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Role of Colonization in the Virulence of *Actinomyces viscosus* Strains T14-Vi and T14-Av

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Germfree rats fed a high-sucrose diet were inoculated with *Actinomyces viscosus* strain T14-Vi (virulent) or T14-Av (avirulent). The mean recovery of strain T14-Vi from six extracted finely ground molars of rats sacrificed after 90 days was 1.1×10^8 colony-forming units (CFU). The mean recovery of strain T14-Av was 5.7×10^7 CFU, which was significantly less. Strain T14-Vi caused severe alveolar bone loss, but only minimal bone loss occurred in rats infected with strain T14-Av. Scanning electron microscopy of teeth of germfree rats revealed that strain T14-Vi colonized in the fissures as well as on tooth surface areas near the gingiva; strain T14-Av also colonized in fissures but was unable to colonize the teeth near the gingiva. In studies with conventional rats fed a high-sucrose diet, streptomycin-resistant strain T14-Vi colonized on the teeth of all rats inoculated with in the order of 10^8 or 10^7 CFU and on the teeth of about half of the rats inoculated with 10^6 or 10^5 CFU. In contrast, streptomycin-resistant strain T14-Av could not be detected on the teeth of any of the rats in groups similarly inoculated. In vitro "resting" cells of both strains suspended in conventional or germfree rat saliva survived to comparable degrees. [^{3}H]thymidine-labeled T14-Vi cells adhered well to hydroxyapatite (HA) beads and to HA beads pretreated with saliva obtained from germfree or conventional rats. In contrast, T14-Av cells adhered less well than did T14-Vi cells to HA, whereas their adherence to saliva-coated HA was negligible. Transmission electron microscopy of negatively stained T14-Vi and T14-Av cells repeatedly passed in 1% phosphotungstic acid revealed fibrils on cells of both strains. T14-Av cells were covered by large amounts of extracellular material which was presumably heteropolysaccharide; little extracellular material was present on the surface of T14-Vi cells. T14-Vi cells had a relatively low affinity for the heteropolysaccharide synthesized by strain T14-Av. Other evidence also suggested that this polysaccharide had a relatively low affinity for saliva-coated HA. Collectively, the evidence indicates that the difference in periodontopathic potential between strains T14-Vi and T14-Av results from their different abilities to colonize teeth. This difference is probably due to the lower adherence of T14-Av cells to teeth rather than to their ability to grow in the mouth. The low affinity of T14-Av cells for tooth surfaces may be due, in part, to the presence of large amounts of cell-surface-associated polysaccharide.

Actinomyces viscosus strain T14-Vi (virulent) colonizes the teeth of germfree rats and induces periodontal disease characterized by severe alveolar bone loss. In contrast, strain T14-Av (avirulent), a laboratory mutant, also colonizes the teeth of germfree rats but does not induce periodontal disease (S. S. Socransky, personal communication).

A number of studies have been aimed at clarifying the difference in virulence between both strains. A cell wall antigen containing 6-deoxytalose as a major component has been isolated from strain T14-Vi. This antigen is also present in the cell wall of strain T14-Av but is not readily detectable (9, 10). Quantitative differences between both strains with respect to a surface antigen which is different from the 6-deoxytalose-containing antigen have been noted by others (1). Also, strain T14-Av has been found to produce much larger amounts of extracellular heteropolysaccharide than does strain T14-Vi (10). Whole cells of strain T14-Vi are more active in stimulating the release of lysosomal enzymes from human polymorphonuclear leukocytes than are cells of strain T14-Av (N. S. Taichman, B. F. Hammond, C. C. Tsai, P. Baehni, and W. P. McArthur, J. Dent. Res. **56**:B156, 1977), and purified cell walls of strain T14-Vi stimulate a

higher blastogenic response in human lymphocytes than do cell walls of strain T14-Av (J. J. Baker, S. P. Chan, B. F. Hammond, and S. E. Mergenhagen, J. Dent. Res. **54**:A178, 1975). On the other hand, both strains have similar complement-fixing efficiencies (17).

To date, possible differences between strains T14-Vi and T14-Av in their abilities to colonize teeth have not been considered. The present study, therefore, deals with various aspects of the colonization of both strains in germfree and conventional rats. Also, both strains have been studied by electron microscopy, and their attachment to hydroxyapatite has been studied in vitro under various conditions.

MATERIALS AND METHODS

Animals. Germfree male and female Sprague-Dawley rats, 2 months of age, raised and maintained at the Forsyth Dental Center, Boston, Mass. (6), were fed diet L-356 (Teklad Test Diets, Madison, Wis.) before use. Conventional male Sprague-Dawley rats, 40 days of age, were obtained from Charles River Breeding Laboratories, Wilmington, Mass. These animals were housed individually in screen-bottomed, stainless steel cages without bedding. Food and drinking water were given ad libitum. Before arrival from Charles River Breeding Laboratories, the animals had been fed a stock diet 4-RF (Country Best; Agway, Inc., Syracuse, N.Y.).

About 24 h before experimental infection as well as during the experiments, germfree and conventional rats were fed a diet containing 34.7% vitamin-free casein, 3.5% brewer's yeast extract, 5% salt mixture W, and 0.8% vitamin mixture, supplemented with 56% sucrose. A 56% sucrose-containing diet was also used by S. S. Socransky et al. in their original studies (personal communication).

Bacterial strains. *A. viscosus* strains T14-Vi and T14-Av from previous studies (10), which were stored in the lyophilized state, were used. In some studies, streptomycin-resistant mutants of these strains were used. The mutants were selected by serial transfer of the strains in broth containing increasing concentrations (up to 2,000 μg/ml) of streptomycin sulfate (resistance indicated by suffix "R"). Cells for animal inoculation were obtained from cultures of strains grown in actinomyces broth containing 0.5% glucose (Baltimore Biological Laboratory [BBL], Baltimore, Md.) for 48 h and incubated in candle jars at 37°C.

In vivo studies. (i) Studies with germfree rats. For inoculation of germfree rats, cells grown in actinomyces broth were placed in sterile glass ampoules which were then heat sealed. These sealed vials were externally sterilized and then introduced into germfree isolators. The contents of these vials were used to infect two separate groups of eight animals (6). Bacterial colonization was monitored by culturing fecal Calgiswab (Wilson Diagnostics, Inc., Glenwood, Ill.) samples on Trypticase soy agar (BBL) plates to which 5% sheep blood was added. Ninety days after inoculation, all rats were killed by carbon dioxide inhalation and decapitated, and the heads were skinned. Three maxillary and three mandibular molars from two quadrants of the mouth were extracted, ground in tissue grinders, and cultured for enumeration of the test organism on the teeth as described earlier (21).

The two remaining quadrants were used to determine alveolar bone loss. They were defleshed by dermestid beetles, washed in 95% ethanol, placed in 25% ammonium hydroxide for 48 h, and then air dried at room temperature. Alveolar bone loss was measured by a modification of the method of Keyes and Gold (14). A Nichrome wire (diameter, 0.4 mm), calibrated into 0.1-mm gradations, was used to measure the vertical distance from the cementum-enamel junction to the bottom of the periodontal pocket. Measurements were made at four corners of each of the first and second molars and at two corners of the third molars with the aid of a binocular dissecting microscope. All values were added and averaged for each molar and for each group of rats. Measurements were done only on maxillary molars, because appreciable mandibular bone loss does not occur until about 4 months after inoculation under the conditions used (Socransky, personal communication).

The localization of both test strains on the teeth of germfree rats was determined by scanning electron microscopy. Two groups of three germfree rats fed high-sucrose diet were inoculated separately with either strain and killed 65 days thereafter. The teeth and surrounding soft tissues were then exposed to Karnovsky fixative (13) before careful dissection of the four molar quadrants. The quadrants were placed in Karnovsky fixative for 24 h, postfixed in 2% osmium tetroxide in s-collidine buffer, dehydrated through increasing concentrations of ethyl alcohol, passed through amyl acetate, and critical-point dried with carbon dioxide. Specimens were mounted on aluminum stubs with silver paint, coated with palladium-gold, and examined with a model JEOL U3 scanning electron miscroscope (Japanese Electron Optics, Tokyo, Japan). The entire surface of each molar was inspected for the presence of cells of either strain T14-Vi or strain T14-Av.

(ii) Studies with conventional rats. Eight groups (five animals per group) of conventional rats were inoculated orally once with 0.2-ml suspensions containing approximately 10^8, 10^7, 10^6, and 10^5 colony-forming units (CFU) of strain T14-ViR or strain T14-AvR. Before inoculation, centrifuged cells from actinomyces broth were suspended or appropriately diluted in quarter-strength Ringer solution (20) and dispersed by mixing on a Vortex mixer (Vortex-Genie; Scientific Industries, Inc., Springfield, Mass.) for 15 s at setting 6. Implantation of the test strains was monitored by culturing oral Calgiswab samples on blood agar plates with 200 μg of streptomycin sulfate per ml. At the end of the experiments, 30 days after inoculation, rats were killed for enumeration of the test organisms on the molars of all four quadrants as outlined earlier (21).

In vitro studies. (i) Collection of saliva. Germfree or conventional rats were sedated by intraperitoneal injection with 30 mg of pentobarbital sodium (Abbott Laboratories, North Chicago, Ill.) per kg of body weight. Secretion of saliva was stimulated by intraperitoneal injection with 10 mg of pilocarpine

nitrate (ICN Pharmaceuticals, Inc., Cleveland, Ohio) per kg of body weight. Pentylenetetrazol, 50 mg/kg of body weight, was injected intraperitoneally 10 min thereafter to reduce animal mortality (15). Saliva was collected for 20 to 30 min after stimulation in a container chilled over ice. In most cases, saliva from four to six animals was pooled to obtain the required quantity for each experiment. It was then placed in a 60°C water bath for 30 min to inactivate salivary enzymes and was then clarified by centrifugation at 12,000 × *g* for 10 min (3).

(ii) Hydroxyapatite (HA) adherence assay. Both test strains were cultivated in Trypticase broth (5) with 5 μCi of [^{3}H]thymidine (New England Nuclear Corp., Boston, Mass.) per ml. Sterile glucose (0.2%) was aseptically added after autoclaving the medium. Cells from 48-h cultures were harvested by centrifugation, washed twice, and suspended in 0.01 M phosphate-buffered saline (PBS), pH 6.0, at a concentration of 2.2×10^8 cells per ml. Samples of such suspensions were lyophilized and stored in screw-capped vials at room temperature. Before use, lyophilized cells were suspended in PBS, washed twice, and then passed 10 to 15 times through a 26-gauge syringe needle to break up cell aggregates. In some experiments, fresh ^{3}H-labeled cells cultivated similarly to the lyophilized cells were used. These cells were washed twice, suspended in PBS at the desired concentration, and dispersed as above. In all assays, suspensions containing 2.2×10^7 cells per ml, with a specific activity of 8×10^4 to 12×10^4 cpm, were used.

A method developed by Clark et al. (2) was modified to study bacterial adherence to HA. Fifteen milligrams of spheroidal HA beads (BDH Biochemicals, Ltd., Poole, England) with diameters of 85 to 125 μm and an approximate surface area of 0.27 cm^2/mg was placed in 500-μl polypropylene micro-test tubes (Dynana Labs., Rochester, N.Y.) and washed three times in PBS. Tubes were filled with approximately 0.5 ml of saliva or PBS, tightly stoppered, and placed at room temperature in a turning apparatus which continuously inverted them 10 times per min for 16 to 18 h. Unabsorbed saliva was removed by washing twice in PBS. Tubes with untreated HA beads or saliva-coated beads were then filled with 0.5 ml of ^{3}H-labeled cell suspensions of strain T14-Vi or T14-Av. The mixtures were continuously turned at room temperature for 2 h. The beads were then allowed to settle for 1 min, and the supernatant, which contained the unabsorbed organisms, was removed. The cells in the supernatant were collected on a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.), and the activity on the filters was counted in a Packard Tri-Carb scintillation spectrometer. Cell adherence to HA was determined after washing the beads twice with PBS to remove the few remaining unabsorbed cells and then transferring them to the scintillation vials for counting. Control bacterial suspensions (0.5 ml) were incubated without HA beads and counted similarly to correct for cell loss due to adsorption to the tubes. The counts per minute of cells adhering to the HA beads were expessed as a percentage of the counts per minute of cells in the control suspension. All assays were carried out in duplicate.

(iii) Studies with extracellular polysaccharide from T14-Av. Isolation of the extracellular polysaccharide of strain T14-Av was accomplished by minor changes in the method of Rosan and Hammond for the rodent strain of *A. viscosus* T6 (16). Supernatants of 16-h cultures of strain T14-Av cultivated in Trypticase soy broth (Difco Laboratories, Detroit, Mich.) were first dialyzed extensively against PBS (0.1 M) and then subjected to acetone precipitation. The resulting precipitate was further treated with trichloroacetic acid and then reprecipitated in acetone, followed by dialysis against distilled water. The dialyzed material (2 mg/ml) was then applied to a column of Sepharose 2B (0.9 by 19.3 cm) previously equilibrated with 0.05 M phosphate buffer (pH 7.5) and eluted with the same buffer at a rate of 1 ml/5 min. All fractions (1 ml) were monitored for *N*-acetylglucosamine (12) and by Ouchterlony gel diffusion plates using rabbit antisera directed against whole cells of T14-Av. A single large hexosamine peak appeared at fraction 10 and disappeared at fraction 20. The appearance/disappearance of the hexosamine peak was paralleled by serological detection of the slime in gel diffusion plates. Fractions 10 through 19 were pooled, and the resulting preparation was a viscous gel rich in *N*-acetylglucosamine (approximately 45%) with small amounts of glucose, galactose, and glycerol and less than 10% protein. The preparation was lyophilized and stored at −20°C. Control experiments with uninoculated broth material yielded negligible amounts of precipitable material, none of which was subsequently detectable in column eluates.

Solutions of 0.1% polysaccharide in PBS were prepared. In view of the low solubility of the polysaccharide, it was dispersed with the aid of sonic oscillation, using an MSE 100-W ultrasonic disintegrator (MSE, Inc., Westlake, Ohio) at 8 μM amplitude for 4 min. To determine the adherence of cells to HA beads pretreated with polysaccharide, samples (0.5 ml) of the polysaccharide solution were added to tubes with HA beads or saliva-coated HA beads prepared as described earlier. After 2 h of incubation, the beads were washed twice with PBS before use in the assay. Cells of strain T14-Vi were also tested in mixture with polysaccharide. Mixtures of equal volumes of polysaccharide solution and cell suspensions (final concentration of 2.2×10^7 CFU/ml) were added to tubes containing uncoated or saliva-coated HA beads.

(iv) Survival of strains T14-ViR and T14-AvR in saliva. Cells obtained from 48-h cultures of either strain and washed twice in quarter-strength Ringer solution were added to 2 ml of clarified, heat-inactivated (3) conventional or germfree rat saliva to a concentration of 10^5 to 10^6 CFU per ml. The saliva used was freshly collected from 2-month-old rats. Duplicate samples (0.1 ml) were taken periodically during aerobic stationary incubation of the mixtures at 37°C and, after mixing with a Vortex mixer for 15 s at setting 6, were cultured on blood agar plates with streptomycin to determine the number of viable organisms present.

(v) Transmission electron microscopy. Strains T14-Vi and T14-Av were freshly isolated from monoinfected germfree rats by culturing on blood agar plates for 48 h. Isolated colonies were then picked, mixed, and stained with 1% phosphotungstic acid for

60 s. Suspensions were then placed on 100-μm carbon grids and examined with a model JEOL JEM 100B transmission electron microscope (Japanese Electron Optics).

RESULTS

Studies with germfree rats. The recoveries of strains T14-Vi and T14-Av from extracted molars of monoinfected germfree rats are shown in Table 1. The mean recovery of strain T14-Vi was about 10^8 CFU, which was twofold higher than that of strain T14-Av. This difference was significant ($P < 0.01$, Student's t test). The measurements of loss of alveolar bone in these animals are shown in Table 2. Strain T14-Vi caused considerable destruction of bone, especially around the first and second molars. In contrast, little or no bone loss was observed in the rats infected with strain T14-Av ($P < 0.01$). The difference in bone destruction in rats infected with either strain is shown in Fig. 1. Strain T14-Vi also caused considerable root surface caries.

Scanning electron microscopy of the molars of germfree rats monoinfected with either strain T14-Vi or strain T14-Av revealed differences between both strains with respect to their localization on the teeth. Strain T14-Vi colonized the fissures as well as the gingival area of the teeth; massive accumulation of cells was often seen in the approximal area near the gingiva. Strain T14-Av, on the other hand, colonized only in fissures and was never seen in the gingival region of the molars. Some typical examples of the colonizations of both strains on the molar teeth are shown in Fig. 2.

Studies with conventional rats. Strain T14-ViR was recovered from most rats 2 and 7 days after their inoculation with varying cell numbers (Table 3). At sacrifice, all rats inoculated with 9.1×10^8 CFU or a 10-fold lower dose were heavily infected with the organism; about half of the rats inoculated with lower cell numbers also harbored this strain. In contrast, only a few rats inoculated with strain T14-AvR were positive for the organism 2 and 7 days after inoculation, whereas at sacrifice none of the rats harbored the test strain.

TABLE 1. *Recovery of A. viscosus strains T14-Vi and T14-Av from monoinfected germfree rats fed high-sucrose diet*[a]

Strain	Recovery from teeth (CFU)	
	Mean	Range
T14-Vi	1.1×10^8	6.5×10^7–1.4×10^8
T14-Av	5.7×10^7	3.1×10^7–1.6×10^8

[a] Arithmetic means from groups of 8 rats of total recoveries from left maxillary and right mandibular ground molar teeth 90 days after inoculation.

TABLE 2. *Bone loss in germfree rats fed high-sucrose diet and inoculated with A. viscosus T14-Vi or T14-Av*[a]

Strain	Mean bone loss (mm) ± SD		
	M_1	M_2	M_3
T14-Vi	0.7 ± 0.2	0.6 ± 0.2	0.1 ± 0.1
T14-Av	0.2 ± 0.1	0.2 ± 0.2	0.1 ± 0.1

[a] Bone loss in groups of 8 rats was determined 90 days after inoculation. M, Right maxillary molars; SD, standard deviation.

Attachment of strains T14-Vi and T14-Av to HA in vitro. Results from experiments in which the adherences of both strains to HA were studied are shown in Table 4. Lyophilized T14-Vi cells adhered better to uncoated HA than did lyophilized T14-Av cells ($P < 0.01$, Student's t test). This difference between the two strains was much smaller with freshly cultivated cells. Treatment of HA with saliva obtained from conventional rats decreased the attachment of lyophilized T14-Vi cells ($P < 0.01$), as compared with untreated HA, but did not affect that of freshly cultivated T14-Vi cells. Both lyophilized and freshly cultivated T14-Av cells adhered poorly to saliva-coated HA. Comparable results were obtained in experiments with saliva obtained from germfree rats or with saliva obtained from conventional rats 28, 40, or 90 days of age (data not shown).

Pretreatment of HA with the extracellular polysaccharide synthesized by strain T14-Av reduced the adherence of lyophilized T14-Vi cells to a negligible level (Table 5). The adherence of lyophilized T14-Vi cells to saliva-coated HA with or without subsequent exposure to polysaccharide was about the same and was higher than that to polysaccharide-coated HA. The adherence of lyophilized T14-Av cells to saliva-coated HA, polysaccharide-coated HA, or saliva-coated HA treated subsequently with polysaccharide was negligible. The adherence of freshly cultivated T14-Vi cells to polysaccharide-coated HA was significantly reduced as compared with untreated HA (Table 5; $P < 0.01$). This reduction was less pronounced than that with lyophilized T14-Vi cells but was consistently observed in each of the different experiments. The adherence of freshly cultivated T14-Vi cells to saliva-coated HA and saliva-coated HA treated with polysaccharide was comparable to their adherence to uncoated HA. When a mixture of freshly cultivated T14-Vi cells and polysaccharide was added to uncoated HA, a significant reduction in cell adherence was observed as compared with the adherence of freshly cultivated T14-Vi cells to uncoated HA (Table 5; $P < 0.01$). This reduction in adherence was similar to the one ob-

served when freshly cultivated T14-Vi cells alone were added to polysaccharide-coated HA. The adherence of the mixture of T14-Vi cells and polysaccharide to saliva-coated HA was similar to that of T14-Vi cells alone to saliva-coated or uncoated HA.

Survival of strains T14-ViR and T14-AvR in saliva. Both strains T14-ViR and strain T14-AvR survived well in saliva obtained from conventional rats (Table 6). At the end of the 24-h experimental period, the recoveries of the test strains were higher than those at the start of the experiment. In contrast, the recoveries of both strains from germfree rat saliva decreased rapidly, and no viable organisms were present after 4 h. This difference between conventional and germfree rat saliva was consistently observed in other experiments.

Transmission electron microscopy. Transmission electron microscopy of negatively stained cells of strain T14-Av prepared by mixing colonies from blood agar plates once in 1% phosphotungstic acid revealed cells heavily coated with extracellular material, and no detailed structures were visible (Fig. 3A). In contrast, similar treatment of strain T14-Vi or strain T14-ViR revealed numerous fibrils extending far away from the cells, whereas much less extra-

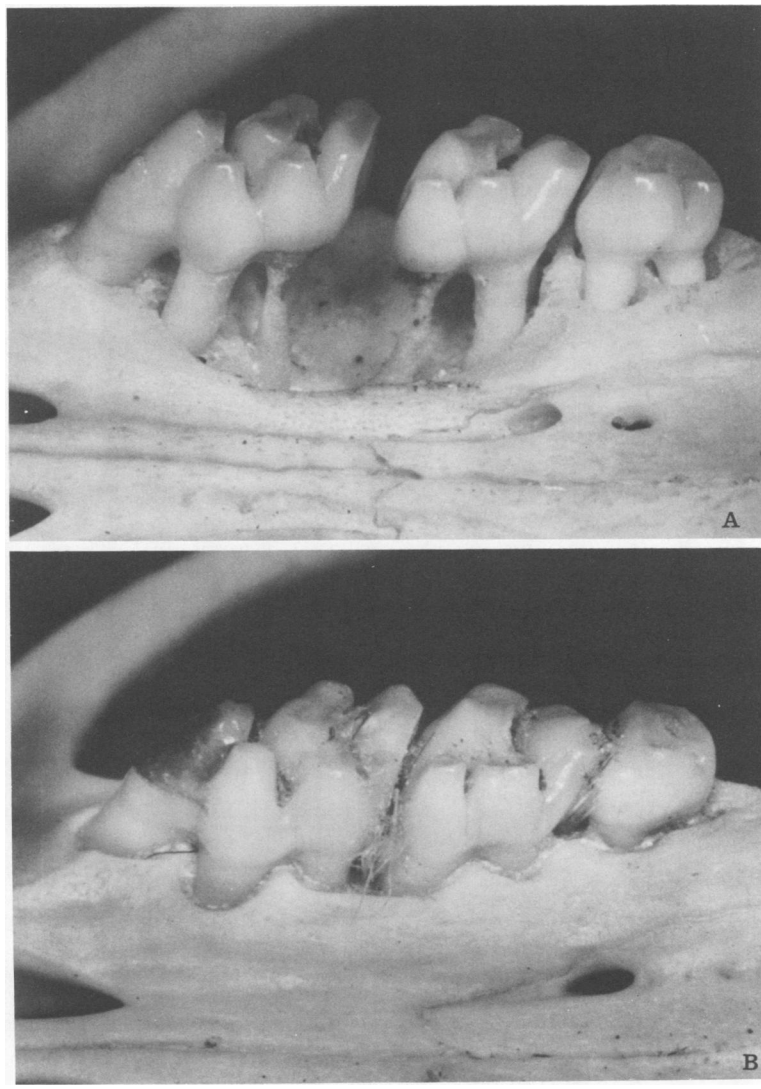

FIG. 1. *Maxillary molars of germfree rats monoinfected for 90 days with A. viscosus. (A) Severe alveolar bone loss and root surface caries induced by strain T14-Vi. (B) Lack of bone loss associated with strain T14-Av.*

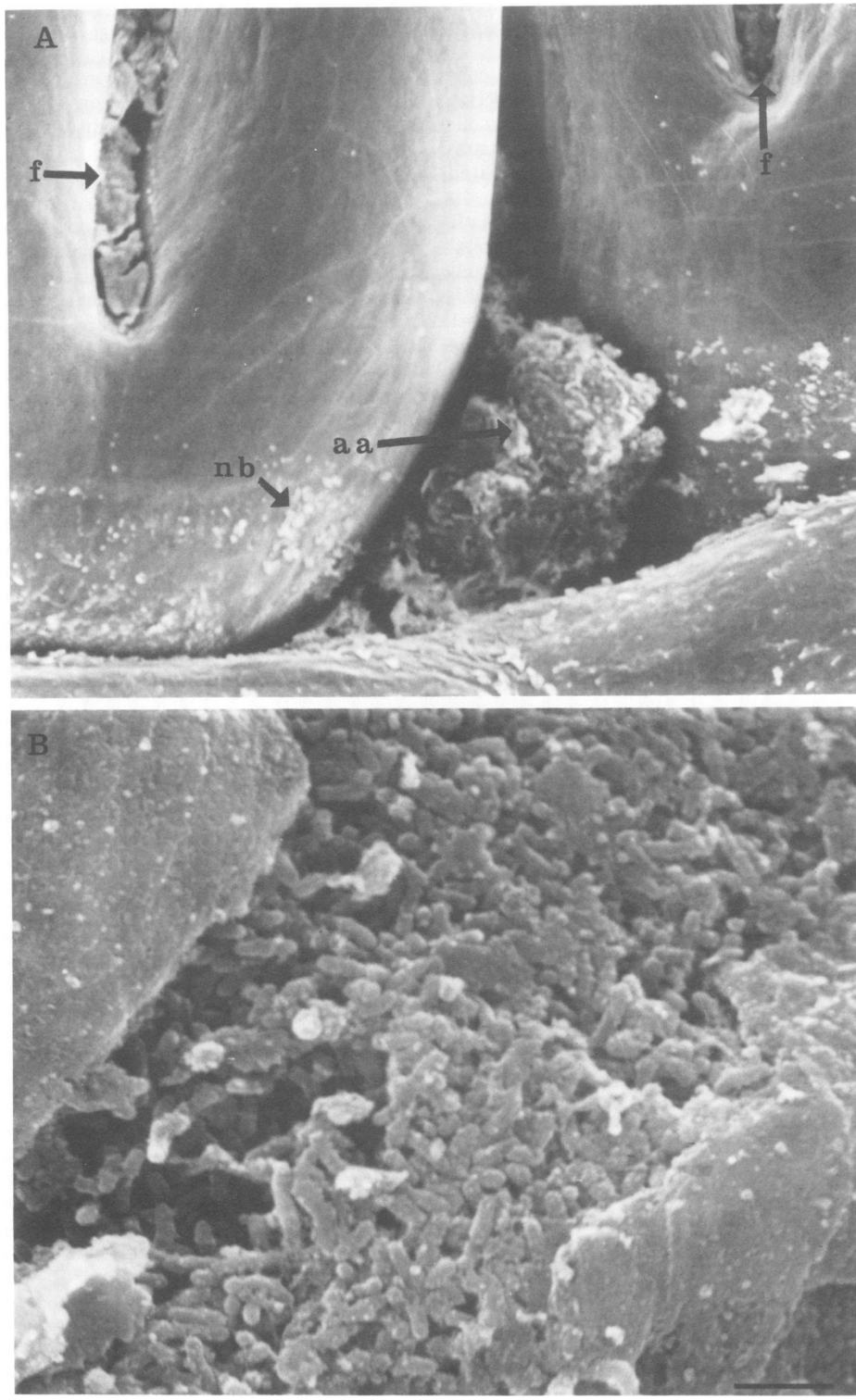

FIG. 2. *Scanning electron photomicrographs of maxillary molars of germfree rats monoinfected with A. viscosus strain T14-Vi or A. viscosus strain T14-Av. (A) Colonization of strain T14-Vi in fissures (f) and massive cell accumulation in approximal area (aa) near the gingiva; nonbacterial cellular remnants (nb) (×60). (B) Higher magnification of approximal area with cell masses of strain T14-Vi; bar = 5.0 μm. (C) Colonization of strain T14-Av in fissures and absence of T14-Av cells in the approximal area; hair impaction (h) (×60).*

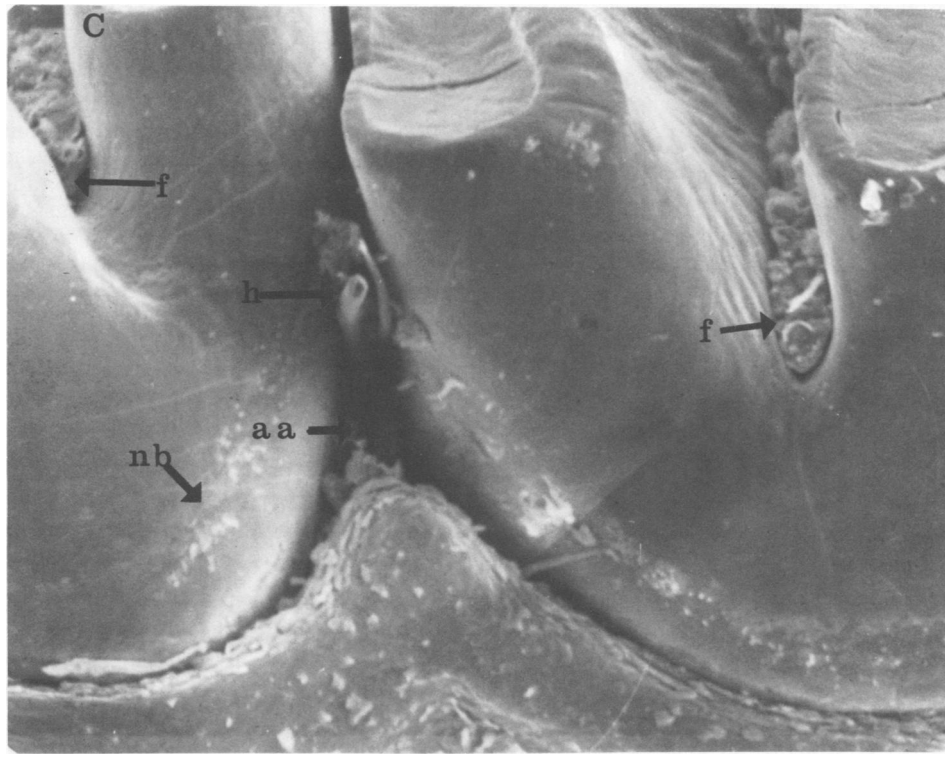

FIG. 2—*Continued*

cellular material was evident (Fig. 3B and C). After cells of strain T14-Av were dispersed and diluted twice in 1% phosphotungstic acid, numerous fibrils could be seen (Fig. 3D). No clear differences were discernible with respect to either the numbers or the sizes of the fibrils on cells of both strains. Colonies of strain T14-Vi on agar could be fragmented with a wire loop, but colonies of strain T14-Av on agar were slimy and could not be fragmented. In accordance with this observation, negatively stained preparations of strain T14-Vi revealed cells connected by fibrils as well as many separate cells. In contrast, T14-Av cells were often aggregated and apparently bound together by extracellular material (Fig. 3E).

DISCUSSION

Strain T14-Vi induced severe bone loss in monoinfected germfree rats, whereas only minimal bone loss occurred in rats infected with strain T14-Av. These observations confirm the findings of S. S. Socransky et al. (unpublished data). In the present study, actual determination of cell numbers on teeth and scanning electron microscopy of infected molars revealed significant differences in colonization between both strains. Thus, fewer cells of strain T14-Av than of strain T14-Vi were present on the molars. More significantly, strain T14-Av, in contrast to strain T14-Vi, did not colonize tooth surface areas near the gingiva. This latter finding appears to explain adequately the difference in periodontopathic potential between the strains. We therefore question the value of the further use of these strains as a model for the study of virulence factors of *A. viscosus* that are not related to its oral colonization.

The differences in colonization between strains T14-Vi and T14-Av were more evident with conventional than with germfree rats. Conventional rats inoculated with the highest cell number of strain T14-Av used, i.e., 10^9 CFU, did not become infected, wheras inoculation with similar or even 100- or 1,000-fold lower cell numbers of strain T14-Vi resulted in satisfactory colonization of the organism on the teeth of many of the rats. Colonization of teeth in vivo by bacteria requires an initial phase of cell attachment, which is followed by proliferation and accumulation of attached cells. Components of saliva may be expected to influence this process, since the acquired pellicle which generally covers the tooth enamel and to which bacteria must adhere to initiate plaque formation is thought to be of mainly salivary origin (19). Furthermore, salivary components can engage in a variety of interactions with oral bacteria which lead to

their presence in the interbacterial plaque matrix as well as the peripheral plaque surface (19). In our studies, strain T14-Av failed to adhere to HA beads exposed to rat saliva, whereas strain T14-Vi adhered well to saliva-coated HA. This suggests that the failure of strain T14-Av to establish on the teeth of conventional rats, or only in the fissures of the molars of germfree rats, may be due to its low affinity for the tooth surface rather than to its inability to initiate growth. This is supported by the observations that the two strains did not appear to differ in growth in vitro and that both strains survived to comparable degrees in rat saliva and, in the case of conventional rat saliva, exhibited limited growth. The colonization of strain T14-Av in fissures but not on other parts of the molars of germfree rats is probably due to the lesser significance of cell affinity as a determinant of bacterial colonization in the more stagnant environment of fissures (22).

The adherence of strains T14-Vi and T14-Av involves interactions between receptors on their cell surface and the tooth surface. Recent studies have demonstrated the presence of fibrils on the cell surface of *A. viscosus* and *A. naeslundii* (1, 4, 7). Receptors responsible for the attachment of *Actinomyces* to teeth may therefore be located on these fibrils and/or in the cell wall. Removal of fibrils has recently been shown to decrease the adherence of *A. naeslundii* to epi-

TABLE 3. *Recovery of A. viscosus strains T14-ViR and T14-AvR from conventional Sprague-Dawley rats fed high-sucrose diet*

Strain	Inoculum (CFU)	Recovery[a] at:		
		2 days	7 days	30 days (sacrifice)
T14-ViR	9.1×10^8	4/5[b]	5/5	5/5 (2.1×10^7)[c]
	9.1×10^7	5/5	4/5	5/5 (1.8×10^7)
	9.1×10^6	5/5	3/5	2/5 (2.4×10^7)
	9.1×10^5	2/5	2/5	2/5 (4.6×10^7)
T14-AvR	6.3×10^8	3/5	2/5	0/5
	6.3×10^7	2/5	0/5	0/5
	6.3×10^6	2/5	0/5	0/5
	6.3×10^5	1/5	0/5	0/5

[a] Recovery at 2, 7, and 30 days postinoculation.
[b] Number of rats infected/number of rats inoculated.
[c] Arithmetic mean of total recoveries (CFU) from 12 ground molar teeth from sacrificed infected rats.

TABLE 4. *Adherence of [^{3}H]thymidine-labeled cells of A. viscosus strains T14-Vi and T14-Av to uncoated and conventional rat saliva-coated HA beads*

Cells	Strain	Mean % adherence ± SD[a] to:	
		Uncoated HA	Saliva-coated HA
Lyophilized[b]	T14-Vi[b]	70 ± 9	48 ± 15
	T14-Av[b]	30 ± 14	2 ± 2
Freshly cultivated[c]	T14-Vi[c]	84 ± 4	83 ± 11
	T14-Av[d]	70 ± 6	4 ± 1

[a] SD, Standard deviation.
[b] Seven different experiments.
[c] Six different experiments.
[d] Two different experiments.

TABLE 6. *Recovery of A. viscosus strains T14-ViR and T14-AvR from pilocarpine-stimulated rat saliva*

Time (h)	Recovery (CFU/ml of saliva)			
	Conventional rat		Germfree rat	
	T14-ViR	T14-AvR	T14-ViR	T14-AvR
0	1.8×10^5	1.2×10^6	1.1×10^5	1.3×10^6
0.5	1.9×10^5	1.4×10^6	1.3×10^4	4.0×10^5
1.0	1.9×10^5	1.5×10^6	5.5×10^1	6.5×10^3
4.0	1.9×10^5	1.8×10^6	0	0
8.0	2.1×10^5	2.6×10^6	0	0
24.0	1.2×10^6	1.0×10^7	0	0

TABLE 5. *Effect of extracellular polysaccharide isolated from A. viscosus strain T14-Av on the adherence of A. viscosus strains T14-Vi and T14-Av to HA*

Cells	Strain	Mean % adherence ± SD[a] to HA beads			
		Uncoated	Saliva coated	Polysaccharide coated	Saliva-polysaccharide coated[b]
Lyophilized	T14-Vi[c]	73 ± 3	42 ± 3	4 ± 3	37 ± 5
	T14-Av[d]	26 ± 15	1 ± 1	1 ± 1	1 ± 1
Freshly cultivated	T14-Vi[e]	92 ± 4	93 ± 3	67 ± 7	94 ± 4
Freshly cultivated + polysaccharide[f]	T14-Vi[e]	64 ± 13	94 ± 3		

[a] SD, Standard deviation.
[b] HA first treated with saliva and then with polysaccharide.
[c] Four different experiments.
[d] Two different experiments.
[e] Three different experiments.
[f] Cells and polysaccharide were mixed for use in the assay.

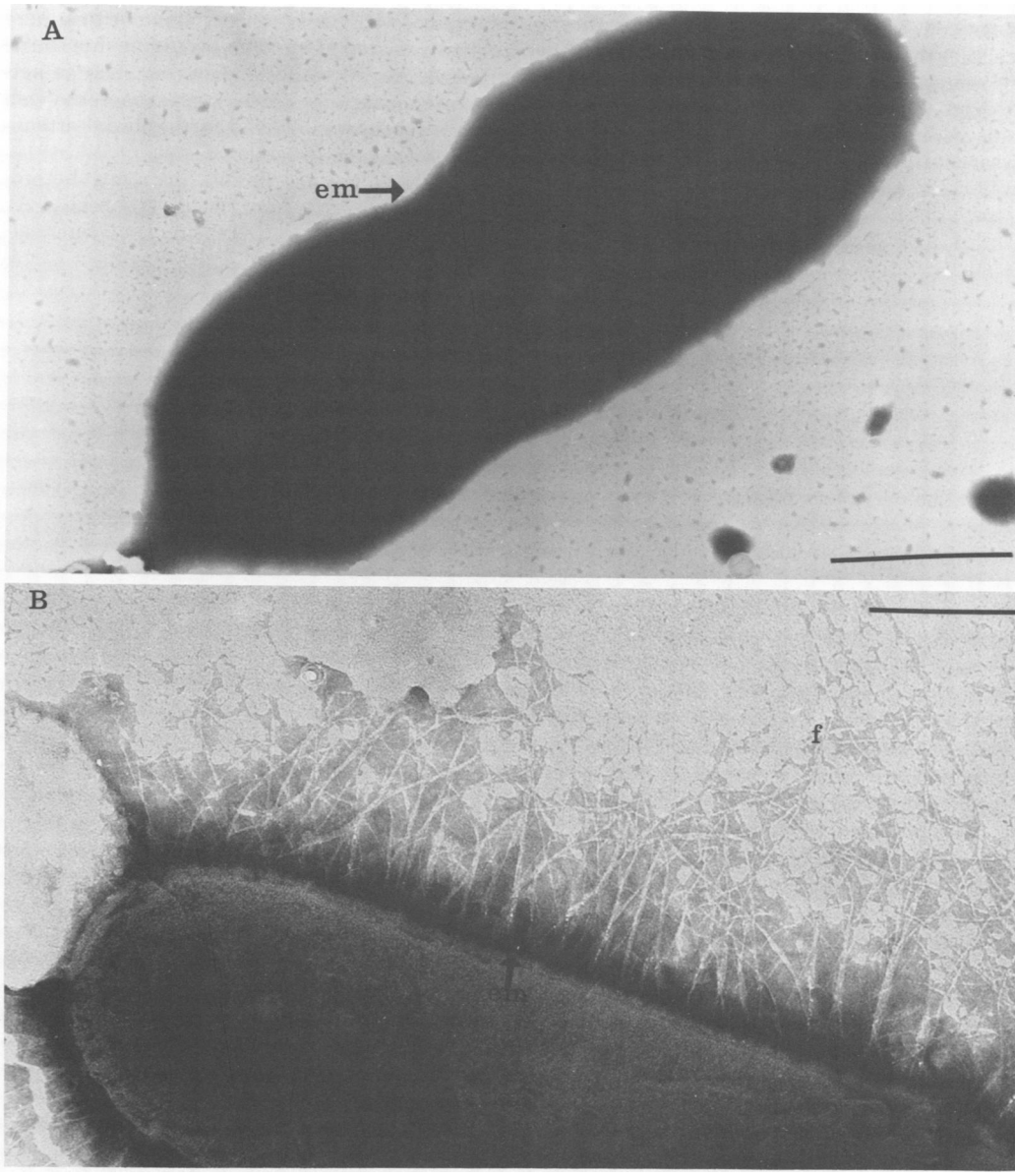

FIG. 3. *Transmission electron photomicrographs of A. viscosus. (A) 1% phosphotungstic acid (PTA)-stained T14-Av; massive amounts of extracellular material (em) are masking cell surface fibrils; bar = 1.0 μm. (B) 1% PTA-stained strain T14-Vi; the long surface fibrils (f) are only partly covered by extracellular material; bar = 0.5 μm. (C) 1% PTA-stained strain T14-ViR; the long surface fibrils extend far beyond the extracellular material; bar = 1.0 μm. (D) 1% PTA-stained strain T14-Av that was dispersed, passed, and diluted twice in 1% PTA before examination; the surface fibrils are embedded in extracellular material; bar = 1.0 μm. (E) 1% PTA-stained strain T14-Av passed and diluted once in 1% PTA before examination; fibrils embedded in extracellular material appear to bind cells together; bar = 1.0 μm.*

thelial cells (R. E. Ellen and D. L. Walker, J. Dent. Res. **56**:A155, 1977). Strain T14-Vi as well as strain T14-Av were found to possess numerous fine fibrils on their surfaces. Their numbers did not appear to differ greatly between the two strains, although another recent study of these strains with transmission electron microscopy of thin cell sections has suggested that strain T14-Vi possesses a greater number of fibrils than does strain T14-Av (1). The observation that

T14-Av cells were extensively covered with extracellular material, whereas only small amounts were seen around T14-Vi cells, correlates well with the reported difference between both strains in the synthesis of extracellular heteropolysaccharide (10). It is of interest, therefore, that fibrils of T14-Av cells were partly embedded in this polysaccharide, whereas many of the fibrils of T14-Vi cells extended far beyond it. This suggests that the markedly reduced ability of strain T14-Av to colonize in vivo or to adhere to saliva-coated HA in vitro could be due to the presence of extracellular material, such as heteropolysaccharide, which masks bacterial cell surface receptors involved in the initial attachment of the cells to teeth.

The in vitro experiments involving heteropolysaccharide synthesized by strain T14-Av lend some support to this contention. Since attempts to obtain T14-Av cells devoid of extra-

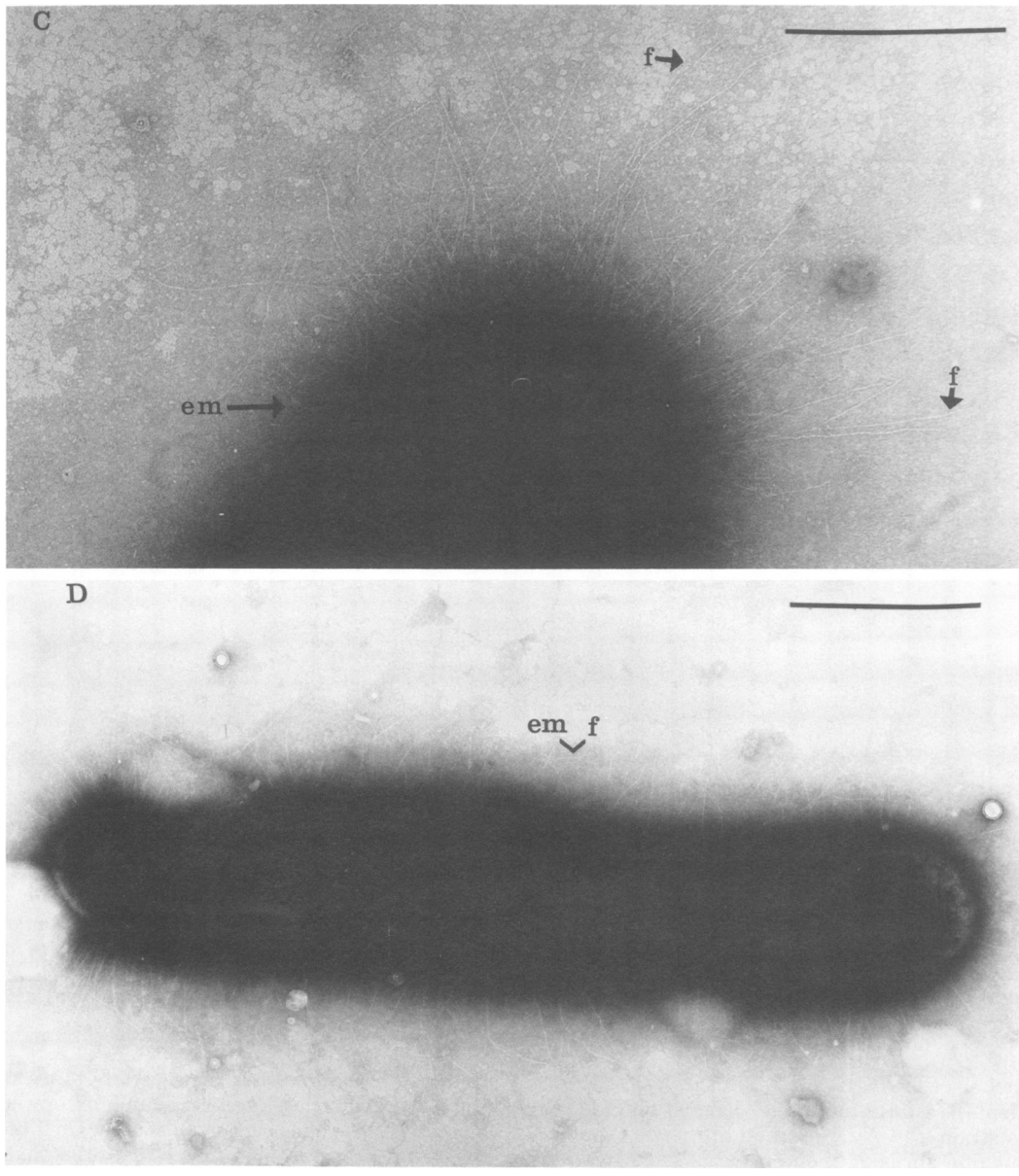

FIG. 3—*Continued*

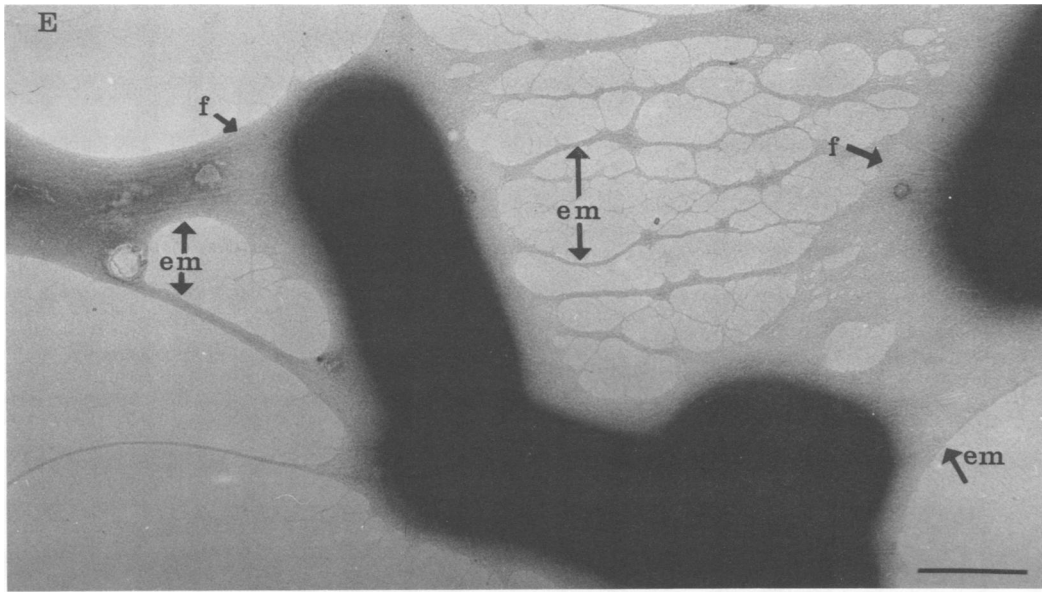

FIG. 3—*Continued*

cellular polysaccharide to study directly its role in the adherence of strain T14-Av were unsuccessful, the influence of this polysaccharide on the adherence of strain T14-Vi was studied. Pretreatment of HA with polysaccharide or the addition of T14-Vi cells mixed with polysaccharide to HA greatly impaired the attachment of T14-Vi cells to HA. This indicates that T14-Vi cells have a low affinity for T14-Av polysaccharide and is consistent with our finding that T14-Vi cells do not aggregate in the presence of polysaccharide (data not given). T14-Vi cells with or without the addition of polysaccharide adhered with equal efficiencies to saliva-coated HA and to saliva-coated HA pretreated with polysaccharide. This suggests the possibility that T14-Av polysaccharide has a low affinity for saliva-coated HA and thus prevents the attachment of T14-Av cells to saliva-coated HA.

Capsular material is commonly associated with the enhanced virulence of microorganisms, e.g., *Streptococcus pneumoniae* and *Klebsiella pneumoniae*. In the case of *S. mutans*, the extracellular glucans synthesized from sucrose appear to enhance colonization by promoting bacterial accumulation on teeth; it is at present not clear whether they also promote the initial attachment of *S. mutans* cells (19). On the other hand, the synthesis of extracellular glucans from sucrose by other oral streptococci, such as *S. sanguis*, does not appear to aid in colonization. Other strains of *A. viscosus* which are periodontopathic in rodents also produce an extracellular slime which closely resembles that produced by strains T14-Vi and T14-Av (8, 11, 16, 18). Our data suggest that extracellular slime impairs the initial attachment of *A. viscosus* to the teeth. The electron microscopic observations with strain T14-Av do not exclude the possibility that heteropolysaccharide could promote the accumulation of cells of *A. viscosus* that are already on the teeth by increasing cell-to-cell binding. Clearly, further studies are required to clarify the relationship between heteropolysaccharide and the adherence of *A. viscosus* to teeth.

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