The *Actinobacillus actinomycetemcomitans* Autotransporter Adhesin Aae Exhibits Specificity for Buccal Epithelial Cells from Humans and Old World Primates

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**Cells of the gram-negative periodontopathogen *Actinobacillus actinomycetemcomitans* express a surface-exposed, outer membrane autotransporter protein, designated Aae, which has been implicated in epithelial cell binding. We constructed a mutant strain of *A. actinomycetemcomitans* that contained a transposon insertion in the *Aae* structural gene (*aae*) and tested the mutant to determine its ability to bind to buccal epithelial cells (BECs) isolated from healthy volunteers. Significantly fewer mutant cells than wild-type cells bound to BECs. A broad-host-range plasmid that contained an intact *aae* gene driven by a heterologous lac promoter restored the ability of the mutant strain to bind to BECs at wild-type levels. This plasmid also conferred upon *Escherichia coli* the ability to express the Aae protein on its surface and to bind to human BECs. Aae-expressing *E. coli* also bound to BECs isolated from six Old World primates but not to BECs isolated from four New World primates or nine other nonprimate mammals, as well as to human gingival epithelial cells but not to human pharyngeal, palatal, tongue, bronchial, or cervical epithelial cells. Our findings indicate that Aae mediates binding of *A. actinomycetemcomitans* to BECs from humans and Old World primates and that this process may contribute to the host range specificity and tissue tropism exhibited by this bacterium.**

*Actinobacillus actinomycetemcomitans* is a gram-negative bacterium that colonizes the oral cavities of humans and Old World primates (3). In humans, *A. actinomycetemcomitans* causes a severe and rapid form of periodontal disease that affects adolescents (23). *A. actinomycetemcomitans* is also a member of the clinically important HACEK group of oral bacteria (Haemophilus paraphrophilus, Haemophilus parainfluenzae, Haemophilus aphrophilus, Haemophilus paraphrophilus, *A. actinomycetemcomitans*, Cardiobacterium hominis, Eikenella corrodens, and Kingella kingae), which has been implicated in the etiology of infective endocarditis (1). *A. actinomycetemcomitans* cells secrete leukotoxin, a 120-kDa lipoprotein that kills polymorphonuclear leukocytes and macrophages of humans and Old World primates (19). Leukotoxin is considered to be an important virulence factor which may also play a role in the observed host range specificity of *A. actinomycetemcomitans*. 

*A. actinomycetemcomitans* forms extremely tenacious biofilms on inert surfaces in vitro (5), a property that may contribute to the ability of *A. actinomycetemcomitans* to colonize surfaces such as teeth and damaged heart tissue. Tight adherence is mediated by adhesive type IV pili which are composed of repeating subunits of a 6.5-kDa protein designated Flp-1 (10, 11). Mutants with mutations in the Flp-1 structural gene (flp-1) fail to form biofilms in vitro and are unable colonize the oral cavity, elicit an immune response, or cause bone loss in a rat model of periodontal disease (18). These findings suggest that biofilm formation plays an important role in the pathogenesis of *A. actinomycetemcomitans*. However, *A. actinomycetemcomitans* is routinely isolated from mucosal surfaces of predentate children as young as 20 days old (13, 20). In adults, *A. actinomycetemcomitans* is recovered more frequently and in higher numbers from oral mucosal surfaces than from subgingival and supragingival plaque, and mucosal surfaces can have high diagnostic value for identifying individuals colonized by *A. actinomycetemcomitans* (2, 15). These findings suggest that the oral mucosa is the initial site colonized by *A. actinomycetemcomitans* and the primary reservoir of *A. actinomycetemcomitans* in the oral cavity.

In vitro, *A. actinomycetemcomitans* is capable of binding to and invading epithelial cells (4, 14, 16), a property that may play a role in the ability of *A. actinomycetemcomitans* to colonize mucosal surfaces. Rose et al. (16) showed that *A. actinomycetemcomitans* cells express a 90-kDa surface-exposed protein, designated Aae, that is homologous to an epithelial cell adhesin (Hap) produced by *H. influenzae* (9). Aae is a member of the autotransporter family of bacterial proteins, which are characterized by a C-terminal domain that becomes integrated into the bacterial outer membrane and an N-terminal domain (the passenger domain) that is exposed on the cell surface (8). Rose et al. (16) showed that Aae mediates weak binding of *A. actinomycetemcomitans* to KB cells, a cell line that was originally thought to be derived from an epidermal carcinoma of the mouth but was subsequently found, based on isoenzyme, HeLa marker chromosome, and DNA fingerprinting analyses, to have been established via contamination by the human cervix carcinoma cell line HeLa (product information sheet ATCC CCL-17; American Type Culture Collection, Manassas, Va.).

In the present study we examined the host range specificity and tissue tropism of *A. actinomycetemcomitans* Aae. By using an *A. actinomycetemcomitans* aae knockout strain and a broad-host-range plasmid that expressed wild-type Aae in both *A. actinomycetemcomitans* and *Escherichia coli*, we obtained evi-
ence that Aae mediates binding of *A. actinomycetemcomitans* to buccal epithelial cells (BECs) isolated specifically from humans and Old World primates.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The bacterial strains used in this study are listed in Table 1. *A. actinomycetemcomitans* strains were cultured in 100-mm-diameter tissue-culture-treated polystyrene petri dishes (model 430167; Corning) containing 20 ml of Trypticase soy broth supplemented with 6 g of yeast extract per liter and 8 g of glucose per liter. Plasmids were mobilized into *E. coli* by using the RK2 ori-selective-defective mutant plasmid pRK2761 as previously described (21). Plasmid-harboring strains of *A. actinomycetemcomitans* were cultured in medium containing 3 μg of chloramphenicol per ml. Inoculated culture vessels were incubated for 24 to 48 h at 37°C in an atmosphere containing 10% CO<sub>2</sub>. Cells were harvested by using a cell scraper, washed three times with phosphate-buffered saline (PBS), and then sonicated on ice for 30 s at 30% capacity with a 30% duty cycle by using a Branson model 450 sonicator equipped with a cup horn in order to disrupt cell aggregates. Previous studies showed that this brief sonication step had no effect on cell viability (4).

**Polystyrene adherence assay.** *A. actinomycetemcomitans* strains were cultured in medium containing 3 μg of chloramphenicol per ml. Inoculated culture vessels were incubated for 24 to 48 h at 37°C in an atmosphere containing 10% CO<sub>2</sub>. Cells were harvested by using a cell scraper, washed three times with phosphate-buffered saline (PBS), and then sonicated on ice for 30 s at 30% capacity with a 30% duty cycle by using a Branson model 450 sonicator equipped with a cup horn in order to disrupt cell aggregates. Previous studies showed that this brief sonication step had no effect on cell viability (4).

**Construction of an aae knockout strain.** Genomic DNA from *A. actinomycetemcomitans* strain CU1000 was amplified by PCR by using forward primer CGCG CAGCTCCCTCATAACGTGTCGTCAGAAGAAGAGAAAGTG and reverse primer GGCGCTGCAGCTACCTAGTAAATCGAGTTTCTGCGCTCCATATAAGAAGAAAGTG (11). This study introduced a BamHI restriction site (underlined) 18 bp upstream of the aae initiation codon (boldface type), and reverse primer GGCGCTGCAGCTACCTAGTAAATCGAGTTTCTGCGCTCCATATAAGAAGAAAGTG (11). This study introduced a BamHI restriction site (underlined) immediately downstream of the aae stop codon (boldface type). The PCR product (2.5 kb) was digested with BamHI and PstI and ligated into the BamHI/PstI sites of plasmid pET-29b. The DNA sequence of the insert from the resulting plasmid (pVK45) was 99.4% identical (37 base changes) to the DNA sequence of *A. actinomycetemcomitans* strain HK1651 (GenBank accession no. X926734). Plasmid pVK45 was mutagenized with an EZ-TN transposon mutagenesis kit (Epitect), which inserted a copy of the 2.0-kb kanamycin resistance transposon R6K at a random location in the plasmid. One plasmid (designated pVK52) contained a transposon insertion near the middle of the *aae* gene (12). Plasmid pVK52 was used to transform strain *A. actinomycetemcomitans* to kanamycin resistance by using a natural transformation protocol supplied by Mrinal Bhattacharjee and David Figurski (Columbia University). Genomic DNA isolated from the transformants was amplified by PCR by using the primers described above. One transformant (designated JK1046) which produced a PCR product that was 2.0 kb larger than that produced by strain IDH781N was selected. The insertion of transposon R6K at the chromosomal *aae* locus of strain JK1046 was confirmed by DNA sequence analysis across the transposon junctions.

**Polystyrene adherence assay.** A quantitative adherence assay was carried out in 96-well polystyrene microtiter plates as previously described (11). Briefly, cells were inoculated into the wells of the microtiter plates and allowed to adhere for 1 h. Loosely adherent cells were removed by washing, and adherent cells were stained with crystal violet for 10 min and then washed extensively. The crystal violet remaining in each well was then solubilized in ethanol, and the absorbance of the solution at 595 nm was measured. Adherence was proportional to optical density. Polystyrene adherence assays were performed three times with similar results.

**Isolation of epithelial cells.** Buccal epithelial cells were collected from healthy human volunteers and from various mammalian species by scraping the inside of a cheek with a sterile tongue depressor. Cells were collected in 5 ml of PBS,

<table>
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<td>M. Bhattacharjee and D. Figurski</td>
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<td>pVK71</td>
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<sup>a</sup> Nal<sup>R</sup>, nalidixic acid resistant; Ap<sup>R</sup>, ampicillin resistant; Km<sup>R</sup>, kanamycin resistant; Cm<sup>R</sup>, chloramphenicol resistant.

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**TABLE 1. Bacterial strains and plasmids**

- **A. actinomycetemcomitans** strains
  - IDH781: Wild-type clinical isolate (serotype d)
  - IDH781N: Spontaneous Nal<sup>R</sup> variant of IDH781
  - JKI046: IDH781N aae::R6K[Kan]; Km<sup>R</sup>
  - JKI047: IDH781N flp-1::Trp0336kan; Km<sup>R</sup>
  - CU1000: Wild-type clinical isolate (serotype f)
  - JK1009: CU1000N flp-1::Trp0336kan; Nal<sup>R</sup> Km<sup>R</sup>

- **E. coli** strains
  - DH5α: Used for functional expression of *aae*
  - BL21 (DE3): Used for overexpression of *aae* passenger domain

- **Plasmids**
  - LITMUS28: LITMUS28 cloning vector; Ap<sup>R</sup>
  - pVK45: LITMUS28 containing *aae*
  - pVK56: pVK45 *aae::R6K[Kan]; Km<sup>R</sup>
  - pJAK16: Broad-host-range expression vector; Cm<sup>R</sup>
  - pVK43: pJAK16 containing flp-1
  - pRA33: pJAK16 containing flp-1
  - pET-29b: pET-29b expression plasmid
  - pVK71: pET-29b expressing Aae passenger domain

- **Reference or source**
  - 7: M. Bhattacharjee and D. Figurski
  - 6: This study
  - 11: This study
  - Novagen
  - New England Biolabs
washed once, and resuspended in PBS. BECs were counted with a hemocytometer and diluted in PBS to obtain a concentration of 10^3 to 10^4 cells/ml.

Human gingival, palatal, and tongue epithelial cells were collected by using a similar method. Cell lines A-549 (ATCC CCL-185; American Type Culture Collection), NHBE (BioWhittaker, Walkersville, Md.), and KB (ATCC CCL-17) were used as sources of human alveolar, bronchial, and cervical epithelial cells, respectively.

Epithelial cell binding assay. Binding of A. actinomycetemcomitans and E. coli to epithelial cells was measured by using the assay described by Fine and Furgang (4). Briefly, 250 μl of epithelial cells was mixed with 250 μl of bacterial cells in a 2-ml polypropylene microcentrifuge tube, resulting in a ratio of 10^3 to 10^4 bacterial cells per epithelial cell. The tube was gently rotated for 90 min at 37°C. The epithelial cells were separated from unbound bacteria by centrifugation through a Ficoll gradient, and the bacteria bound to the epithelial cells were diluted and plated on agar for enumeration. Attachment of bacterial cells to epithelial cells was confirmed by direct microscopic visualization of rhodamine-labeled E. coli against a fluorescein-cytokeratin-labeled BEC background. All assays were performed in duplicate and on at least three separate occasions. The significance of differences in binding was determined by using an unpaired, two-tailed t test (P < 0.05).

Preparation of anti-Aae antiserum. The N-terminal portion of aae that encodes the surface-exposed passenger domain of the Aae adhesin (corresponding to bp 160 to 1,878 in GenBank accession no. AY487820) was amplified by PCR by using genomic DNA isolated from A. actinomycetemcomitans strain CU1000 as a target. The forward primer (TCAACCGGCACATATGTCAGAGTTTAA) introduced an NdeI restriction site (underlined) upstream of codon 54, and the reverse primer (GCTCGGTACCTGGGTTATATATCGTTGGG) introduced a KpnI restriction site (underlined) downstream of codon 626. The PCR product (1,754 bp) was digested with NdeI and KpnI and ligated into the NdeI/KpnI sites of the T7 expression vector pET-29b (Novagen), resulting in plasmid pVK71. Recombinant Aae passenger domain protein was purified from cultures of E. coli strain BL21(DE3) carrying pVK71 by using an Ni affinity column (Pharmacia model 154-0990). Immunization and bleeding of specific-pathogen-free female New Zealand White rabbits were carried out by Pocono Rabbit Farm and Laboratory (Canadensis, Pa.). The specificity of the resulting antiserum was confirmed by probing filters containing immobilized Aae passenger domain with both preimmune and postimmune sera.

Microscopic assays for functional analysis. Human BECs and E. coli cells were mixed and incubated as described above. BECs incubated in the absence of bacterial cells were used as controls in all experiments. After Ficoll gradient centrifugation, BECs were washed with PBS and heat fixed on glass microscope slides.

For immunofluorescence microscopy, microscope slides were treated with anti-Aae antiserum (diluted 1:160) for 60 min at room temperature to label bacterial cells. The slides were then washed twice in PBS and once in distilled water and air dried. The slides were then treated with a 1:20 dilution of tetramethyl rhodamine isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Sigma catalog no. T6778) for 60 min, washed with water, and air dried. To label BECs, slides were treated with a 1:50 dilution of fluorescein isothiocyanate-conjugated anti-cytokeratin monoclonal antibody (Sigma catalog no. F3418) for 60 min, washed with water, and air dried. The slides were viewed at a magnification of ×400 by using an Olympus BX50WI fluorescence microscope equipped with fluorescein and rhodamine filters. Individual tetramethyl rhodamine isothiocyanate and fluorescein isothiocyanate images of each field were digitized and combined in Adobe Photoshop 4.0 to obtain a single dual-fluorescence image.

For crystal violet staining, microscope slides were treated with Gram crystal violet solution (Fisher catalog no. 23-291472) for 60 s and then rinsed extensively with distilled water and air dried. The slides were viewed at a magnification of ×400 by using an Olympus BX50WI microscope under bright-field conditions. Images of each field were recorded and digitized with a computer.

Nucleotide sequence accession number. The DNA sequence of aae from A. actinomycetemcomitans strain CU1000 has been deposited in the GenBank database under accession no. AY487820.

RESULTS

A. actinomycetemcomitans aae mutant is deficient in human buccal epithelial cell binding. We constructed an isogenic mutant of the transformable A. actinomycetemcomitans strain IDH781N which contained a transposon insertion at codon position 376 of aae. This aae mutant strain (designated JK1046) and parental strain IDH781N were tested to determine their abilities to bind to BECs isolated from healthy volunteers (Fig. 1A). Significantly more wild-type cells than mutant cells bound to BECs (210 ± 14 and 14 ± 4 bacterial cells per BEC, respectively; P < 0.01). A plasmid carrying a wild-type aae gene under control of a heterologous IPTG-inducible tac promoter (plasmid pVK43) restored the ability of JK1046 to bind to BECs (Fig. 1A). The bars indicate the mean numbers of A. actinomycetemcomitans (Aa) cells per BEC for duplicate samples, and the error bars indicate ranges. Plasmid pJAK16 was the vector, and plasmid pVK43 was pJAK16 containing aae. (B) Biofilm formation in the wells of a 96-well microtiter plate. The optical density at 590 [OD (590 nm)] was proportional to biofilm formation. Strain JK1047 is an isogenic flp-1 mutant of strain IDH781N that lacks adhesive pili. Plasmid pRA33 is pJAK16 containing flp-1. (C) Binding to BECs isolated from humans and various mammalian species. Solid bars, wild-type strain IDH781; open bars, aae mutant strain JK1046. The asterisks indicate Old World primates. The bars indicate the mean numbers of A. actinomycetemcomitans (Aa) cells per BEC for duplicate samples, and the error bars indicate ranges. The values for species lacking bars were < 1 bacterial cell per 1,000 BECs.
the mutant strain to bind to BECs at wild-type levels (Fig. 1A). The aae mutant exhibited wild-type levels of biofilm formation when it was tested in a 96-well microtiter plate biofilm assay (Fig. 1B). In contrast, a strain which contained a transposon insertion in \textit{flp-1} (strain JK1047) exhibited significantly reduced biofilm formation in the microtiter plate assay (Fig. 1B). These findings indicate that Aae mediates binding of \textit{A. actinomycetemcomitans} to oral epithelial cells but not to abiotic surfaces.

\textbf{Host range specificity of Aae.} We tested \textit{A. actinomycetemcomitans} wild-type strain IDH781N and the aae mutant strain JK1046 to determine their abilities to bind to BECs isolated from six Old World primates, four New World primates, and nine nonprimate mammals (Fig. 1C). Strain IDH781N bound to BECs isolated from Old World primates, rats, and cows. In contrast, strain JK1046 bound at lower levels to BECs isolated from humans and Old World primates and at wild-type levels to BECs isolated from rats and cows. These data suggest that Aae recognizes a receptor present on the surface of BECs of humans and Old World primates and that one or more other adhesins mediate binding of \textit{A. actinomycetemcomitans} to BECs derived from rats and cows.

\textbf{Expression of aae in \textit{E. coli}.} Plasmid pVK43 was transformed into \textit{E. coli} strain DH5\textalpha. Transformants expressed Aae protein on the surface, as determined by immunofluorescence microscopy with anti-Aae antiseraum and a rhodamine-labeled anti-rabbit immunoglobulin G secondary antibody (Fig. 2A and B). Microscopic examination of both rhodamine-labeled \textit{E. coli} against a fluorescein-cytokeratin-labeled BEC background (Fig. 2C and D) and crystal violet-stained bacterial cells (Fig. 2E and F) confirmed that \textit{E. coli} cells expressing Aae bound to human BECs.

When increasing numbers of Aae-expressing \textit{E. coli} cells were added to BECs, a plateau in the number of bacterial cells per BEC was observed (Fig. 3). These data indicate that \textit{E. coli} cells expressing Aae fully saturated the BEC binding sites. The plateau level reached by both the Aae-expressing \textit{E. coli} strain and the \textit{A. actinomycetemcomitans} \textit{flp-1} knockout strain (JK1047) was 1 to 2 logs lower than the plateau level reached by wild-type \textit{A. actinomycetemcomitans} strain IDH781N (Fig. 3). These data suggest that the increased BEC binding exhibited by wild-type strain IDH781N may be due to bacterial autoaggregation mediated by Flp-1 pili (11). Flp-1 protein may also bind to a distinct BEC receptor. The aae mutant strain JK1046 bound to BECs only when high numbers of bacterial cells were added (Fig. 3). This binding may have been nonspecific or may have resulted from the presence of a second BEC adhesin on the surface of \textit{A. actinomycetemcomitans} cells.

When tested with BECs isolated from various mammalian species, Aae-expressing \textit{E. coli} bound only to BECs isolated from humans and Old World primates (Fig. 4). The level of binding of \textit{E. coli} carrying plasmid vector pJAK16 was <1 bacterial cell per 100 BECs. These data confirmed both the host range specificity of Aae and the presence of one or more different \textit{A. actinomycetemcomitans} surface adhesins that recognize BEC receptors of rats and cows.

\textbf{Aae-mediated binding exhibits tissue tropism.} When tested with human epithelial cells derived from various other anatomical sites, the Aae-expressing strain of \textit{E. coli} bound to human gingival epithelial cells (3 ± 1 bacterial cells per epithelial cell) but not to human alveolar, bronchial, palatal, tongue, or cervical epithelial cells (<1 bacterial cell per 100 epithelial cells). It is interesting that \textit{E. coli} cells expressing Aae did not bind to tongue epithelial cells, although the tongue is the oral site that is most frequently colonized by \textit{A. actinomycetemcomitans} (2, 15). This observation suggests that binding of \textit{A. actinomycetemcomitans} to tongue epithelium may be mediated by an adhesion other than Aae.

\textbf{DISCUSSION}

In this report we present physical, microscopic, and genetic evidence that the Aae adhesin mediates binding of \textit{A. actinomycetemcomitans} to BECs isolated from humans and Old World primates but not to BECs isolated from New World primates or nonprimate mammals. Inactivation of aae in wild-type \textit{A. actinomycetemcomitans} strain IDH781N failed to reduce binding of bacterial cells to inert surfaces or to BECs isolated from rats and cows, indicating that other adhesins or surface molecules mediate these interactions. Functional ex-
Our findings provide a molecular basis for the susceptibility of humans to colonization by *A. actinomycetemcomitans*. A complete understanding of the Aae-receptor interaction on a molecular level could be used to develop novel antiadhesive interventions that could have broad therapeutic and preventive applications for diseases caused by *A. actinomycetemcomitans*.

ACKNOWLEDGMENTS

We thank Mrinal Bhattacharjee and David Figurski (Columbia University) for providing the protocols and accompanying bacterial strains and plasmds used for natural transformation of *A. actinomycetemcomitans* and Karen Rice (Southwest National Primate Research Center, San Antonio, Tex.), Keith Mansfield (New England Regional Primate Research Center, Southborough, Mass.), Larry Katz (New Jersey Agricultural Station, Rutgers University, New Brunswick, N.J.), and Helen Schreiner (New Jersey Dental School) for help in obtaining epithelial cells.

REFERENCES


### FIG. 4. Binding of *E. coli* strain DH5α carrying plasmid pVK43 to epithelial cells isolated from humans and various mammalian species.

The asterisks indicate Old World primates. The bars indicate mean numbers of *E. coli* cells per epithelial cell for duplicate samples, and the error bars indicate ranges. The values for species lacking bars were <1 bacterial cell per 1,000 BECs.

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