each gel section was collected and transferred to a centrifuge tube. The soluble material in each section was eluted by suspending the gel in distilled water. The gel was then removed from the eluate by centrifugation (12,000 × *g* for 10 min). Following pH measurement, the eluate from each gel section was buffered by the addition of 1/10 volume of 10× phosphate-buffered saline (7.65% NaCl, 0.72% Na_2HPO_4, 0.2% KH_2PO_4, pH 7.2), and a sample of each eluate was tested for AIF activity by the inhibition zone method described previously. AIF-active eluates of sections from multiple IEF runs were pooled, dialyzed against distilled water at 4°C, and lyophilized.

(ii) Gel filtration chromatography. A column (100 by 2.0 cm) of Sephadex G-100 was poured and equilibrated with Tris buffer (0.4 M Tris hydrochloride, pH 7.4). Portions (200 mg, dry weight) of the lyophilized AIF-active eluates from the preparative IEF gels were suspended in 0.5 ml of Tris buffer and applied to the G-100 column. The column fractions were monitored for AIF activity and absorbance at 280 nm. AIF-positive column fractions were pooled, dialyzed against distilled water, and lyophilized.

(iii) PAGE. For preparative polyacrylamide gel electrophoresis (PAGE), anionic, nondissociating polyacrylamide gels (7.5%) were cast in 5-mm-diameter tubes by the method described by Maizel (16). The lyophilized AIF material eluted from the G-100 column was suspended in anionic PAGE sample buffer (0.06 M Tris hydrochloride, pH 7.5) containing sucrose (10%) and phenol red tracking dye (0.01%) at 5 mg/ml. An equal amount (50 μl) of the AIF suspension was loaded onto each of 12 gels and electrophoresed for 1 h at 5 mA per gel in a water-cooled (10°C) tube gel electrophoresis apparatus. At the completion of electrophoresis one of the gels was stained for protein with Coomassie brilliant blue and a second gel was sectioned. Any protein in each of the sections from the second gel was electroeluted (see below). The eluates obtained from these gel sections were tested for AIF activity. The region of each of the 10 remaining unsectioned gels which corresponded to the section of the stained gel containing the AIF-active protein band was collected, and the protein was electroeluted. The electroeluted protein was dialyzed against distilled water and then lyophilized.

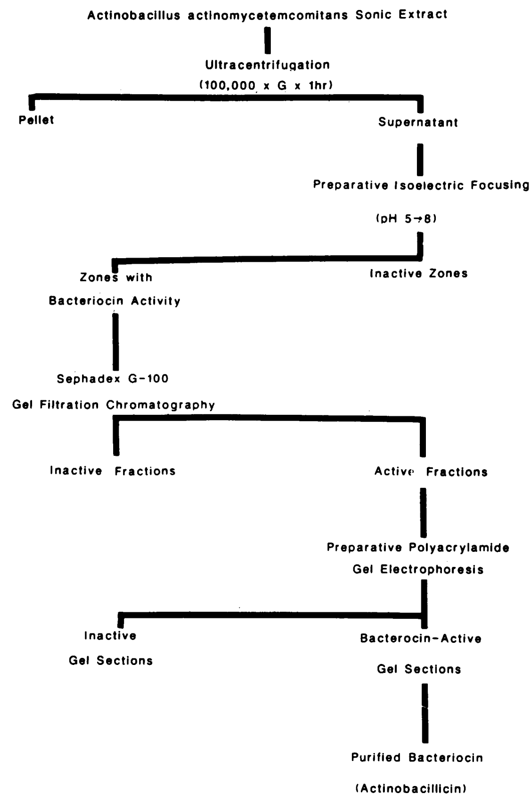

FIG. 1. Flow chart of AIF purification.

(iv) Electroelution of AIF from polyacrylamide gel. The recovery of the protein from the gels following preparative PAGE was accomplished by a modification of the electroelution procedures outlined by Maniatis et al. (19) for recovering DNA from agarose gels. The gel sections containing the AIF-active protein were placed in a dialysis membrane tube which was filled with elution buffer (100 mM Tris hydrochloride, pH 8.0) to the exclusion of any air prior to being sealed. The resulting sealed tube containing the gel sections was placed on a horizontal gel electrophoresis tray and oriented so that it was perpendicular to the path of the current which was subsequently applied across the tray. An 0.8% agarose

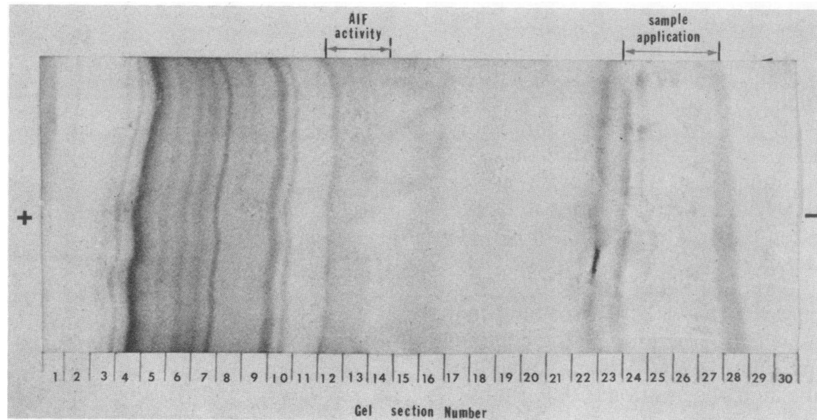

FIG. 2. Preparative IEF. A stained transfer blot of a preparative IEF gel of the crude strain 511 SE 100,000 × *g* supernatant is shown above. The gel in the section labeled sample application was removed from the tray, mixed with 3 ml of a strain 511 SE 100,000 × *g* supernatant suspension, and returned to the same position in the gel tray prior to focusing. The anode (+) is toward the left, and the gel sections having AIF activity are indicated above the blot. The pH gradient is shown in Fig. 3.

gel (agarose ME; FMC Corp., Rockland, Maine) in elution buffer was cast on the electrophoresis tray so that the only path of conduction between the anodal and cathodal ends of the gel was through the dialysis membrane tube. The electrophoresis tray was then placed across the glass cooling plate of a Multiphore electrophoresis unit (LKB, Inc.). A film of 0.1% Triton X-100 was placed on the cooling plate to ensure good heat transfer between the cooling plate and the electrophoresis tray. The buffer chambers of the Multiphore unit were filled with elution buffer so that the buffer contacted both the electrodes and the ends of the agarose gel. Electroelution of the proteins from the polyacrylamide gel sections was carried out for 2.5 h at 100 V with a temperature of 8°C being maintained in the cooling plate. During the final 2 min of electrophoresis, the polarity was reversed to remove the eluted protein from the wall of the dialysis membrane. Following electroelution, the buffer within the dialysis membrane tube was collected, dialyzed against distilled water, and then lyophilized.

Analytical PAGE. At each step of the purification, samples were solubilized in 2% sodium dodecyl sulfate (SDS)–0.5 M urea–1% mercaptoethanol and then analyzed by SDS-PAGE in 8% polyacrylamide–SDS gels as described by Maizel (16).

Protein determinations. Protein measurements were made with a dye binding assay (BioRad Laboratories) according to the instructions of the manufacturer.

RESULTS

The procedures used in purifying the AIF from the crude strain 511 SE 100,000 × *g* supernatant are outlined in Fig. 1. The initial purification was accomplished by preparative IEF. This procedure had the dual advantages of allowing relatively large sample sizes to be run and achieving a high degree of purification in a single step. The banding pattern of the focused *A. actinomycetemcomitans* proteins in the preparative gel can be seen in the transfer blot shown in Fig. 2. It is evident from a comparison of this transfer blot and the pH profile of the gel (Fig. 3) that the overwhelming majority of the *A. actinomycetemcomitans* proteins focus in a pH range of 5.1 to 5.9. In contrast, the AIF activity localized in a discrete region of the gel (sections 12 to 14) having a pH of 6.1 to 62.5 (Fig. 3). When the AIF-active material which

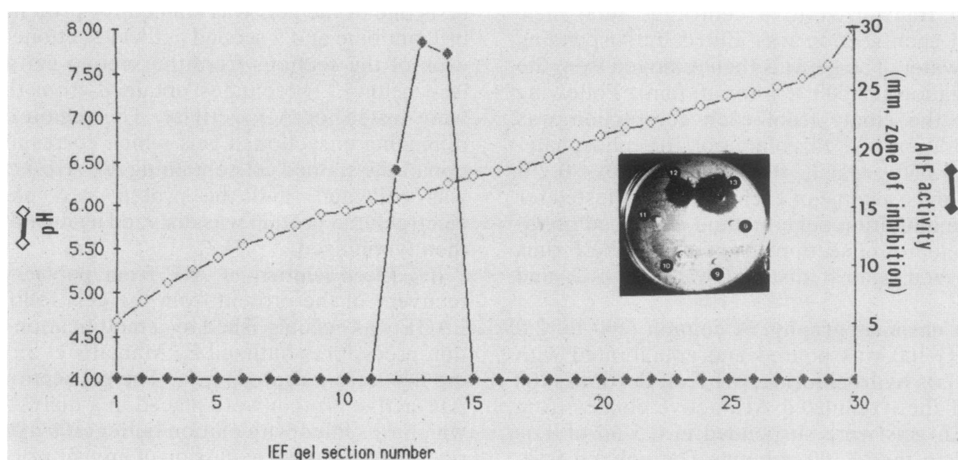

FIG. 3. pH and AIF activity profile of preparative IEF gel. The pH and AIF activity of the eluates of each IEF gel section were measured. Symbols: ◇, pH of gel section; ◆, AIF activity as measured by diameter of zone of inhibition in an *S. sanguis* lawn. Inset: AIF assay plate showing zones of inhibition in *S. sanguis* lawn around wells containing plates from IEF gel sections 12 and 13. No inhibition was produced by eluates from sections 8 to 11.

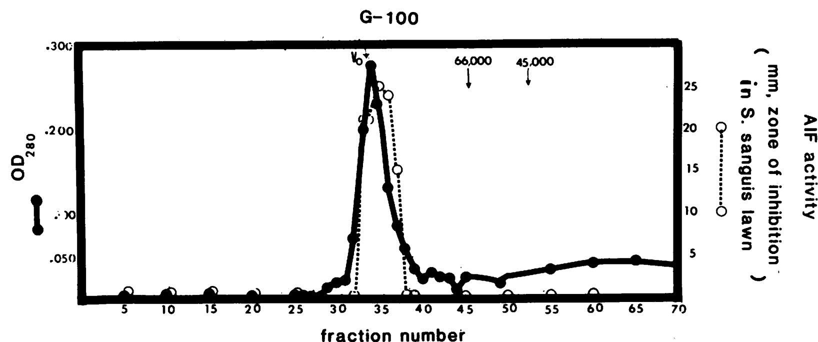

FIG. 4. Sephadex G-100 gel filtration chromatography. Lyophilized AIF-active material (200 mg, dry weight) eluted from the preparative IEF gel was suspended in 0.5 ml of Tris buffer and eluted with the same buffer through a Sephadex G-100 column (100 by 2 cm). The resulting fractions were monitored for absorbance at 280 nm (●) and AIF activity (○).

eluted from these sections of the preparative IEF gel was chromatographed through a Sephadex G-100 column, the AIF activity eluted as a single peak corresponding to a molecular weight (MW) in excess of 100,000 (Fig. 4). After electrophoresis (under nondissociating conditions) through an anionic polyacrylamide gel, the pooled, concentrated, AIF-active material collected from the G-100 column appeared as one intensely staining major band and four to five fainter, minor bands (Fig. 5A). The association of the AIF activity with the single major band in the gel is demonstrated in Fig. 6, in which the eluates of sections from a similar but unstained gel were tested for AIF activity. SDS-PAGE analysis of the denatured proteins of the AIF-active G-100 column fractions is shown in Fig. 5B. In addition to multiple faint bands, two distinctive, intensely staining bands of MWs 50,000 and 70,000 are evident.

An electrophoretically homogeneous preparation was achieved by preparative PAGE of the AIF-active material recovered from the G-100 column. Gel sections corresponding to the region of the AIF-active band in the stained gel (Fig. 6) were pooled and electroeluted. Analysis of the eluted material by PAGE under nondissociating conditions disclosed a single protein band (Fig. 7A); however, PAGE analysis of the same material under dissociating conditions revealed two protein bands of 50,000 and 70,000 MW (Fig. 7B). To verify that the bacteriocin activity resided with the isolated protein, the AIF specific activity of the protein was compared with that of the crude SE 100,000 × *g* supernatant starting material. A concentration of 39 μg of the crude SE protein per ml was required for 50% inhibition of an *S. sanguis* suspension (Table 1). In comparison, only 22 ng of the purified AIF protein per ml was sufficient to achieve the same inhibition. This is equivalent to a 1,758-fold increase in specific activity.

DISCUSSION

The data presented in this paper demonstrate that an activity present in crude extracts of *A. actinomycetemcomitans*, which is bactericidal for *S. sanguis*, copurified with a specific dimeric *A. actinomycetemcomitans* protein. The *A. actinomycetemcomitans* product responsible for this activity was designated actinobacillicin in an accompanying communication due to its numerous bacteriocinlike properties (6). This designation is further supported in the present paper by the identification of the bactericidal activity with a specific *A. actinomycetemcomitans* protein.

Our observation that the purified actinobacillicin can be dissociated into two constituent protein subunits is not

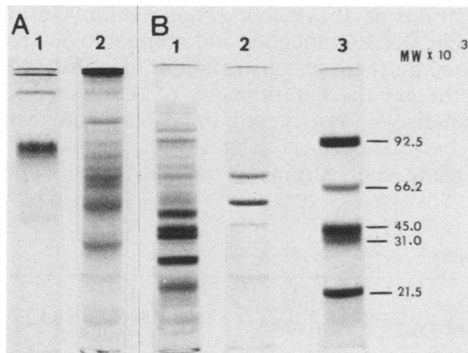

FIG. 5. PAGE analysis of partially purified strain 511 AIF eluted from G-100 column. (A) Nondissociating anionic PAGE. Lanes: 1, AIF (partially purified from G-100 column); 2, crude strain 511 SE 100,000 × *g* supernatant (starting material). (B) SDS-PAGE. Lanes: 1, crude strain 511 SE 100,000 × *g* supernatant (starting material); 2, AIF (partially purified from G-100 column); 3, MW standards: β-galactosidase (116,250), phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), and soybean trypsin inhibitor (21,500).

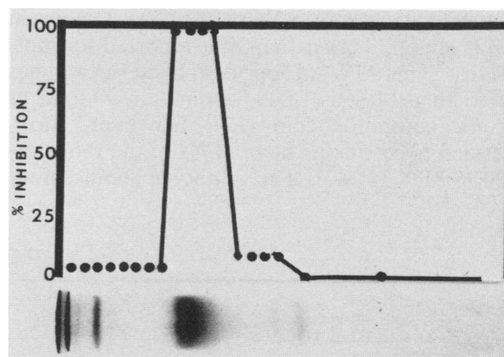

FIG. 6. Localization of AIF activity in a nondissociating anionic polyacrylamide gel. Samples of partially purified AIF (eluted from G-100 column) were loaded onto each of two nondissociating anionic polyacrylamide gels. Following electrophoresis, one gel was stained and the other was sectioned. Any protein in each section of the gel was eluted and assayed for AIF activity by measuring its effect on substrate utilization by an indicator cell (*S. sanguis*) suspension. The inhibitory effect of the eluate from each region of the gel is indicated.

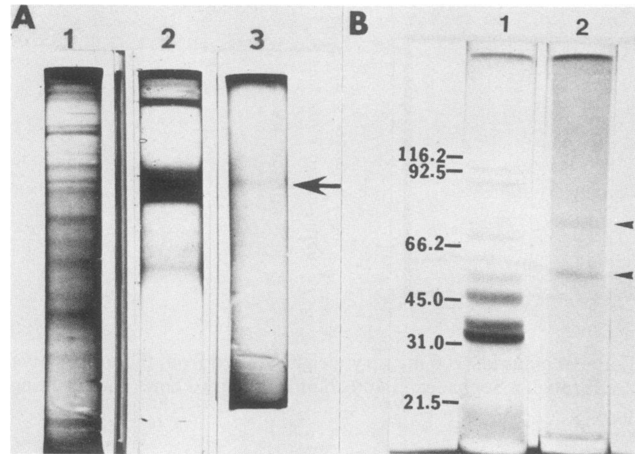

FIG. 7. PAGE analysis of purified 511 AIF eluted from preparative polyacrylamide gels. (A) Nondissociating anionic PAGE. Lanes: 1, crude strain 511 SE 100,000 × *g* supernatant (starting material); 2, AIF (partially purified from G-100 column); 3, AIF (purified from preparative PAGE). (B) SDS-PAGE. Lanes: 1, crude 511 SE 100,000 × *g* supernatant (starting material); 2, AIF (purified from preparative PAGE). MW (10^3) standards are described in the legend to Fig. 5.

without precedent among several well-studied bacteriocins such as colicins E2 and E3, cloacin DF13 (3, 10, 11, 28), colicin B (26), and pyocin AP41 (27). For colicin E2 and E3 and cloacin DF13, it has been shown that the bacteriocidal activity resides with the large protein subunit and the smaller protein acts by modulating the activity of the larger subunit. It is not known whether the same relationship exists between the two proteins composing the actinobacillicin.

The behavior of the actinobacillicin in gel filtration chromatography and SDS-PAGE may reflect the dimeric nature of the molecule. In its native state, the actinobacillicin may exist as a dimer consisting of two protein subunits with a combined MW of 120,000. This is consistent with its behavior in the G-100 column. Under dissociating conditions, the two protein subunits of 50,000 and 70,000 MW were resolvable in SDS-PAGE analysis.

With regard to the size of the actinobacillicin, it should be noted that the aggregate MW of the two protein subunits (120,000) is slightly higher than that reported for most other bacteriocins. The MW of purified bacteriocins has been reported to be as little as 1,000 for mutacin (8) to as much as 150,000 for staphylococcin (12). However, most well-characterized bacteriocins have MWs in the range of 30,000 to 80,000 (7, 13, 14, 37). The actinobacillicin subunits are similar in size to the larger subunits of colicins E2 and E3 (50,000 [10, 11]), colicin E1 (55,000; S. A. Schwartz and D. R. Helinski, Abstr. Ann. Meet. Am. Soc. Microbiol. 1968, abstr. GP42, p. 53), the larger subunit of cloacin DF13 (58,000 [3]), pesticin (65,000 [9]), colicin Ia (77,600 [15]), and colicin Ib (79,600 [15]).

Classically, bacteriocins were considered to be bacterially derived antibacterial proteins with a narrow spectrum of activity which was restricted to species related to that producing the bacteriocin. However, as more and more bacteriocinlike bacterial products have been discovered and characterized, it has become apparent that this restrictive definition no longer holds true (14). The literature is replete with examples of bacteriocins which act against taxonomically unrelated organisms (5, 8, 14). Therefore, our finding that organisms which are taxonomically remote from *A. actinomycetemcomitans*, such as *S. sanguis* and *Actinomyces viscosus*, are sensitive to actinobacillicin is in keeping with our current understanding of bacteriocins.

Given the existence of an *A. actinomycetemcomitans* product (actinobacillicin) which is inhibitory to strains of *S. sanguis* and the reported decrease in the *S. sanguis* population when *A. actinomycetemcomitans* is present in plaque, it is tempting to speculate on the possibility of a cause and effect relationship between these two phenomena. Mature plaque may be looked upon ecologically as a climax community of microorganisms. It is into this milieu that *A. actinomycetemcomitans* inserts itself when it initially colonizes plaque. In attempting to colonize this environment, *A. actinomycetemcomitans* strains must disturb the ecological equilibrium and compete with the preexisting plaque organisms (such as *S. sanguis* and *A. viscosus*) for both nutrients and surface area. By inhibiting some of these organisms, the actinobacillicin may ease these ecological pressures on the superinfecting *A. actinomycetemcomitans* and thereby facilitate successful colonization.

It remains to be proven whether the production of a bacteriocin by *A. actinomycetemcomitans* per se has an impact on the plaque ecology. However, one intriguing possibility raised by these studies is that of using a bacteriocin produced by one *A. actinomycetemcomitans* strain to interfere with the colonization of other *A. actinomycetemcomitans* strains. Previously, we reported that in addition to inhibition of *S. sanguis*, there are antagonisms between different strains of *A. actinomycetemcomitans* (6). By using recombinant DNA techniques and immunologic probes prepared from purified bacteriocins, it may be possible to mobilize the genetic determinants of the bacteriocins into other (non-disease-associated) organisms and identify the resulting bacteriocin-producing strains. Thus far, the efficacy of using such strains in an ecological approach to

TABLE 1. Purification summary[a]

Step	Protein (mg)	ID_{50}[a] (μg/ml)	Sp act[b] (ID_{50} units/μg)	Purification (fold)[c]	ID_{50} units of activity[d]	% Recovery[e]
Starting material (SE 100,000 × *g* supernatant)	3,108	39.0	0.0256	1	79,565	100
G-100 (partially pure) AIF	1.74	0.050	20.0	781	34,800	44
Preparative PAGE (pure AIF)	0.174	0.022	45.0	1,758	7,830	10

[a] ID_{50} = μg protein per ml required to cause a 50% inhibition in glycolysis in an *S. sanguis* suspension under standardized conditions.
[b] Specific activity = ID_{50} units per μg of protein.
[c] Purification = (specific activity of preparation ÷ specific activity of starting material) × 100.
[d] ID_{50} units = μg of protein × specific activity.
[e] Recovery = ID_{50} units in preparation ÷ total (ID_{50} units in starting material) × 100.

controlling periodontopathic plaque bacteria has scarcely been explored.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant DE-5640 from the National Institute of Dental Research and a grant from the Colgate-Palmolive Company.

We thank Martha Pina for her assistance in the preparation of this manuscript.

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