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Characteristics of Adherence of *Actinobacillus actinomycetemcomitans* to Epithelial Cells

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***Actinobacillus actinomycetemcomitans* smooth variants [SUNY 75(S), SUNY 465, 652] were investigated for their ability to adhere to KB epithelial cells. Both the type of medium (broth versus agar) and anaerobicity influenced adherence levels and cell surface characteristics. Optimal adherence was observed with all three strains after growth of the bacterial cells in broth under anaerobic conditions, a condition which was associated with extracellular microvesicles. Adherence of SUNY 75(S) also was correlated with extracellular amorphous material, whereas adherence of SUNY 465 was also associated with fimbriation which accompanied a smooth to rough phenotype shift. The relationship between adherence and extracellular vesicles, extracellular amorphous material, and fimbriation suggests that all of these components may function in *A. actinomycetemcomitans* adherence to epithelial cells. The phenotype shift observed in SUNY 465 cells is further evidence that *A. actinomycetemcomitans* SUNY 465 is predisposed to variant shifts which are associated with changes in adherence and invasion properties.**

Actinobacillus actinomycetemcomitans is considered an important pathogen in both adult and juvenile periodontal disease(s) (21, 26, 27, 33). Nevertheless, knowledge concerning *A. actinomycetemcomitans* colonization and persistence in the oral cavity is extremely limited. Adhesion of *A. actinomycetemcomitans* to epithelial cells, teeth, and other oral bacteria and its invasion into the gingiva are probably initial and essential steps in the pathogenesis of periodontitis.

A significant feature of *A. actinomycetemcomitans* is its colony variation. Most freshly isolated species exhibit rough-surfaced colonies which are associated with fimbriae (9, 17, 18, 23). Upon subculture, a change in colonial morphology from rough- to smooth-surfaced colonies occurs (18). Reversion to rough-surfaced variants has been described for only one *A. actinomycetemcomitans* strain (14). Smooth-surfaced variants are not highly fimbriated (9, 18), yet they exhibit adhesive properties, suggesting that nonfimbrial adhesins function in *A. actinomycetemcomitans* adherence (9). Previously, we used an in vitro invasion model to show that *A. actinomycetemcomitans* strains with smooth colony morphology invade KB monolayers more proficiently than do those with rough morphology (14). Since adhesion is most probably a prerequisite for the penetration of invasive organisms into epithelial cells, knowledge of the adhesive properties of smooth-surfaced *A. actinomycetemcomitans* strains is germane.

Very little research has been carried out on *A. actinomycetemcomitans* adhesion. A few studies have examined the binding of *A. actinomycetemcomitans* to hydroxyapatite and saliva-coated hydroxyapatite (11, 19). They indicate that *A. actinomycetemcomitans* adhesion to solid surfaces is probably mediated by more than one mechanism and suggest that fimbriae may be involved in one of the mechanisms (11, 19). Studies on adherence of *A. actinomycetemcomitans* to epithelial cells are even more limited than those on adherence to solid surfaces. One study showed that a fimbriated *A. actinomycetemcomitans* strain adhered better to mouse epithelial cells than did one which was not fimbriated (19). Another study showed that *A. actinomycetemcomitans* 9710, like many of the other oral bacteria studied, adhered poorly to HeLa cells (30). However, treatment of the HeLa monolayer with either human saliva or serum resulted in increased adherence of *A. actinomycetemcomitans* (30).

A modification of the previously used invasion model was used in this study to study the adhesive nature of smooth-surfaced *A. actinomycetemcomitans* variants. This report supplies important basic information about *A. actinomycetemcomitans* adhesion to epithelial cells and provides a solid foundation for future studies of adhesion.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *A. actinomycetemcomitans* SUNY 75(S) (S stands for smooth) and SUNY 465 were obtained from J. J. Zambon, State University of New York, Buffalo. Strain 652 was obtained from A. Tanner, Forsyth Dental School, Boston, Mass. All three strains exhibit a smooth colony morphology. VT 749, a rough variant of SUNY 465, was generated in broth in our laboratory (14). Cells were stored frozen in Trypticase soy broth which contained 0.6% yeast extract (TSB-YE) (Difco Laboratories, Detroit, Mich.) and 10% dimethyl sulfoxide (Sigma Chemical Co., St. Louis, Mo.). Bacteria were grown in TSB-YE and on Trypticase soy agar with 0.6% yeast extract at 37°C in an atmosphere of 10% CO_2 in air. Stationary broth cultures were overnight, statically grown 5-ml cultures with an optical density of 0.3 at 495 nm. Exponential broth cultures were cells from statically grown overnight cultures which were diluted fivefold and incubated further until an optical density of 0.2 at 495 nm was reached. In studies which compared stationary-phase versus exponentially growing broth cultures, exponentially growing cultures with an optical density of 0.175 were used. Stationary-phase agar cultures were aerobically grown, overnight broth cultures which were streaked onto agar and cultured under aerobic and anaerobic conditions for 48 h.

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Colonies were suspended in broth for enumeration and immediately pelleted prior to assay. Adherence was determined with both exponentially growing and stationary-phase broth cultures but with only stationary-phase agar cultures. Bacteria were cultured anaerobically in a GasPak anaerobic system (Becton Dickinson, Cockeysville, Md.) at 37°C. Studies which compared culture conditions and growth phases were carried out concurrently.

Cell culture. Cell lines used in this study were KB (derived from a human oral epidermoid carcinoma) and HeLa (carcinoma of the human cervix). Both cell lines were obtained from J. Moehring, University of Vermont, Burlington. The cells were cultured in RPMI 1640 medium (Sigma) which contained 5% fetal bovine serum (Flow Laboratories, McLean, Va.) and gentamicin (25 μg/ml) (GIBCO Laboratories, Grand Island, N.Y.) in 75-cm^2 flasks at 37°C in a humidified atmosphere of 5% CO_2 in air. Cultures were split by treatment with 0.5 mM EDTA (GIBCO) followed by trypsin (GIBCO) to detach cells.

Adherence assays. Approximately 6×10^5 KB cells in antibiotic-free RPMI 1640 medium were seeded onto 12-mm-diameter glass coverslips in wells of 24-well (16-mm-diameter) tissue culture plates and incubated for 18 h in the standard assay. An inverted microscope was used to determine that the monolayers were confluent. Bacteria were pelleted, and the pellets were drained well and suspended in antibiotic-free RPMI 1640 medium. Bacteria (6×10^7) were added to confluent KB monolayers to attain a multiplicity of infection (MOI) of 100:1. The plates were incubated at 37°C for 2 h in an atmosphere of 5% CO_2 in air. The medium was removed from the infected monolayers, which were subsequently washed three times with Ca^{2+}–Mg^{2+}–phosphate-buffered saline (PBS) to remove nonadherent bacteria. A 0.1-ml volume of a 0.5% Triton X-100 (Sigma) solution in PBS was added to release the cells from the wells. PBS (2 ml) was added to each well to dilute the detergent, appropriate dilutions were spread onto TSB-YE agar, and the CFU which resulted were enumerated. Triplicate or quadruplicate measurements were routinely carried out for each determination. Unless otherwise noted, adherence assays were carried out for 2 h at a MOI of 100:1 with exponentially growing (optical density, 0.2) aerobic broth cultures. An inverted microscope was used to monitor KB monolayers throughout the course of experiments. The term "three strains" refers to SUNY 75(S), SUNY 465, and strain 652.

KB monolayers were omitted in some experiments to test the ability of *A. actinomycetemcomitans* to adhere to the glass coverslip. Other assays were carried out concurrently at 37 and 4°C in the absence of CO_2.

Visualization of adherence with the light microscope was achieved as follows. KB monolayers were not treated with detergent after the 2-h incubation period. Instead, the monolayers were washed and stained with DiffQuik (Dade Diagnostics, Aguada, Puerto Rico), a modification of Wright-Giemsa stain.

SDS-PAGE. Bacteria were pelleted, lysed in sample buffer, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 5 to 15% gradient. The gels were stained with both brilliant blue R-250 and silver.

Electron microscopy. Bacterial colonies were removed from agar plates with a spatula. Broth cultures were pelleted by centrifugation. Colonies or pellets were dispersed or diluted and negatively stained with 2% methylamine tungstate (pH 7.0) (Polysciences, Inc., Warrington, Pa.) by mixing 1 drop of bacterial suspension with 1 drop of stain. Bacteria were suspended and stirred on an epoxy resin slab for 30 s. A drop of suspension was applied to a carbon-coated 200-mesh copper grid for 30 s, and any excess suspension was removed by blotting. Specimens were examined on a JEOL 100CX II TEMSCAN at 80 kV.

Determination of hydrophobicity. The cell surface hydrophobicity of the *A. actinomycetemcomitans* strains was assessed by the hexadecane method of Rosenberg et al. (20). Bacteria were grown and treated as described for adherence assays.

Preparation of rabbit antiserum. SUNY 465 cells were harvested during exponential growth, treated with formalin, and injected into New Zealand White adult rabbits in accordance with established protocols (2). The titer in serum was measured by an enzyme-linked immunosorbent assay (2), and high-titer serum was pooled, treated at 56°C for 30 min to destroy complement, and stored at −20°C. Pooled serum which had been collected from the same rabbits prior to immunization with SUNY 465 served as preimmune serum.

Sugar treatment. Sugars used were D-mannose, mannan, D-glucose, D-galactose, L-fucose, and L-rhamnose. These sugars are some that have been identified as the carbohydrate involved in lectin-mediated adherence by certain bacterial species (1, 10, 15, 16, 31). The sugar being tested was dissolved in RPMI 1640 medium at a concentration of 4%. Bacteria were suspended in RPMI 1640 medium with or without sugar, kept at room temperature for 15 min, and added to the assay medium to yield a final sugar concentration of 1%. Bacteria were also suspended at room temperature with or without sugar for 30 min. The bacteria were washed with PBS, centrifuged (twice) to remove unbound sugar, resuspended in fresh RPMI 1640 medium, and added to KB cells.

RESULTS

Development of standard adherence assay. Preliminary experiments carried out with semiconfluent cell monolayers (10^5 KB cells per well) and aerobically grown broth cultures revealed that all three bacterial strains adhered to both glass and plastic. Both SUNY 75(S) and SUNY 465 adhered better to glass coverslips (no KB cells) than to confluent KB monolayers (on glass coverslips). Thus, the number of *A. actinomycetemcomitans* cells which adhered to epithelial cells could be defined only when no glass or plastic was exposed. Therefore, all subsequent studies were carried out with confluent monolayers. Determinations for adherence of bacteria to mammalian cells customarily use a MOI of 50 or 100 bacteria to 1 mammalian cell in a 1- to 2-h assay (8, 22, 24, 29). When we used 6×10^7 bacteria and 6×10^5 KB cells (confluent monolayer) per well in a 2-h assay, the number of *A. actinomycetemcomitans* cells associated with the KB cells was well defined for all three strains. Under these (and all other) adhesion conditions, adherence could be assessed accurately, because it was determined that invasion was minimal (fewer than 1% of the total cell-associated *A. actinomycetemcomitans* organisms were internalized). Typical adherence values (CFU recovered) ranged from 25×10^5 to 50×10^5 for SUNY 75(S), from 5×10^5 to 10×10^5 for SUNY 465, and from 1×10^5 to 3×10^5 for strain 652. Values varied from day to day, but within experiments replicate values were consistent and relative adherence levels [SUNY 75(S) > SUNY 465 > strain 652] were maintained. VT 749, the broth-generated rough variant of SUNY 465, adhered 5- to 10-fold better than SUNY 465 did. Light microscopy revealed no qualitative differences in the adhesion patterns of the three smooth strains. In all cases there were individual cells, as well as some chains and clumps (results not shown). Sublethal sonication of bacteria before use

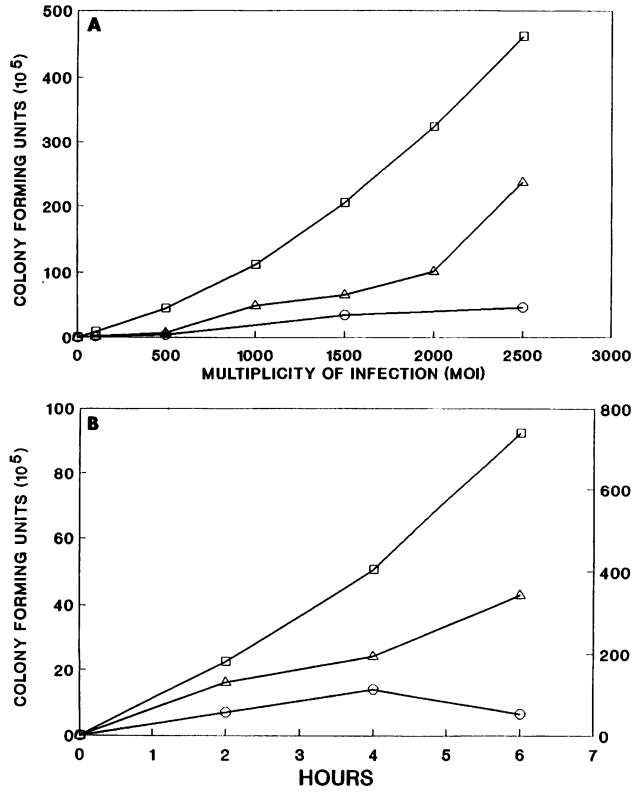

FIG. 1. Kinetic parameters of adherence of *A. actinomycetemcomitans* to KB cell monolayers following aerobic growth in TSB-YE. Symbols: △, SUNY 465; □, SUNY 75(S); ○, strain 652. (A) Effect of bacterial concentration (MOI) in a 2-h assay. (B) Effect of incubation time at an MOI of 800:1. In panel B, *y*-axis 1 (left) represents SUNY 465 and strain 652 values, whereas *y*-axis 2 (right) represents SUNY 75(S) values. Points represent the mean ± standard deviation of triplicate samples from a typical experiment. The standard deviation was less than 25% of the mean in all cases.

in the assay did not disrupt *A. actinomycetemcomitans* aggregation.

Effect of incubation period and bacterial number on adherence to KB monolayers. Adherence of all three strains increased with increasing bacterial concentrations after aerobic growth in broth (Fig. 1A). At all bacterial concentrations, SUNY 75(S) adhered better than SUNY 465, which adhered better than strain 652. At a MOI of 1,500:1, adherence by strain 652 had leveled off, whereas for SUNY 75(S) and SUNY 465 it was still increasing. The kinetics of adherence of the three *A. actinomycetemcomitans* strains is shown in Fig. 1B. In these determinations a MOI of 800:1 in a 2-h assay was used, because at these concentrations the differences in adhesion were defined, but saturation of the epithelial cells was not evident for any strain. Adherence of both SUNY 75(S) and SUNY 465 increased with time over the 6-h assay. Beyond 6 h, the number of CFU recovered decreased for SUNY 75(S) but increased for SUNY 465. The decreased number of SUNY 75(S) was due to loss of KB cells (as determined by microscopic examination), which was most probably the result of highly acidic medium in the SUNY 75(S) wells. In contrast to SUNY 75(S) and SUNY 465, adherence by strain 652 plateaued after 4 h. In other kinetic experiments with SUNY 465 at lower MOI (100:1 and 250:1) and time points less than 2 h, a lag period was detected before adherence accelerated (data not shown).

TABLE 1. Comparison of adherence of *A. actinomycetemcomitans* strains to KB monolayers at 37 and 4°C

Strain	CFU recovered (10^5)[a] at:		CFU ratio (37°C/4°C)[b]
	37°C	4°C	
SUNY 75(S)	50.0 ± 9.5	5.9 ± 1.1	8.5
SUNY 465	14.4 ± 4.8	3.48 ± 1.0	4.1
652	6.6 ± 2.2	0.16 ± 0.05	41.3

[a] Adherence is defined as the CFU recovered after a 2-h assay at a MOI of 100:1 under the conditions indicated. Bacteria were cultured aerobically in broth. Values represent the mean ± standard deviation of quadruplicate samples from a typical experiment.

[b] CFU recovered after assay at 37°C divided by the CFU recovered after assay at 4°C.

Comparison of adherence at 37 and 4°C. Adherence to KB monolayers by all three strains cultured aerobically in broth was substantially better at 37°C than at 4°C. The differences were 8- and 6-fold for SUNY 75(S) and SUNY 465, respectively, whereas a 40-fold difference was exhibited by strain 652 (Table 1). Controls were bacteria which were only incubated for 2 h at 37 and 4°C. There was no decrease in the number of cells after incubation at 4°C, which indicated that the decrease in adherence which occurred at 4°C did not result from decreased viability. As indicated above, at 37°C both SUNY 75(S) and SUNY 465 cells adhered at least as well to glass coverslips as to KB cells. By contrast, strain 652 adhered over fivefold better to KB cells than to glass coverslips. Adherence of SUNY 75(S), SUNY 465, and strain 652 to glass coverslips (no KB cells) was 127, 219, and 15%, respectively, of that with a confluent monolayer (standard assay). All three strains also adhered better to glass coverslips at 37°C than at 4°C (data not shown).

Effect of growth phase on adherence of aerobically grown broth cultures. Adherence of exponentially growing and stationary aerobic broth cultures was compared (Table 2). Both SUNY 75(S) and strain 652 were more adherent when harvested during exponential growth then when harvested during the stationary phase. By contrast, SUNY 465 stationary-phase cultures were more adherent than were exponentially growing cultures. Specifically, adherence of exponentially growing cultures of SUNY 75(S), SUNY 465, and strain 652 was 213, 54, and 157%, respectively, of that of stationary cultures (Table 2).

TABLE 2. Effect of growth phase and culture conditions on *A. actinomycetemcomitans* adherence

Condition	CFU (10^5) of:		
	SUNY 75(S)	SUNY 465	652
Growth phase (aerobic broth)[a]			
Exponential (mid)	51.2 ± 12.4	11.2 ± 4.2	8.8 ± 2.4
Stationary	24.0 ± 5.0	20.8 ± 6.4	5.6 ± 1.8
Culture conditions[b]			
Broth (late exponential)			
Aerobic	47.3 ± 7.5	20.7 ± 1.9	14.6 ± 6.5
Anaerobic	71.5 ± 8.6	31.2 ± 10.1	35.4 ± 4.2
Agar (stationary)			
Aerobic	3.42 ± 1.17	4.24 ± .07	8.08 ± 2.31
Anaerobic	4.65 ± 1.93	12.72 ± 4.06	10.18 ± 2.86

[a] Values represent the mean ± standard deviation of quadruplicate samples from a typical experiment.

[b] Values represent the mean ± standard deviation of quadruplicate samples from four individual experiments.

Comparison of adherence of aerobically and anaerobically grown broth cultures. All three strains adhered better when grown in broth anaerobically than when grown aerobically (Table 2). Adherence by anaerobically grown SUNY 75(S) and 465 cells was about 1.5-fold greater than by those aerobically grown, whereas anaerobically grown strain 652 cells adhered 2.4-fold better than did those grown aerobically.

Adherence of aerobically and anaerobically grown stationary-phase agar cultures. Adherence of SUNY 75(S) and SUNY 465 was substantially lower after growth on agar than after growth in broth under both aerobic and anaerobic conditions (Table 2). The difference on agar was not as clear-cut for strain 652, since aerobically grown stationary-phase broth cultures of strain 652 exhibited very low adherence levels (Table 2). In contrast to what was observed after broth culture, only SUNY 465 showed a difference in adherence when cultured anaerobically versus aerobically on agar. More specifically, after both aerobic and anaerobic growth on agar, SUNY 75(S) no longer exhibited the high levels of adherence which occurred after broth culture. Instead, its adherence was actually lower than that of the other two strains (Table 2). SUNY 465 cells cultured anaerobically on agar adhered threefold better than did those cultured aerobically (Table 2), and cultures generated from assays contained a heterogeneous population with numerous colonies exhibiting a rough phenotype rather than the usual homogeneous smooth phenotype. There was a good correlation between the number of rough colonies and the extent of adherence; the greater the number of rough colonies, the greater the extent of adherence. By contrast, no morphological differences were observed between anaerobically grown agar cultures of either SUNY 75(S) or strain 652.

Surface ultrastructure of *A. actinomycetemcomitans* strains. Electron microscopy revealed that the three strains had distinct surface characteristics. Under the various growth conditions, small vesicles (ExVes), fibrillar membranous extensions with knob-like ends (MemVes), extracellular amorphous material (ExAmMat), and fimbriae were observed in association with one or more of the strains (Fig. 2). Details are presented below. A comparison of the surface ultrastructures and adherence levels exhibited by the three strains under the various culture conditions appears in Table 3.

(i) SUNY 75(S). After aerobic growth in broth, SUNY 75(S) cells were enmeshed (singularly and in groups) in ExAmMat which completely obscured the cell surface (Fig. 2A). After anaerobic growth in broth, SUNY 75(S) cells did not exhibit ExAmMat; however, ExVes were associated with their surface (Fig. 2B). After both aerobic (Fig. 2C) and anaerobic (Fig. 2D) growth on agar, SUNY 75(S) cells were associated with large amounts of MemVes but no ExAmMat or ExVes.

(ii) SUNY 465. After aerobic growth in broth, SUNY 465 cells were associated with small amounts of ExVes (Fig. 2E); the number of ExVes increased substantially after anaerobic broth culture (Fig. 2F). After aerobic growth on agar, SUNY 465 had copious amounts of MemVes but few if any ExVes (Fig. 2G). MemVes were not prominent after anaerobic growth on agar. Instead, fimbriae in peritrichous array were associated with many SUNY 465 bacteria (Fig. 2H).

(iii) Strain 652. After aerobic growth in broth, the surface of strain 652 had little or no surface-associated material (Fig. 2I). However, after anaerobic growth in broth its surface was associated with a number of ExVes (Fig. 2J). A few ExVes were also associated with strain 652 after both aerobic (Fig. 2K) and anaerobic (Fig. 2L) agar growth.

In summary, ExVes were associated with all strains under certain growth conditions, whereas only SUNY 75(S) and SUNY 465 ever exhibited MemVes. Fimbriae were observed only on SUNY 465 and only when the cells were grown anaerobically on agar. VT 749, the broth-generated rough variant of SUNY 465, exhibited no fimbriae (data not shown). ExAmMat was associated only with SUNY 75(S) and only after aerobic growth in broth.

SDS-PAGE protein profiles of whole-cell extracts. Whole-cell extracts of cultures grown under the conditions noted above were analyzed by SDS-PAGE to determine whether there was any correlation between protein-banding patterns and adherence levels. The protein profiles of aerobic broth cultures of the three strains exhibited numerous bands. The banding patterns were for the most part the same, although occasional banding differences occurred (data not shown). There was no clear-cut correlation between the presence or absence of specific protein bands and the high degree of adherence exhibited by SUNY 75(S). The protein profiles were modified in response to other culture conditions, which also, in certain instances, affected adherence. However, in many instances it was impossible to interpret the data accurately because of the abundance of bands in the gels, including many doublets revealed by silver staining.

Cell surface hydrophobicity. More than 89% of the cells of all three strains remained in the aqueous phase after aerobic and anaerobic growth on agar, which indicates that their cell surfaces possess strongly hydrophilic properties (Table 3). Whereas more than 87% of the cells of SUNY 465 and strain 652 remained in the aqueous phase after aerobic and anaerobic broth culture, for SUNY 75(S) only about 80% did. This suggests that the surface of SUNY 75(S) cells may be slightly less hydrophilic than that of the other two strains after broth culture (Table 3).

Influence of SUNY 465 immune serum on adherence. Subagglutinating concentrations of SUNY 465 immune serum produced a modest decrease in the adherence of SUNY 465 to KB cells (data not shown). A maximum decrease (26%) was observed at a 1:400 dilution. At a 1:1,600 dilution a 19% decrease in adherence occurred, whereas at a 1:3,200 dilution the number of CFU recovered was the same after pretreatment with immune and preimmune serum.

Consequences of sugar treatments. Cells were subjected to treatments with various sugars to determine whether carbohydrate-specific lectins on the surface of *A. actinomycetemcomitans* cells are involved in adherence. The sugars tested, i.e., mannose, mannan, glucose, galactose, fucose, and rhamnose, are some that are specific for lectins (including fimbriae) on the surface of *Escherichia coli* (1, 16), *Fusobacterium* species (15), *Vibrio cholerae* (10), and a *Capnocytophaga* species (a gram-negative oral bacterium that mediates coaggregation with gram-positive oral species) (31). None of the sugars caused decreased adherence of any of the three strains.

DISCUSSION

This report is the first in-depth comparative study describing the adherence of *A. actinomycetemcomitans* organisms to epithelial cells. All three smooth *A. actinomycetemcomitans* strains adhered to KB epithelial cells. The adherence characteristics and cell surface of strain 652, a nonleukotoxic strain, differed markedly from those of the other strains. Whether a correlation exists between the leukotoxin phenotype and adherence remains to be determined. The kinetics and saturation studies showed that adherence by strain 652 reached saturation. Although the degree of adherence by SUNY 75(S) was greater than that by SUNY 465, saturation was not attained with either strain. We believe that saturation of SUNY 75(S)

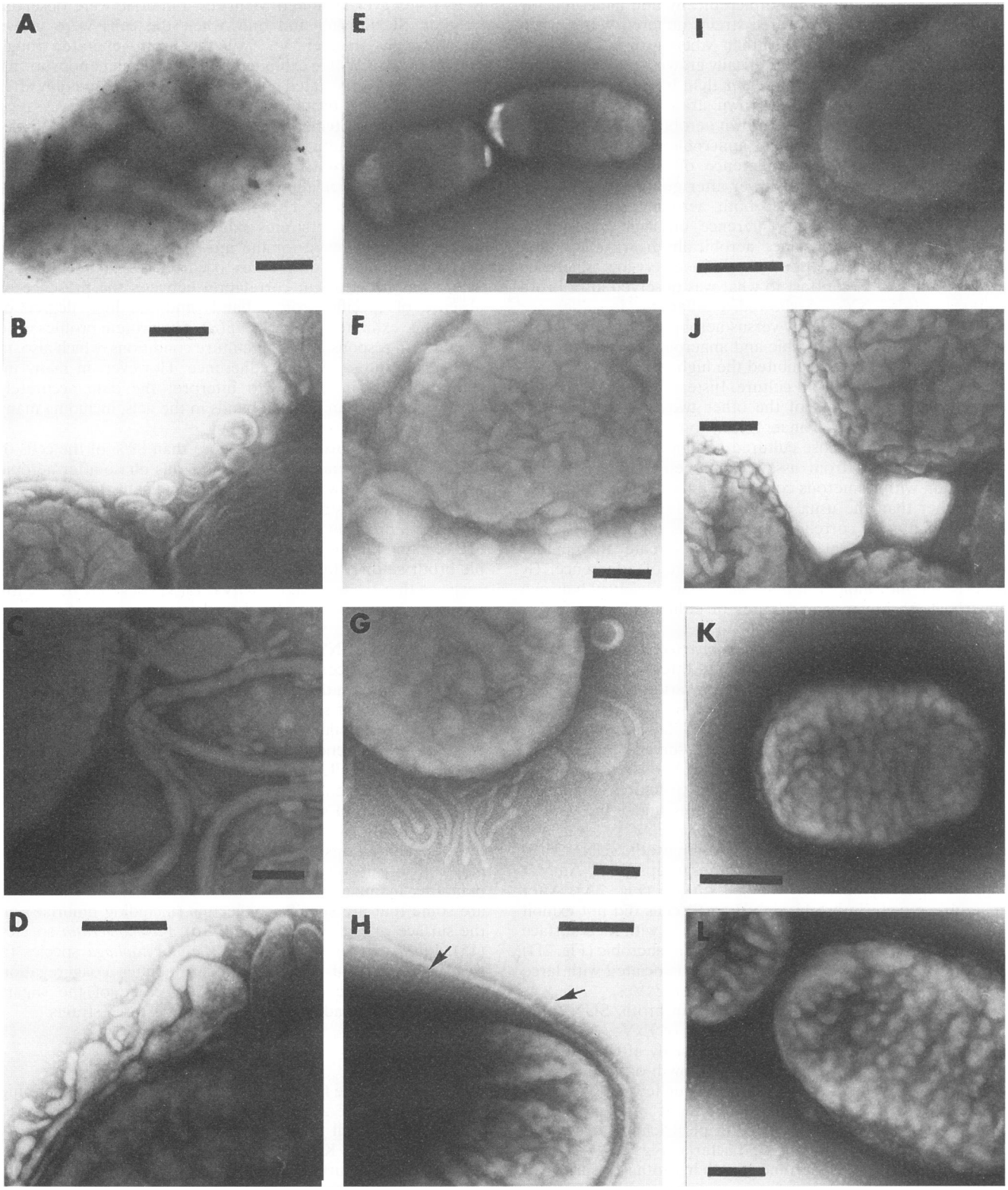

FIG. 2. Electron micrographs of *A. actinomycetemcomitans* strains under various culture conditions. (A to D) SUNY 75(S) cells incubated in broth aerobically (panel A) and anaerobically (panel B) and on agar aerobically (panel C) and anaerobically (panel D). (E to H) SUNY 465 cells incubated in broth aerobically (panel E) and anaerobically (panel F) and on agar aerobically (panel G) and anaerobically (panel H). (I to L) Strain 652 incubated in broth aerobically (panel I) and anaerobically (panel J) and on agar aerobically (panel K) and anaerobically (panel L). Note the ExAmMat associated with SUNY 75(S) in panel A and the peritrichous fimbriae (arrows) associated with SUNY 465 in panel H. Also note the microvesicle material (ExVes and MemVes) associated with all strains, even in the nonleukotoxic strain 652, following anaerobic growth in broth (panel J). Bars: panels A and E, 0.5 μm; B, D, F, and H to L, 0.25 μm; C and G, 0.1 μm.

TABLE 3. Comparison of *A. actinomycetemcomitans* surface ultrastructure, hydrophobicity, and adherence under various culture conditions

Strain	Culture condition	Surface structure	Hydrophobicity (%)[a]	Adherence[b]
SUNY 75(S)	Aerobic broth	ExAmMat	79 ± 4	13+
	Anaerobic broth	ExVes	81 ± 7	18+
	Aerobic agar	MemVes	100 ± 1	1+
	Anaerobic agar	MemVes	98 ± 2	1+
SUNY 465	Aerobic broth	ExVes	93 ± 2	5+
	Anaerobic broth	ExVes	88 ± 1	8+
	Aerobic agar	MemVes	100 ± 1	1+
	Anaerobic agar	Fimbriae	100 ± 1	4+
652	Aerobic broth	ExVes?	87 ± 3	4+
	Anaerobic broth	ExVes	88 ± 7	10+
	Aerobic agar	ExVes (↓)	89 ± 2	2+
	Anaerobic agar	ExVes (↓)	89 ± 1	2+

[a] Percentage of bacteria remaining in the aqueous phase after hexadecane partitioning. Values represent the mean ± standard deviation of triplicate (aerobic broth) or duplicate (all others) samples from two individual experiments.

[b] Adherence to KB epithelial cells exhibited by bacteria after growth under various conditions. Adherence was scored from 1+ to 18+ on the basis of relative adherence levels, with 1+ representing the lowest level of adherence observed and 18+ representing the highest.

and SUNY 465 was not achieved because of bacterium-bacterium interactions. Bacterium-bacterium interactions could result in an underestimation of adherence because individual cells and aggregated cells both produce a single CFU. All *A. actinomycetemcomitans* strains tend to clump and form chains which cannot be broken up by vortexing or sublethal sonication. Since our standard adherence assay involved low MOI and short periods, bacterium-bacterium interactions were minimal so that KB cell-bacterium interactions were measured.

In addition to adhering to epithelial cells, all three smooth *A. actinomycetemcomitans* strains adhered to glass. In fact, both SUNY 75(S) and SUNY 465 adhered better to glass coverslips than to KB cells. By comparison, strain 652 adhered substantially better to the epithelial cells, further evidence that this nonleukotoxic strain (which had few if any ExVes and no MemVes) may adhere by a different mechanism(s) from those used by the other two. Many bacterial species adhere as well at 4°C as they do at 37°C. All three *A. actinomycetemcomitans* strains were significantly more adherent at 37°C than at 4°C, which suggests that metabolically active *A. actinomycetemcomitans* strains are a prerequisite for optimal adherence.

Both the type of medium (broth or agar) and the anaerobicity influenced adherence levels and cell surface characteristics of all strains. A relationship between adherence levels and specific cell surface components (ExAmMat, ExVes, and fimbriae) was observed, which suggested that these components play a role in *A. actinomycetemcomitans* adherence to epithelial cells. After aerobic growth in broth, SUNY 75(S) cells were associated with ExAmMat and adhered significantly better than the other two strains, which were not associated with ExAmMat. A recent report from our laboratory confirms the adhesive nature of ExAmMat (13). We demonstrated that the high adherence level exhibited by SUNY 75(S) cells after aerobic growth in broth was decreased substantially after the cells were washed with PBS (13). Furthermore, adherence of other *A. actinomycetemcomitans* strains was increased in the presence of SUNY 75(S) ExAmMat (13).

The present study also suggested a correlation between adherence and ExVes. In this regard, we recently reported that ExVes isolated from SUNY 75(S) cells increase adherence of both SUNY 465 and strain 652 to epithelial cells (13). Interestingly, adherence by *A. actinomycetemcomitans* strains associated with copious amounts of MemVes was low. Since ExVes bud off from MemVes, this finding was somewhat surprising, and at present we have no explanation for it. One possibility is that MemVes and ExVes have identical properties but that the fibrillar network of the MemVes interferes in some way with the adherence process. Another possibility is that the adherence capability associated with ExVes arises during the budding-off process. Recently it has been shown that components associated with fibrillar projections which emanate from the surface of *Salmonella typhimurium* are involved in adherence and invasion-signaling mechanisms (3). *A. actinomycetemcomitans* MemVes and the fibrillar projections of the *Salmonella* strain appear morphologically similar, which suggests that MemVes of *A. actinomycetemcomitans* could be involved in a similar signaling mechanism. Studies to determine the nature of ExVes-associated adherence are under way. Extracellular vesicles associated with *Porphorymonas gingivalis*, an oral pathogen also implicated in periodontal disease, function in attachment (25), and it has been reported that they bear fimbriae (6).

SUNY 465 immune serum raised against whole bacteria produced only a modest decrease in adherence. This suggests that the adhesin(s) may not be very immunogenic. The immunological response of many patients with periodontitis is directed primarily toward a protein with a molecular mass of 29 kDa, with little reactivity toward other antigens (32). The immunogenic response to this antigen may be so great that it dominates the immunological mechanism and necessitates the generation of immune sera raised to specific cell surface components. Previously we reported that SUNY 465 immune serum inhibited invasion of SUNY 465 by as much as 90% (28). Since SUNY 465 immune serum had little effect on adherence, it indicates that *A. actinomycetemcomitans* adhesion and invasion mechanisms do not share a common antigen.

Gibbons et al. reported that the cell surface of *A. actinomycetemcomitans* was very hydrophilic (4), whereas others who used different hydrocarbons found *A. actinomycetemcomitans* to be hydrophobic (7, 12). In this study we used methods identical to those of Gibbons et al. (4). All three *A. actinomycetemcomitans* strains were very hydrophilic, and there was essentially no correlation between hydrophobic tendencies and adherence to epithelial cells. Our studies suggest that although hydrophobic interactions may contribute in a subordinate way to the overall adherence mechanism, it is unlikely that hydrophobicity per se is a major factor in *A. actinomycetemcomitans* adherence. Widespread discrepancy with regard to cell surface hydrophobicity of *A. actinomycetemcomitans* is most probably the result of the use of different experimental conditions (4, 7, 12; see above) and indicate that caution must be used when comparisons are made and conclusions are drawn.

These studies and those of others demonstrate that *A. actinomycetemcomitans* cells have fimbriae and suggest that fimbriae most probably play some role in *A. actinomycetemcomitans* adherence (5, 9, 17, 23). It has been reported that an 11-kDa protein of *A. actinomycetemcomitans* may function as an adhesin associated with fimbriae (5). The *A. actinomycetemcomitans* strains we studied exhibited an 11-kDa band; however, we observed no changes in this band, even with SUNY 465 cells when they exhibited fimbriae and adherence was elevated. None of the sugars we tested blocked adherence of *A. actinomycetemcomitans*. Additional sugars must be tested to

determine whether *A. actinomycetemcomitans* fimbriae (or other as yet unidentified lectins) interact with specific carbohydrates in adherence processes.

Smooth-to-rough variant shifts are indicative of shifts from nonfimbriated to fimbriated *A. actinomycetemcomitans* cells (18). The smooth-to-rough variant shift which we observed after anaerobic growth of SUNY 465 cells on agar was accompanied by cell fimbriation and increased adherence. Under the same conditions, SUNY 75(S) cells did not undergo a variant shift, nor did they become fimbriated, and their adherence level was increased by only 50%. VT 749, the broth-generated, poorly invasive (14) rough variant of SUNY 465, is highly adherent but does not exhibit fimbriae. Scannapieco et al. reported that after agar culture, SUNY 75(R) cells grown anaerobically had more fimbriae than those grown aerobically but that neither had fimbriae after broth culture (23). The fact that we observed no fimbriae attached to SUNY 75(S) cells after anaerobic growth on agar and that we (and many other laboratories) observed no fimbriae on *A. actinomycetemcomitans* cells after growth in broth may have many explanations. The fragility of *A. actinomycetemcomitans* fimbriae (23) would favor their loss during manipulation. Furthermore, visualization of fimbriae might be impaired by other cell surface components (such as ExAmMat). Scannapieco et al. reported that *A. actinomycetemcomitans* surfaces were frequently obscured with amorphous material which hampered visualization (23). It is also possible that certain strains have a greater potential to exhibit fimbriae (or more fimbriae) than others. Inouye et al. have purified fimbriae with a 54-kDa subunit protein from *A. actinomycetemcomitans* 310, isolated from a patient with rapid progressive periodontitis (9).

In summary, certain *A. actinomycetemcomitans* strains are capable of undergoing a variant shift which is associated with changes in adherence and invasion properties. We propose that fimbriae most probably function in adherence of rough variants, whereas nonfimbrial components (such as vesicles) are probably involved in adherence of smooth, highly invasive strains. Since many clinical isolates of *A. actinomycetemcomitans* exhibit a rough colony morphology, it seems likely that the variant shift is relevant to the in vivo situation. Certain oral environments might favor rough, highly adherent variants which advance colonization. Other conditions in the ever-changing oral environment might trigger a shift to smooth variants which promote invasion. Variant shifts of this nature could contribute to the cyclical nature of periodontal disease.

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