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Aae, an Autotransporter Involved in Adhesion of *Actinobacillus actinomycetemcomitans* to Epithelial Cells

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The periodontal pathogen *Actinobacillus actinomycetemcomitans* possesses myriad virulence factors, among them the ability to adhere to and invade epithelial cells. Recent advances in the molecular manipulation of this pathogen and the sequencing of strain HK 1651 (http://www.genome.ou.edu/act.html) have facilitated examination of the genetics of its interaction with epithelial cells. The related gram-negative organism, *Haemophilus influenzae*, possesses autotransporter adhesins. A search of the sequence database of strain HK 1651 revealed a homologue with similarity in the pore-forming domain to that of the *H. influenzae* autotransporter, Hap. *A. actinomycetemcomitans* mutants deficient in the homologue, Aae, showed reduced binding to epithelial cells. A method for making *A. actinomycetemcomitans* SUNY 465 transiently resistant to spectinomycin was used with conjugation to generate an isogenic *aae* mutant. An allelic replacement mutant was created in the naturally transformable *A. actinomycetemcomitans* strain ATCC 29523. Lactoferrin, an important part of the innate host defense system, protects against bacterial infection by bactericidal and antiadhesion mechanisms. Lactoferrin in human milk removes or cleaves Hap and another autotransporter, an immunoglobulin A1 protease, from the surface of *H. influenzae*, thereby reducing their binding to epithelial cells. Human milk whey had similar effects on Aae from *A. actinomycetemcomitans* ATCC 29523 and its binding to epithelial cells; however, there was little effect on the binding of SUNY 465. A difference in the genetic structure of *aae* in the two strains, apparently due to the copy number of a 135-base repeated sequence, may be the cause of the differential action of lactoferrin. *aae* is the first *A. actinomycetemcomitans* gene involved in adhesion to epithelial cells to be identified.

The gram-negative bacterium *Actinobacillus actinomycetemcomitans* is strongly implicated in the etiology of severe forms of juvenile and adult periodontitis. Colonization of the gingival sulcus and then the periodontal pocket by bacteria from dental plaque is the initial step in the development of periodontal disease. The ability of bacteria to adhere to surfaces in the oral cavity is essential for colonization. Earlier studies in our laboratory have shown that bacterial surface proteins and structures are important in the adhesion of *A. actinomycetemcomitans* to epithelial cells (33, 38). More recently, genes involved in the formation of long fibrils and bundled pili that are involved in the adherence of *A. actinomycetemcomitans* to solid surfaces have been discovered (9, 27, 44). The authors speculate that these genes may control the binding of *A. actinomycetemcomitans* to the tooth surface as a tenacious biofilm. This is possibly an early step of successful colonization of the oral cavity by *A. actinomycetemcomitans*.

Once established in the oral cavity, *A. actinomycetemcomitans* has been found inside gingival tissues (11, 49) and mucosal epithelium apart from the gingiva (47). The adhesive and invasive nature of *A. actinomycetemcomitans* has been examined with an in vitro model (35, 36, 52). No genes responsible for the attachment to soft tissue have been uncovered, whereas two genes related to invasion have been identified (29, 42, 48). One is homologous to *apaH*, a gene that encodes RGD, a sequence known to bind integrins (48). The *apaH* gene is a homolog of *ialA*, *ygdP*, and *invA*, genes associated with invasion by *Bartonella bacilliformis* (40), *Escherichia coli* K1 (8), and *Rickettsia prowazekii* (21), respectively. The proteins produced by these genes are members of the Nudix family of hydrolases which catalyze the dinucleoside polyphosphates, a class of signaling nucleotides (8, 12, 42). It has also been reported that *A. actinomycetemcomitans* invasion involves genes with sequence homology to *spa* genes, which are involved in protein export (29). The search for more adhesins and invasins has now been made easier with the advent of functional genomics and the whole-genome sequencing of *A. actinomycetemcomitans* and the closely related organism, *Haemophilus influenzae*.

One class of gram-negative adhesins that has been found in organisms of the family *Pasteurellaceae* (genera *Haemophilus*, *Actinobacillus*, and *Pasteurella*) is the autotransporter or type V secretion system (reviewed in reference 24). The family *Pasteurellaceae* contains several pathogens of the upper respiratory tract and oral cavity (31). The autotransporter proteins Hap (55) and Hia (54) of *H. influenzae* are implicated as adhesins in the adhesion of that organism to epithelial cells. The close relationship of *A. actinomycetemcomitans* to *H. influenzae* prompted a search for autotransporter adhesins in *A. actinomycetemcomitans*.

Proteins of the type V secretion system are termed "autotransporters" because they mediate their own transport from the periplasm to the exterior surface of the outer membrane (24). The extreme N terminus of an autotransporter is a signal sequence that targets the newly synthesized polypeptide to a component of the general (or type II) secretion pathway. The signal peptidase of the type II system cleaves the signal sequence and exports the remainder of the protein to the periplasm. The C-terminal region of the autotransporter then

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TABLE 1. Bacterial strains used in this study

Strain	Characteristics	Source or reference
A. actinomycetemcomitans SUNY 465	Clinical isolate, smooth phenotype, invasive; one copy of the *aae* repeat	60
A. actinomycetemcomitans VT 1006	SUNY 465 carrying plasmid pPK1	51
A. actinomycetemcomitans VT 1565	*aae* mutant of SUNY 465	This study
A. actinomycetemcomitans ATCC 29523	Naturally transformable; four copies of the *aae* repeat	This study
A. actinomycetemcomitans VT 1568	*aae* mutant of ATCC 29523	This study
A. actinomycetemcomitans ATCC 29522	Three copies of the *aae* repeat	American Type Culture Collection
A. actinomycetemcomitans SUNY 523	Two copies of the *aae* repeat	60
E. coli JM109	Cloning host for blue-white screen	Lab stock
E. coli DH5α*λpir*	Cloning host for mobilizable plasmids	Lab stock
E. coli SM10*λpir*	Conjugation host for mobilizable plasmids	Lab stock
E. coli VT 1561	JM109 with 1.5-kb fragment in pGEM-T Easy	This study
E. coli VT1562	DH5α*λpir* with Km^r in pGP704; mobilizable plasmid	This study
E. coli VT 1563	DH5α*λpir* with 1.5-kb fragment in pVT1562	This study
E. coli VT1564	SM10*λpir* with 1.5-kb fragment in pVT1562	This study
E. coli VT 1566	JM109 with 3.1-kb fragment (whole *aae* gene) in pGEM-T Easy	This study
E. coli VT1567	JM109 with Km^r inserted at the *Hin*dIII site in pVT1567 (disrupts *aae* gene)	This study

forms a β-barrel pore in the outer membrane through which the N-terminal "passenger domain" is threaded for presentation on the surface of the cell.

Comparison of autotransporter proteins from *H. influenzae* to the *A. actinomycetemcomitans* genome database revealed an open reading frame, termed *aae*, with characteristics of an autotransporter. We report here that mutation of this gene in two strains of *A. actinomycetemcomitans* resulted in a defect in adhesion to epithelial cells.

Lactoferrin is an iron-binding glycoprotein present in human milk and saliva and serves as part of the innate host defense system, possessing antibacterial (6) and antifungal (50) effects. Unsaturated lactoferrin (iron free and anion free) is able to kill *A. actinomycetemcomitans* (28), probably through damage to the cell envelope (18). Iron- containing lactoferrin interferes with the binding of *A. actinomycetemcomitans* to monolayers of fibroblasts and epithelial cells (2). Interestingly, lactoferrin cleaves two autotransporters from the surface of *H. influenzae* and thereby inhibits its adhesion to epithelial cell monolayers (45). Our studies for both of these phenomena showed similar effects of lactoferrin on *A. actinomycetemcomitans* strain ATCC 29523 cells and Aae protein but not on either SUNY 465 or its Aae.

MATERIALS AND METHODS

Bacterial strains, plasmids, and KB cells. The bacterial strains used in this work are listed in Table 1. *A. actinomycetemcomitans* strains were grown using Trypticase soy broth plus yeast extract (TSB-YE; 30 g of Trypticase soy broth plus 6 g of yeast extract per liter) in a humidified 10% CO_2 incubator at 37°C. *E. coli* strains were grown in Luria-Bertani broth (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter) at 37°C. For solid medium, liquid medium was augmented with 15 g of agar per liter.

KB, the epithelial cell line used, was derived from an oral epidermoid carcinoma and obtained from J. Moehring, University of Vermont. The cell culture medium was RPMI 1640 (Sigma, St. Louis, Mo.) plus 5% fetal bovine serum (Gibco-BRL, Grand Island, N.Y.). KB cells were cultured in a humidified 10% CO_2 incubator at 37°C.

PCR amplification of *aae*. Nucleotide sequences of PCR primers are indicated by dashed underlines in Fig. 1. Primers INT5 (AAG TTG CCC GAG TAA ATC G) and INT3 (CCG GGA CTT CTC ACG TTT AAC) were used to amplify an internal fragment of the *aae* gene with genomic DNA (obtained using PureGene [Gentra Systems, Minneapolis, Minn.]) as a template. After an initial denaturing period at 94°C (5 min), 40 cycles of denaturation at 94°C (15 s), annealing at 52.5°C (15 s), and elongation at 72°C (2 min) were performed in a Genius thermocycler (Techne, Princeton, N.J.). The 1.5-kb fragment was cloned into pGEM-T Easy (Promega, Madison, Wis.) to form plasmid pVT1561.

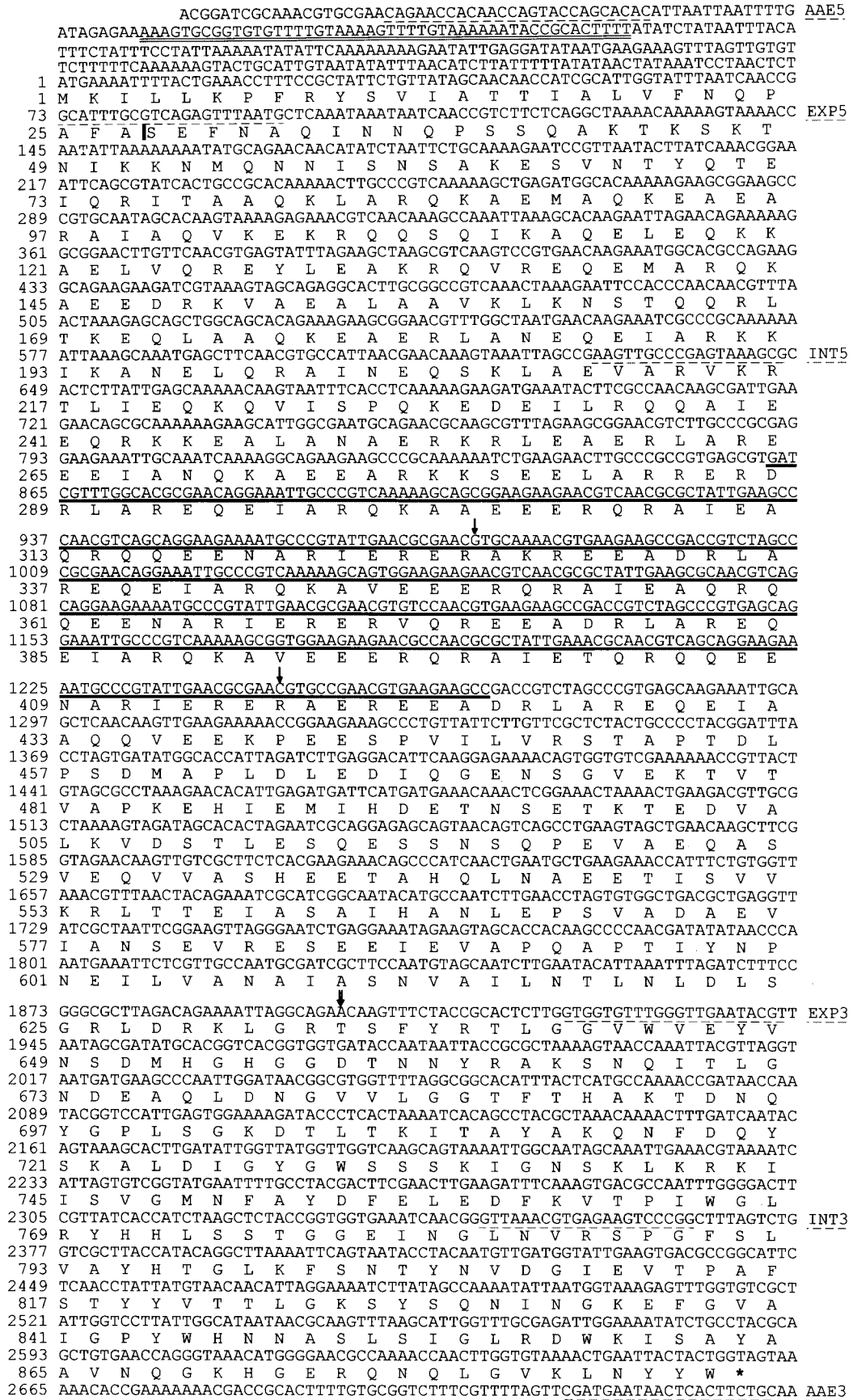
To amplify the entire gene, primers Aae5 (CAG AAC CAC AAC CAG TAC CAG CAC AC) and Aae3 (GCA GAA GTG AGT TAT TCA TCG) were used with the same thermocycler conditions described above, except that the annealing temperature was 60°C and the elongation time was 4 min. The 3.1-kb fragment was cloned into pGEM-T Easy to form plasmid pVT1566.

Sequencing of *aae*. A region that included the entire open reading frame was sequenced at the Vermont Cancer Center Sequencing Facility at the University of Vermont, using pVT1566 as template and, for sequencing primers, first the SP6 and T7 primers from pGEM-T Easy and then the primers depicted in Table 2.

Plasmid-loss generation of isogenic mutant. A mutagenesis system based on that described by Mintz and Fives-Taylor (39) was used to generate an *aae* mutant that is isogenic to SUNY 465. The *A. actinomycetemcomitans-E. coli* shuttle plasmid pPK1 (51) was used to make strain SUNY 465 transiently spectinomycin resistant by transformation (53). Plasmid pPK1 is a derivative of the shuttle plasmid pDL282, which was derived by the ligation of the cryptic *A. actinomycetemcomitans* plasmid pVT736-1 with a pUC19 derivative containing an Sp^r gene (see reference 51 for details). Whereas pPK1 maintains the ability to replicate in both *E. coli* and *A. actinomycetemcomitans*, it does not contain the plasmid maintenance genes of pVT736-1; therefore, it is lost if the strain is grown for 10 generations, about 16 h, in liquid medium not containing spectinomycin. Therefore, *A. actinomycetemcomitans* strains containing pPK1 can be easily cured of the plasmid by removing the selective pressure of spectinomycin. Construction of the mobilizable plasmid for site-directed mutagenesis of *aae* was as follows. The large fragment (containing the origin of replication and mobility element) from a *Pst*I digest of pGP704 (37) was ligated to the *aphA* (kanamycin resistance [Km^r])-containing *Pst*I fragment from p34S-Km3 (14). This plasmid, pVT1562, was transformed by electroporation into *E. coli* strain DH5α*λpir*. The internal fragment of *aae* was cut from pVT1561 with *Eco*RI and ligated into the *Eco*RI site in pVT1562 to generate pVT1563, also in the host DH5α*λpir*. For conjugation, plasmid pVT1563 was transformed by electroporation into the conjugation host, SM10α*λpir*, and the resulting strain was called VT 1564. The recipient strain, VT 1006, was a SUNY 465 derivative containing plasmid pPK1 (51).

To perform the mutagenesis, 1.0 ml of exponentially growing recipient cells (VT 1006) and 1.0 ml of exponentially growing donor cells (VT 1564) were pelleted in a centrifuge and each was resuspended in 50 μl of TSB-YE. Donor cells (10 μl) and recipient cells (50 μl) were mixed, poured onto a TSB-YE plate, and incubated in 10% CO_2 at 37°C for 5 h. Thereafter, bacteria were scraped from the plate, resuspended in 1 ml of TSB-YE, diluted in TSB-YE, and plated on several TSB-YE plates containing 100 μg of kanamycin per ml and 100 μg of spectinomycin per ml. These plates were incubated in 10% CO_2 at 37°C for 48 h.

Isolated colonies of putative transconjugants were grown separately overnight in 0.2 ml of TSB-YE with 100 μg of kanamycin per ml and replica plated on


ACGGATCGCAAACGTGCGAACAGAACCACAACCAGTACCAGCACACATTAATTAATTTTG AAE5
ATAGAGAAAAAGTGCGGTGTGTTTTGTAAAAGTTTTGTAAAAAATACCGCACTTTTATATCTATAATTTACA
TTTCTATTTCCTATTAAAAATATATTCAAAAAAAAGAATATTGAGGATATAATGAAGAAAGTTTAGTTGTGT
TCTTTTTCAAAAAAGTACTGCATTGTAATATATTTAACATCTTATTTTTATATAACTATAAATCCTAACTCT
1 ATGAAAATTTTACTGAAACCTTTCCGCTATTCTGTTATAGCAACAACCATCGCATTGGTATTTAATCAACCG
1 M K I L L K P F R Y S V I A T T I A L V F N Q P
73 GCATTTGCGTCAGAGTTTAATGCTCAAATAAATAATCAACCGTCTTCTCAGGCTAAAACAAAAAGTAAAACC EXP5
25 A F A S E F N A Q I N N Q P S S Q A K T K S K T
145 AATATTAAAAAAAATATGCAGAACAACATATCTAATTCTGCAAAAGAATCCGTTAATACTTATCAAACGGAA
49 N I K K N M Q N N I S N S A K E S V N T Y Q T E
217 ATTCAGCGTATCACTGCCGCACAAAAACTTGCCCGTCAAAAAGCTGAGATGGCACAAAAAGAAGCGGAAGCC
73 I Q R I T A A Q K L A R Q K A E M A Q K E A E A
289 CGTGCAATAGCACAAGTAAAAGAGAAACGTCAACAAAGCCAAATTAAAGCACAAGAATTAGAACAGAAAAAG
97 R A I A Q V K E K R Q Q S Q I K A Q E L E Q K K
361 GCGGAACTTGTTCAACGTGAGTATTTAGAAGCTAAGCGTCAAGTCCGTGAACAAGAAATGGCACGCCAGAAG
121 A E L V Q R E Y L E A K R Q V R E Q E M A R Q K
433 GCAGAAGAAGATCGTAAAGTAGCAGAGGCACTTGCGGCCGTCAAACTAAAGAATTCCACCCAACAACGTTTA
145 A E E D R K V A E A L A A V K L K N S T Q Q R L
505 ACTAAAGAGCAGCTGGCAGCACAGAAAGAAGCGGAACGTTTGGCTAATGAACAAGAAATCGCCCGCAAAAAA
169 T K E Q L A A Q K E A E R L A N E Q E I A R K K
577 ATTAAAGCAAATGAGCTTCAACGTGCCATTAACGAACAAAGTAAATTAGCCGAAGTTGCCCGAGTAAAGCGC INT5
193 I K A N E L Q R A I N E Q S K L A E V A R V K R
649 ACTCTTATTGAGCAAAAACAAGTAATTTCACCTCAAAAAGAAGATGAAATACTTCGCCAACAAGCGATTGAA
217 T L I E Q K Q V I S P Q K E D E I L R Q Q A I E
721 GAACAGCGCAAAAAAGAAGCATTGGCGAATGCAGAACGCAAGCGTTTAGAAGCGGAACGTCTTGCCCGCGAG
241 E Q R K K E A L A N A E R K R L E A E R L A R E
793 GAAGAAATTGCAAATCAAAAGGCAGAAGAAGCCCGCAAAAAATCTGAAGAACTTGCCCGCCGTGAGCGTGAT
265 E E I A N Q K A E E A R K K S E E L A R R E R D
865 CGTTTGGCACGCGAACAGGAAATTGCCCGTCAAAAAGCAGCGGAAGAAGAACGTCAACGCGCTATTGAAGCC
289 R L A R E Q E I A R Q K A A E E E R Q R A I E A
937 CAACGTCAGCAGGAAGAAAATGCCCGTATTGAACGCGAACGTGCAAAACGTGAAGAAGCCGACCGTCTAGCC
313 Q R Q Q E E N A R I E R E R A K R E E A D R L A
1009 CGCGAACAGGAAATTGCCCGTCAAAAAGCAGTGGAAGAAGAACGTCAACGCGCTATTGAAGCGCAACGTCAG
337 R E Q E I A R Q K A V E E E R Q R A I E A Q R Q
1081 CAGGAAGAAAATGCCCGTATTGAACGCGAACGTGTCCAACGTGAAGAAGCCGACCGTCTAGCCCGTGAGCAG
361 Q E E N A R I E R E R V Q R E E A D R L A R E Q
1153 GAAATTGCCCGTCAAAAAGCGGTGGAAGAAGAACGCCAACGCGCTATTGAAACGCAACGTCAGCAGGAAGAA
385 E I A R Q K A V E E E R Q R A I E T Q R Q Q E E
1225 AATGCCCGTATTGAACGCGAACGTGCCGAACGTGAAGAAGCCGACCGTCTAGCCCGTGAGCAAGAAATTGCA
409 N A R I E R E R A E R E E A D R L A R E Q E I A
1297 GCTCAACAAGTTGAAGAAAAACCGGAAGAAAGCCCTGTTATTCTTGTTCGCTCTACTGCCCCTACGGATTTA
433 A Q Q V E E K P E E S P V I L V R S T A P T D L
1369 CCTAGTGATATGGCACCATTAGATCTTGAGGACATTCAAGGAGAAAACAGTGGTGTCGAAAAAACCGTTACT
457 P S D M A P L D L E D I Q G E N S G V E K T V T
1441 GTAGCGCCTAAAGAACACATTGAGATGATTCATGATGAAACAAACTCGGAAACTAAAACTGAAGACGTTGCG
481 V A P K E H I E M I H D E T N S E T K T E D V A
1513 CTAAAAGTAGATAGCACACTAGAATCGCAGGAGAGCAGTAACAGTCAGCCTGAAGTAGCTGAACAAGCTTCG
505 L K V D S T L E S Q E S S N S Q P E V A E Q A S
1585 GTAGAACAAGTTGTCGCTTCTCACGAAGAAACAGCCCATCAACTGAATGCTGAAGAAACCATTTCTGTGGTT
529 V E Q V V A S H E E T A H Q L N A E E T I S V V
1657 AAACGTTTAACTACAGAAATCGCATCGGCAATACATGCCAATCTTGAACCTAGTGTGGCTGACGCTGAGGTT
553 K R L T T E I A S A I H A N L E P S V A D A E V
1729 ATCGCTAATTCGGAAGTTAGGGAATCTGAGGAAATAGAAGTAGCACCACAAGCCCCAACGATATATAACCCA
577 I A N S E V R E S E E I E V A P Q A P T I Y N P
1801 AATGAAATTCTCGTTGCCAATGCGATCGCTTCCAATGTAGCAATCTTGAATACATTAAATTTAGATCTTTCC
601 N E I L V A N A I A S N V A I L N T L N L D L S
1873 GGGCGCTTAGACAGAAAATTAGGCAGAACAAGTTTCTACCGCACTCTTGGTGGTGTTTGGGTTGAATACGTT EXP3
625 G R L D R K L G R T S F Y R T L G G V W V E Y V
1945 AATAGCGATATGCACGGTCACGGTGGTGATACCAATAATTACCGCGCTAAAAGTAACCAAATTACGTTAGGT
649 N S D M H G H G G D T N N Y R A K S N Q I T L G
2017 AATGATGAAGCCCAATTGGATAACGGCGTGGTTTTAGGCGGCACATTTACTCATGCCAAAACCGATAACCAA
673 N D E A Q L D N G V V L G G T F T H A K T D N Q
2089 TACGGTCCATTGAGTGGAAAAGATACCCTCACTAAAATCACAGCCTACGCTAAACAAAACTTTGATCAATAC
697 Y G P L S G K D T L T K I T A Y A K Q N F D Q Y
2161 AGTAAAGCACTTGATATTGGTTATGGTTGGTCAAGCAGTAAAATTGGCAATAGCAAATTGAAACGTAAAATC
721 S K A L D I G Y G W S S S K I G N S K L K R K I
2233 ATTAGTGTCGGTATGAATTTGCCTACGACTTCGAACTTGAAGATTTCAAAGTGACGCCAATTTGGGGACTT
745 I S V G M N F A Y D F E L E D F K V T P I W G L
2305 CGTTATCACCATCTAAGCTCTACCGGTGGTGAAATCAACGGGTTAAACGTGAGAAGTCCCGGCTTTAGTCTG INT3
769 R Y H H L S S T G G E I N G L N V R S P G F S L
2377 GTCGCTTACCATACAGGCTTAAAATTCAGTAATACCTACAATGTTGATGGTATTGAAGTGACGCCGGCATTC
793 V A Y H T G L K F S N T Y N V D G I E V T P A F
2449 TCAACCTATTATGTAACAACATTAGGAAAATCTTATAGCCAAAATATTAATGGTAAAGAGTTTGGTGTCGCT
817 S T Y Y V T T L G K S Y S Q N I N G K E F G V A
2521 ATTGGTCCTTATTGGCATAATAACGCAAGTTTAAGCATTGGTTTGCGAGATTGGAAAATATCTGCCTACGCA
841 I G P Y W H N N A S L S I G L R D W K I S A Y A
2593 GCTGTGAACCAGGGTAAACATGGGGAACGCCAAAACCAACTTGGTGTAAAACTGAATTACTACTGGTAGTAA
865 A V N Q G K H G E R Q N Q L G V K L N Y Y W *
2665 AAACACCGAAAAAAACGACCGCACTTTTGTGCGGTCTTTCGTTTTAGTTCGATGAATAACTCACTTCTGCAA AAE3

TSB-YE with 100 μg of kanamycin per ml and TSB-YE with 100 μg of spectinomycin per ml.

Natural transformation. The entire *aae* gene amplified by PCR and cloned into pGEM-T Easy was restricted with *Hin*dIII, and the Km^r gene excised from p34S-Km3 with *Hin*dIII was inserted. The resulting plasmid, pVT1567, was restricted with *Dra*I and run on a 0.5% agarose gel, and the large fragment containing the disrupted *aae* gene was extracted from the gel (Qiagen, Valencia, Calif.). The DNA was brought to 200 μl with TSB-YE. A 0.6-ml aliquot of overnight culture was centrifuged, and the bacterial pellet was resuspended in 25 μl of the DNA. This suspension was incubated at room temperature for 1 h. A 0.55-ml aliquot of warm (37°C), fresh TSB-YE was added, and the tubes were incubated at 37°C. After incubation for 2 h, 0.1 ml was plated on TSB-YE plus 100 μg of kanamycin per ml and the plates were incubated for 48 h at 37°C in 10% CO_2. Several of the Km^r colonies were streaked onto a plate containing TSB-YE plus 100 μg of kanamycin per ml and incubated for 2 days. All of these putative *aae* mutants grew and were used to inoculate TSB-YE broth for the extraction of chromosomal DNA (as above) and subsequent analysis by Southern blotting. A verified clone was saved as VT 1568.

Adhesion assays. Adhesion assays were performed as described previously (34). Briefly, bacteria at a multiplicity of infection of 100:1 were applied to confluent monolayers of KB cells and incubated for 2 h. The monolayers were washed twice with phosphate-buffered saline (PBS) plus $MgCl_2$ and $CaCl_2$, and the bacteria were released with 0.1% Triton X-100, which was subsequently diluted with PBS and plated on TSB-YE for quantification.

Expression of the passenger domain and antibody production. Primers EXP5 (CCA TGG CTG CAT TTG CGT CAG AGT TTA ATG) and EXP3 (GGA TCC ACG TAT TCA ACC CAA ACA CCA C) were used to amplify the part of *aae* encoding the passenger domain of Aae (the underlined sequences are engineered *Nco*I and *Bam*HI sites, respectively). The thermocycling parameters were an initial 5 min at 94°C, and then 40 cycles of denaturation at 94°C (15 s), annealing at 60°C (15 s), and elongation at 72°C (1.75 min). Recombinant passenger domain polypeptide (rAaePD) was purified using the HisBind kit (Novagen, Darmstadt, Germany) and used for antibody production (Covance, Princeton, N.J.). Crude antiserum was purified before use on a protein A column (Sigma) as specified by the manufacturer.

Protein gels and Western blots. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with a Protean II gel apparatus (Bio-Rad, Philadelphia, Pa.), and proteins were transferred to nitrocellulose in a transfer apparatus (Hoeffer Scientific Instruments, San Francisco, Calif.). The membranes were blocked for 1 to 2 h in 5% nonfat dry milk in Tris-buffered saline (TBS) and then washed three times for 10 min each in TBS plus 0.1% Tween 20 (Sigma) (TBST). The membrane was incubated for 1 h with primary antibody (as indicated in the figure legends), diluted in TBST, and washed three times as above. A final 1-h incubation was performed with a 1:10,000 dilution of horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, Pa.) in TBST and was followed by three washes as above. Detection was done with chemiluminescent ECL Western blotting reagents (Amersham Pharmacia, Piscataway, N.J.) as specified by the manufacturer.

Southern blots. Site-directed mutagenesis was verified using Southern blot analysis. Genomic DNA was digested for 1 h with restriction enzymes as indicated in the figure legends and subjected to electrophoresis in 0.5% agarose gels. Depurination, denaturation, and neutralization of the DNA in the gel, transfer of the DNA to a Hybond N^+ nylon membrane (Amersham Pharmacia), probe construction, and visualization were carried out using the ECL Direct nucleic acid-labeling kit (Amersham Pharmacia).

Bacterial ELISA. A standard method for examining the surface expression of bacteria proteins is the bacterial enzyme-linked immunosorbent assay (ELISA). The assays were carried out as described previously (16). Briefly, bacteria were dried onto the ELISA plates by overnight incubation at 37°C and probed using anti-rAaePD as the primary antibody followed by peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) as the secondary antibody. Hydrogen peroxide-containing buffer was used to generate the colored reaction, which was stopped with sulfuric acid and quantified in a plate reader (BioTek, Winooski, Vt.).

TABLE 2. Primers used for the sequencing of *aae* from strain SUNY 465

Name	Direction	Sequence
AAE5	Forward	CAG AAC CAC AAC CAG TAC CAG CAC AC
EXP5	Forward	GCA TTT GCG TCA GAG TTT AAT G
INT5	Forward	AAG TTG CCC GAG TAA AGC G
S5-2	Forward	TCG CTC TAC TGC CCC TAC GGA TTT AC
S5-3	Forward	GAA ATT CTG GTT GCC AAT GC
S5-4	Forward	CCG GCA TTC TCA ACC TAT TAT G
AAE3	Reverse	GCA GAA GTG AGT TAT TCA TCG
INT3	Reverse	CCG GGA CTT CTC ACG TTT AAC
EXP3	Reverse	ACG TAT TCA ACC CAA ACA CCA C
S3-2	Reverse	GAG CTG CAA TTT CTT GCT CAC
S3-4	Reverse	CCT CTG CCA CTT TAC GAT CTT C

Immunofluorescence microscopy. Surface expression of proteins was also tested by immunofluorescence microscopy. Bacteria were dried onto glass coverslips, fixed in 3.7% formaldehyde, and washed in PBS. Coverslips were incubated with the anti-rAaePD antibody for 20 min, washed with PBS, incubated for 20 min with fluorescein isothiocyanate-conjugated secondary antibody, and washed with PBS. Previously, antibodies to whole bacteria of strain SUNY 465 were created and purified (38). An aliquot of the purified antibody was conjugated to a blue fluorophore (Molecular Probes, Eugene, Oreg.). After incubation for 20 min with the blue fluorophore-conjugated antibody and a final wash in PBS, coverslips were inverted onto a drop of VectaShield (Vector Laboratories, Burlingame, Calif.) and sealed with nail polish. Digital micrographs were recorded with a charge-coupled device camera (Diagnostic Instruments, Sterling Heights, Mich.) attached to a fluorescence microscope (Nikon Instruments, Melville, N.Y.).

Adhesin capture. The adhesin capture assay, a method of determining the interaction between bacterial proteins and KB cells, was essentially the same as that described elsewhere (10). KB cells were released from a monolayer by trypsin-EDTA treatment, centrifuged at 500 × g for 5 min, and resuspended in 200 μl of RPMI 1640. Extracts of *A. actinomycetemcomitans* were prepared by centrifugation of 10^{10} cells at 5,000 × *g* for 20 min, resuspension of the pellet in 200 μl of water, and incubation of the resuspended cells in a boiling-water bath for 10 min. After centrifugation, the supernatant was added to 10^6 KB cells that were resuspended in 200 μl of RPMI 1640 and incubated for 90 min at 37°C in 5% CO_2. After the incubation, the assay milieu was centrifuged at 500 × *g* for 5 min to pellet KB cells. KB cells were washed twice (500 × *g* for 5 min) to remove loosely bound proteins, resuspended in SDS-PAGE loading buffer, and lysed by incubation in a boiling-water bath for 10 min. Western blot analyses were performed as described above.

Cleavage of Aae by human milk whey. To determine if the similarity between Aae and the autotransporters of *H. influenzae* extends beyond sequence homology, the possible cleavage of Aae by human milk whey was examined. Milk whey, a gift from Andrew Plaut, New England Medical Center, Boston, Mass., was obtained by centrifugation of human milk twice to remove cells and lipids and diluted such that the final concentration of lactoferrin in the whey was 1.0 mg/ml. *A. actinomycetemcomitans* (approximately 10^7 cells) was mixed with the milk whey at a lactoferrin concentration of 0.5 mg/ml and diluted with RPMI 1640 to a volume of 100 μl. Mixtures were incubated at 37°C on a rotator for 1 h. Cells were centrifuged at 20,800 × *g* for 10 min, and supernatants were saved. Cell pellets were resuspended in 150 μl of loading buffer, and the supernatant concentrates were brought to 150 μl with loading buffer. Samples were incubated for 10 min in a boiling-water bath and examined by SDS-PAGE on a 7.5% gel. Western blot analyses with anti-rAaePD antibody were performed as above.

FIG. 1. Nucleotide sequence of *aae* and the amino acid sequence of Aae. The PCR primers are indicated by dashed underlines, and the name of the primer is to the right. The doubly underlined sequence in the untranslated 5′ region is an inverted repeat presumed to be a terminator of the upstream gene. The vertical bar (|) in the amino acid sequence (after position 27) indicates the signal peptidase cleavage site. The solid underline beginning at position 862 indicates the repeat region. The single arrows (↓) at positions 977 and 1246 indicate the first and last bases, respectively, of the SUNY 465 deletion. The double arrow (⇓) between positions 1900 and 1901 indicates the insertion site for the sequence AATTAGACAGAA in SUNY 465.

Effect of whey on adhesion. Adhesion assays were performed as described above, except that *A. actinomycetemcomitans* was pretreated with whey as follows. Bacteria were incubated with 0.5 mg of whey per ml diluted with RPMI 1640 or with RPMI 1640 alone at a final volume of 100 μl for 90 min, as for the whey cleavage assay. After centrifugation at 20,800 × *g* for 10 min, supernatants were removed and cells were resuspended in RPMI 1640 and added to KB monolayers (10^8 bacteria/well).

Nucleotide sequence accession number. The GenBank accession number for the *aae* gene identified in this study is AY262734.

RESULTS

Identification of an *H. influenzae* autotransporter homologue in the *A. actinomycetemcomitans* genome database. Searches of the *A. actinomycetemcomitans* HK 1651 genome database at the University of Oklahoma (46) using the BLAST (1) program with the autotransporter proteins Hap and Hia from *H. influenzae* as the query sequence revealed one nucleotide sequence with significant homology (Fig. 1). This search produced the sequence in *A. actinomycetemcomitans* most closely related to *H. influenzae* autotransporters, but whether that sequence was actually most closely related to autotransporters when compared to a larger database of proteins was unknown. To answer this question, a search of the GenBank database using the *A. actinomycetemcomitans* translated open reading frame (termed "Aae") as query was performed. The results showed that this sequence was most homologous to IgA1 proteases and adhesion and penetration proteins of *Haemophilus* and *Neisseria* species. The significant homology to *Neisseria* autotransporters was not surprising since there appears to have been considerable horizontal transfer of DNA between *Haemophilus* and *Neisseria* species (13).

An interesting feature of Aae is that it does not appear to be a full-length version of the proteins to which it is homologous; the other proteins range from 1393 to 1764 amino acids, whereas Aae has 886 amino acids. The region of highest homology is the C terminus, the region in which autotransporters have a series of transmembrane domains that form a pore in the outer membrane. IgA1 protease, composed of 1552 amino acids, has some homology to the N-terminal region of Aae, but there is no homologue for its active site in the Aae sequence. Despite the homology in the C-terminal domain, there was no significant homology between the N-terminal region of Aae and other proteins in the database. Another interesting feature of the Aae sequence is that it has a region with three 45-amino-acid imperfect repeats (indicated by the solid underline in Fig. 1).

Since a signal sequence at the N terminus is an essential feature of autotransporters and the sequence of Aae appeared to be an N-terminal truncation of its homologues, the sequence was examined for a signal sequence by using the PSORT program (http://psort.nibb.ac.jp/form.html) (41) and the method of von Heijne (57). PSORT predicted the existence of a cleaved signal sequence ending at position 27 (Fig. 1). Although Aae appeared to be an autotransporter, it did not possess the proteolytic domain of the *Haemophilus* and *Neisseria* autotransporters to which it is most homologous. That it could be an adhesin was suggested by the fact that the *H. influenzae* autotransporter adhesin, Hap, when uncleaved (and still cell-associated) mediates adhesion to cultured epithelial cells (25, 55). Another *H. influenzae* autotransporter adhesin, Hia, has no known proteolytic activity and remains cell associated (54).

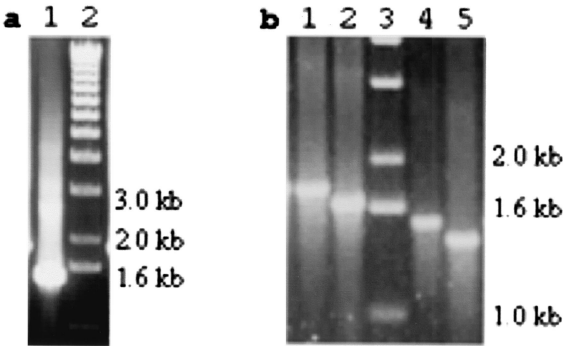


FIG. 2. PCR of the INT5-INT3 fragment from several *A. actinomycetemcomitans* strains demonstrating four *aae* alleles. (a) SUNY 465 (lane 1); DNA markers (lane 2). (b) Strains 652, DB7A-173, SUNY 523, and SUNY 524 (lanes 1, 2, 4, and 5, respectively); DNA markers (lane 3).

Cloning and sequencing of the *aae* gene in SUNY 465. An internal fragment of *aae* was amplified by PCR from genomic DNA from strain SUNY 465, an invasive strain of *A. actinomycetemcomitans* (36), using primers INT5 and INT3. Interestingly, the apparent length of the fragment was about 300 bases shorter than predicted from the HK 1651 database (Fig. 2a). The sequencing of SUNY 465 *aae* revealed a 270-base deletion (two copies of the 135-bp sequence) in the repeat region. The deletion in the SUNY 465 *aae* gene (relative to the HK 1651 *aae* gene) is indicated in Fig. 1 by the thin arrows above the first and last bases in the *aae* sequence. A PCR screen of 30 strains of *A. actinomycetemcomitans* with the same primers showed at least two other alleles, presumably containing two and four copies of the repeat region (Fig. 2b). The use of PCR primers that more closely flank the repeat region showed that the length polymorphism was due to a different number of repeats in the various fragments (data not shown).

The entire open reading frame was amplified using PCR with primers AAE5 and AAE3 and cloned into pGEM-T Easy. Sequencing of the cloned gene revealed that besides the expected deletion in the repeat region, there was a 12-base insertion of the sequence AATTAGACAGAA. The double arrow in Fig. 1 indicates the site of the insertion. This inserted sequence is close to an exact repeat (it differs in 1 base) of the 12 bases (AATTAGGCAGAA) immediately preceding it. Both the deletion and insertion are multiples of three bases, suggesting that the downstream region, most notably the autotransporter pore domain, is essential to the function of the protein.

Construction of an isogenic mutant of strain SUNY 465. A total of 48 putative transconjugant colonies from the initial kanamycin- and spectinomycin-containing plate were picked for further study. All 48 strains were Sp^s, indicating loss of pPK1. Insertion of the plasmid was confirmed by Southern blot analysis (data not shown).

Analysis of SUNY 465 and its *aae* mutant by using a Coomassie blue-stained SDS-PAGE gel revealed a band above the 121-kDa marker in the wild type that is not present in the mutant (Fig. 3). The presence of a band at ~130 kDa was

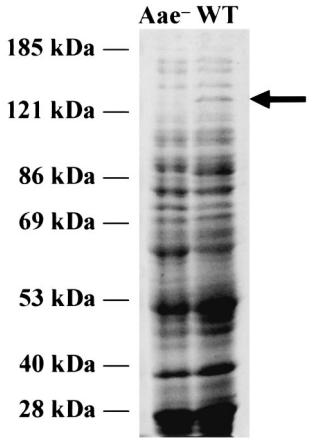


FIG. 3. SUNY 465 and Aae mutant extracts separated by SDS-PAGE (7.5% polyacrylamide) and stained with Coomassie blue. A high-molecular-mass band (arrow, ~130 kDa) is absent in the Aae mutant (Aae-) but present in SUNY 465 wild type (WT).

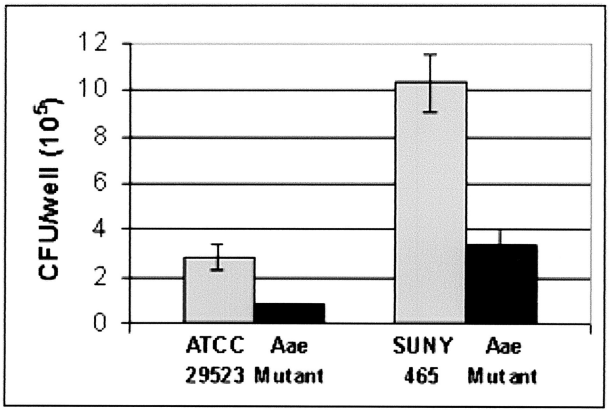


FIG. 4. Adhesion of wild-type *A. actinomycetemcomitans* and Aae mutant strains to KB monolayers. SUNY 465 and ATCC 29523 strains were used. Experiments were performed with quadruplicate wells for each strain. Results shown are from a typical experiment; bars represent the standard deviation of the replicates.

unexpected, since the predicted molecular mass of Aae is 90 kDa (see Discussion).

Construction of an allelic replacement in strain ATCC 29523. One method used to verify the function of a gene is to generate mutations in related strains and determine if the phenotypes change accordingly. An *aae* allelic replacement mutant was generated using linearized DNA in the naturally transformable *A. actinomycetemcomitans* strain ATCC 29523. The *aae* gene within the pGEM-T Easy plasmid was disrupted by insertion of a Km^r cassette at the *Hin*dIII site within the gene. The plasmid was cut with *Dra*I, and the large fragment containing the disrupted gene was used in the natural transformation of ATCC 29523. Disruption of the gene was confirmed by Southern blot analysis using the INT5-INT3 internal fragment as the probe (data not shown).

Adhesion assays with the *aae* mutants of SUNY 465 and ATCC 29523. Adhesion assays were performed to determine if the putative autotransporter, Aae, is involved in the adhesion of *A. actinomycetemcomitans* to epithelial cells. Figure 4 shows that there was close to a 70% reduction in adhesion of the *aae* mutant compared with that of the wild type for each strain. These data, together with the Aae sequence similarity to autotransporter adhesins, suggested that Aae is an adhesin.

Expression of the passenger domain and antibody production. To better characterize Aae, the passenger domain was expressed using the pET28a vector and the *E. coli* BL21(DE3) host (Novagen). Primers EXP5 and EXP3 were constructed such that EXP5 contained an *Nco*I site and EXP3 contained a *Bam*HI site in order to make use of the His_6 C-terminal tag for purification. The His_6 tag was placed at the C terminus because the native protein is predicted to be attached to the cell at the C terminus, with the N terminus being free. If the extreme N terminus is important to adhesion, the presence of a His_6 tag in that position could interfere with that adhesion process.

A Coomassie blue-stained SDS-PAGE gel (Fig. 5a) and the corresponding Western blot (Fig. 5b) generated using anti-His_6 antibodies showed that the promoter in the BL21(DE3) host is leaky; however, ample protein was being produced. Both the gel and the blot indicated that the apparent molecular mass of Aae is ~90 kDa, not the expected 63 kDa. A Coomassie blue-stained gel with the first four fractions from the purification of rAaePD revealed, in addition to the ~90-kDa band, a band at ~65 kDa (Fig. 5c). To determine if the lower band

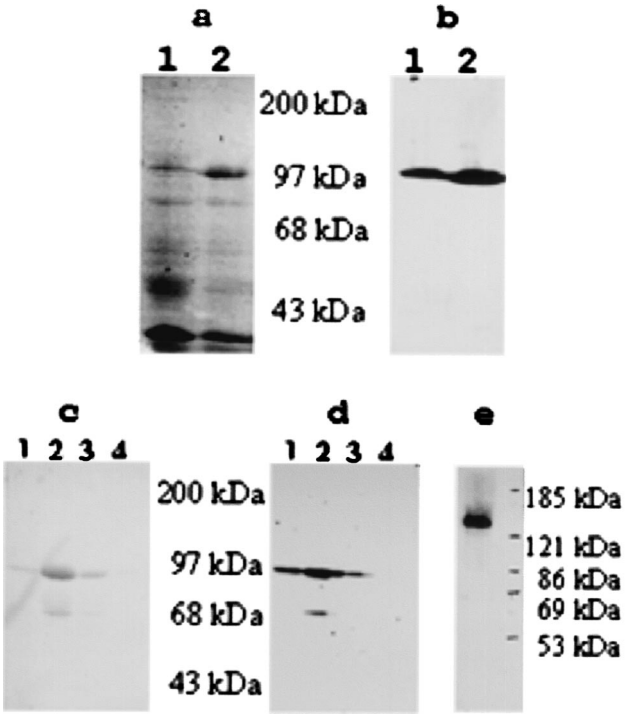


FIG. 5. Expression and purification of the passenger domain. (a) Extracts of the host strain uninduced (lane 1) and induced by 1 mM isopropyl-β-D-thiogalactopyranoside (lane 2), run on a 7.5% polyacrylamide gel. (b) Western blot of the same gel probed with a 1:1,000 dilution of anti-His_6 antibody (Novagen), showing the existence of the His_6 tag. (c) A Coomassie blue gel of the first four fractions from the purification of rAaePD. (d) Corresponding Western blot with conditions as for panel b. (e) Western blot of a SUNY 465 extract, with gel and probe conditions as for panel b. Note that the band is about ~130 kDa, as seen in Fig. 3.

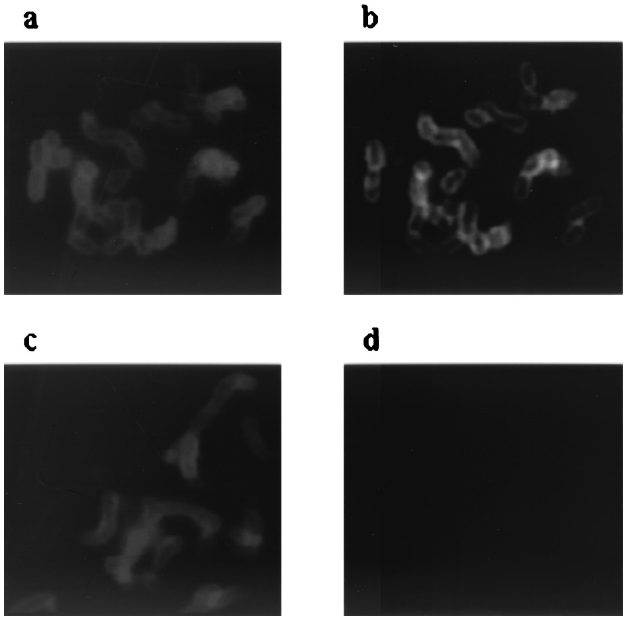


FIG. 6. Immunofluorescence microscopy of SUNY 465 and the Aae mutant. (a and b) Wild-type cells treated with anti-SUNY 465 antibody (a) and anti-rAaePD antibody (b). (c and d) Aae mutant treated with anti-SUNY 465 antibody (c) and anti-rAaePD antibody (d). Primary antibodies were used at a 1:10,000 dilution, secondary antibodies were used at a 1:100 dilution, and blue fluorophore-conjugated antibody was used at a 1:100 dilution.

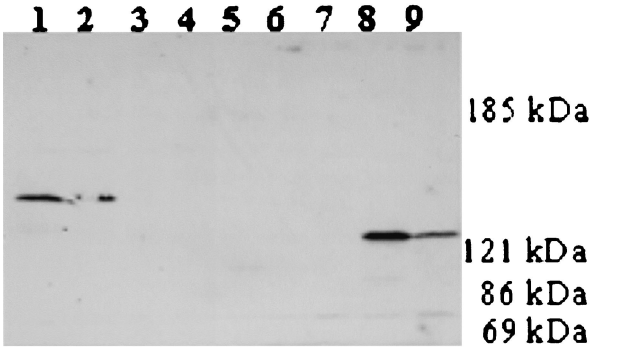


FIG. 7. Adhesin capture. Lanes: 1 and 2 ATCC 29523 wild type; 3 and 4, Aae mutant of ATCC 29523; 5, KB not exposed to bacterial extracts; 6 and 7, Aae mutant of SUNY 465; 8 and 9 SUNY 465 wild-type. This was a 5% gel; the anti-rAaePD antibody was used at a 1:20,000 dilution.

(especially evident in lane 2) was a contaminant or a degradation product, a Western blot analysis was performed (Fig. 5d). The 65- kDa band reacted with the anti-6-HIS antibody, indicating that it resulted from breakdown of the rAaePD peptide. The specificity of the anti-rAaePD antibody is shown in Fig. 5e by the presence of a single band with a molecular mass of about 130 kDa, the same size as the band representing Aae on the gel in Fig. 3.

Bacterial ELISA and immunofluorescence microscopy. The location of Aae is predicted to be on the bacterial surface; thus a bacterial ELISA, which detects surface components, was performed. Cells from both wild-type and *aae* mutant strains of SUNY 465 were dried onto the ELISA plate and probed with the anti-rAaePD antibody. The surface-associated antibody reaction was three times greater with the wild-type cells than with the *aae* mutant cells (data not shown).

A confirmatory test of the presentation of Aae on the bacterial surface was carried out using SUNY 465 wild-type and Aae mutant and both anti-rAaePD and anti-SUNY 465 whole-cell antibody in immunofluorescence microscopy. Figure 6 shows that the Aae mutant reacted with only the anti-SUNY 465 antibody (Fig. 6c); no reactivity occurred with anti-rAaePD (Fig. 6d). By contrast, the wild type reacted with both anti-rAaePD (Fig. 6b) and SUNY 465 whole-cell antibody (Fig. 6a). Taken together, these data showed that the Aae protein is on the surface of *A. actinomycetemcomitans*.

Adhesin capture assay. Aae was shown to be present on the bacterial surface; it could therefore interact directly with epithelial cells. To investigate this possibility, we used the adhesin capture method. KB cells were incubated with extracts of wild-type and *aae* mutant bacteria, washed, and analyzed by SDS-PAGE followed by Western blotting with anti-rAaePD antibody as the probe. As shown in Fig. 7, strong Aae bands were generated by both wild-type strains (lanes 1 and 2 and lanes 8 and 9), but neither of the Aae mutants (lanes 3 to 8) nor KB cells alone (lane 5) generated Aae bands. Whereas the bands representing Aae from the two wild types were different and indicate molecular masses of 140 and 130 kDa for SUNY 465 and strain ATCC Aae, respectively, the sizes were those expected for each strain based upon experimental data. Strain ATCC 29523 was determined by PCR analysis to have the same large allele as strain HK 1651 (data not shown). These data indicated that Aae is in fact an adhesin that interacts directly with KB cells.

Effects of human milk whey on Aae. Another feature of autotransporter adhesins of *H. influenzae* is that they are cleaved by the lactoferrin in human milk (45). Using samples kindly provided by A. G. Plaut, we incubated wild-type *A. actinomycetemcomitans* with human milk whey, fractionated treated and untreated cells, and carried out PAGE and Western blot analysis on the fractions. Aae cleavage by components of the whey would be indicated by the presence of a "small" band in supernatants of whey-treated cells that was not present in supernatants of untreated cells. Since the cleavage site specificity of lactoferrin is not yet known, the expected size of the cleavage product could only be estimated to be less than ~90 kDa (the apparent molecular mass of the recombinant passenger domain). Figure 8 shows bands at ~50 and 64 kDa in the whey-treated supernatants of SUNY 465 and ATCC 29523, respectively, indicating cleavage of the Aae passenger domain. In lanes representing untreated cells and pellet fractions, no similar low-molecular-weight bands were evident. No other lower-molecular-weight bands were seen on Coomassie blue-stained gels or Western blots, suggesting that the shift in molecular mass is not due to nonspecific degradation.

Adhesion assays with length polymorphism strains. A comparison of the effects of whey on adhesion by strains containing one to four copies of the Aae repeat revealed a substantial decrease in adhesion by strains with three and four copies compared with that of SUNY 465, a strain with only one copy (Fig. 9). SUNY 523, a strain with two copies (i.e. only one

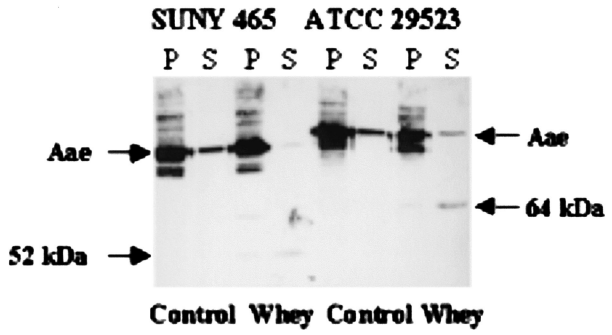


FIG. 8. Cleavage of Aae by human milk whey. Pellets and supernatants from bacteria incubated with RPMI medium (Control) or milk whey diluted to 0.5 mg of lactoferrin per ml with RPMI medium (Whey) are shown. The anti-rAaePD antibody was used at a 1:40,000 dilution. P, pellet; S, supernatant.

additional copy), adhered at essentially the same level as SUNY 465, suggesting that a single extra copy of the repeat could not effectively reduce adhesion.

DISCUSSION

Most research into bacterial pathogens, especially gram-negative organisms, has concentrated on those in the gastrointestinal tract (19) and has led to considerable knowledge of the pathogenic molecules and mechanisms used by these organisms. There has also been an effort to elucidate the mechanisms of oral bacterial pathogenesis (32, 35), but, by comparison, such knowledge for these organisms is limited.

The advent of genomics has enabled researchers to look for homologues of known virulence factors in other organisms, such as oral organisms. While there may be dead ends to this sort of comparative genomics, there are also many big rewards. We have used this approach to find an autotransporter in *A. actinomycetemcomitans* by using the closely related *H. influenzae* as the comparison organism. This is probably the key: the use of a reasonably close relative for the comparison organism.

An open reading frame, *aae*, with homology to autotransporter genes of *H. influenzae* and *Neisseria* species was discovered in the *A. actinomycetemcomitans* database. Although this open reading frame at first appeared to encode an N-terminal truncation with respect to its homologues, the Aae protein did carry an N-terminal signal sequence. Antibodies made against the putative passenger domain reacted with epitopes on the surface of wild-type strains but not with *aae* mutant strains, indicating that the passenger domain is presented on the surface of the bacteria.

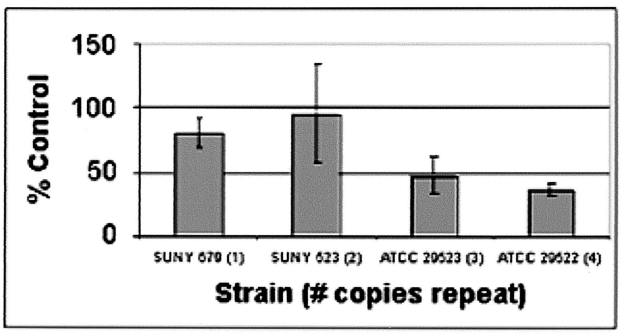


FIG. 9. Effect of whey on adhesion of length polymorphism strains. Adhesion to monolayers of KB cells is shown as a percentage of untreated SUNY 465. The number in parentheses next to the strain name indicates the number of copies of the repeat in the strain. Each strain was tested in quadruplicate. Results shown are from a typical experiment; bars represent the standard deviation of the replicates.

One difference between Aae and its homologues in *Neisseria* and *Haemophilus* is its anomalous apparent molecular mass on denaturing polyacrylamide gels. This is not an uncommon phenomenon (15, 58). One possible explanation for the anomalous apparent molecular mass of rAaePD is the net charge of the residues in the passenger domain. There are 106 negatively charged and 87 positively charged residues in the expressed protein, resulting in a net charge of −19. Replacement of acidic residues with basic residues in the human papillomavirus type 16 E7 protein generates a protein with the predicted mobility rather than the anomalous high mobility of the native protein (5).

Mutants with mutations in *aae* derived from two different strains of *A. actinomycetemcomitans* showed a marked decrease in adhesion to KB cells. Upstream of the start codon of *aae* is an inverted repeat (Fig. 1, double underline) that is probably a terminator for the upstream gene (an open reading frame homologous to *bipA*, a transcription factor). The open reading frame immediately downstream from *aae* (homologous to *bcp*, a gene encoding a protein that comigrates with bacterioferritin) is transcribed in the opposite direction. These facts suggest that *aae* is transcribed alone. If so, this would imply that the mutation in *aae* is solely responsible for the adhesion defect.

Data presented here support the premise that adhesion to epithelial cells can occur in the absence of fimbriae; in *Porphyromonas gingivalis*, the adhesion is due to gingipains (10). There appears to be a connection between the expression of fimbrillin (a fimbria-associated protein) and gingipains, making it difficult to separate the roles of each in adhesion. Rough strains of *A. actinomycetemcomitans* that form transparent colonies tend to be more fimbriated than are smooth strains (26). In *A. actinomycetemcomitans*, smooth strains show a wide variation in adhesion to epithelial cells (34), possibly due to extracellular vesicles and amorphous material (33). This further complicates dissection of the role of nonfimbrial adhesins in *A. actinomycetemcomitans*. Do the vesicles and amorphous material, which are likely to be composed of outer membrane material, contain large amounts of Aae or other nonfimbrial adhesins? These earlier studies showed that the amorphous material and vesicles that could be washed off the more adhesive strains could increase adhesion of the other strains (33), suggesting that nonfimbrial adhesins are contained in that extracellular material. In either event, the role of extracellular material in the adhesion of *A. actinomycetemcomitans* to epithelial cells needs to be investigated further.

It is also interesting that both the gingipains of *P. gingivalis* and Aae of *A. actinomycetemcomitans* appear to be heat stable, given the method of lysis used (10). The oral cavity is subject to mechanical stress from several sources: the tongue, hot and cold food and drink, and in some cases even oral hygienic techniques. Bacteria that cause periodontal disease are able to

withstand this assault and remain attached to tissues in the oral cavity. It is perhaps not surprising, then, that adhesins of periodontal pathogens are very stable.

The secreted gingipains that mediate the adhesion of *P. gingivalis* possess both adhesin and proteinase activities (43). The Hap autotransporter of *H. influenzae* also has these traits (25). Is it possible that Aae is also a proteinase, given the similar niche and adhesion strategies of these two pathogens? The sequence of Aae rules out an IgA1-like active site, such as Hap, but Aae does possess a potential zinc finger domain, HEETAH, similar to that of a dipeptidyl peptidase III enzyme (20). Mutational analysis of the HELLGH zinc-binding domain of dipeptidyl peptidase III shows a requirement for a third amino acid between the glutamic acid and second histidine residues, rather than the more common HEXXH zinc finger domain. More studies are required to determine if Aae has proteolytic activity. It may simply be that Aae is more similar in a functional way to the cell-associated *H. influenzae* autotransporter adhesin, Hia (54).

Lactoferrin is an important part of the host's innate defenses (18, 30). The binding of iron by lactoferrin is very strong and suggests that one of its antimicrobial activities is the sequestering of iron away from the microorganisms. The N-terminal domain of lactoferrin is a cationic peptide called lactoferricin, which can cause damage to the outer membrane of gram-negative bacteria (7, 17, 59). Lactoferrin also has the ability to bind to *A. actinomycetemcomitans* in a nonbactericidal manner (2). Low concentrations of lactoferrin in the oral cavity may be a risk factor in *A. actinomycetemcomitans*-associated periodontal disease (22).

Earlier reports by others showed that lactoferrin decreased *A. actinomycetemcomitans* adhesion to fibroblasts and the basement membrane (3, 4). Consistent with that is our results here, which showed that lactoferrin decreased the adhesion of *A. actinomycetemcomitans* strains ATCC 29522 and ATCC 29523 to epithelial cells. Our finding that lactoferrin did not affect the adhesion of strains SUNY 465 and SUNY 523 is a discrepancy that we believe may be explained by the presence of fewer copies of the Aae repeat in these strains. One notable feature of lactoferrin is the N-terminal peptide called lactoferricin, which is highly charged. The 15-residue part of lactoferricin that has been shown to interact with bacteria has a net charge of +6 (23). Each copy of the repeat at the protein level contains 55% charged residues, with a net charge of −6. The shorter form of Aae in SUNY 465 may effectively reduce its ability to interact with lactoferricin. This may be the first hint of the significance of the length polymorphisms of the *aae* gene.

Identification of an autotransporter adhesin of *A. actinomycetemcomitans* is just the first step in gaining an understanding of the genetic mechanisms involved in the interactions of this organism with epithelial cells. With the anti-rAaePD antibody and expressed recombinant passenger domain, it should be possible to identify the ligand on the surface of KB cells to which Aae binds by using the same method used to find the receptor for the *Yersinia* invasin (56). Gram-negative bacteria that have autotransporters often have several different ones, indicating that further searching of the HK 1651 database is warranted.

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