Oral Microbiology Immunology 2004: 19: 16–25
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Oral Microbiology and Immunology

Two epithelial cell invasion-related loci of the oral pathogen *Actinobacillus actinomycetemcomitans*

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Li L, Matevski D, Aspiras M, Ellen RP, Lépine G. Two epithelial cell invasion-related loci of the oral pathogen Actinobacillus actinomycetemcomitans.
Oral Microbiol Immunol 2004: 19: 16–25. © Blackwell Munksgaard, 2004.

Two invasion-related loci, *apiA* and the two-gene operon *apiBC*, were isolated from the oral pathogen *Actinobacillus actinomycetemcomitans* UT32. *apiA* encodes a 32.5 kDa protein that migrates on SDS-PAGE as a 101 kDa protein as detected by Western blot analysis or silver staining of an outer membrane-enriched fraction of *Escherichia coli* transformants. *E. coli* expressing ApiA have a different phenotype than the host vector, in broth and on solid media, and a colony morphology that resembles that of fresh *A. actinomycetemcomitans* isolates. These *E. coli* transformants bound to chicken collagen type II, human collagen type II, III, V and fibronectin. *apiB* and *apiC* encode proteins of 130.1 and 70.6 kDa, respectively. ApiBC conferred on *E. coli* a slightly enhanced ability to bind to collagen type III. ApiA- and ApiB-deficient mutants were constructed in *A. actinomycetemcomitans*. The ApiB-mutant had 4-fold diminished invasion of KB cells; the ApiA-mutant had increased invasion. Both loci were found in all *A. actinomycetemcomitans* strains, although polymorphism was detected only for *apiBC*. The deduced sequences of these invasion-related proteins are homologous to members of the YadA adhesin/invasin family.

Key words: *Actinobacillus actinomycetemcomitans*; epithelial cell invasion; invasion-related genes; periodontal pathogen

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Accepted for publication July 15, 2003

In the human oral cavity, which is constantly bathed by saliva, bacterial adhesion to other bacteria, to epithelial cells and to extracellular matrix (ECM) components is a prerequisite to initiate mucosal colonization and infection. Some periodontal pathogens have evolved to penetrate the stratified squamous epithelium of the gingival sulcus. Invasion of periodontal tissues, initiated by bacterial adhesion, has been described in several types of periodontal diseases (10, 11, 24, 25, 39, 40). Bacteria have been found in association with the periodontal pocket epithelium (10, 39), gingival connective tissue (11, 24, 25, 39, 40), alveolar bone (39) and oral epithelium (41). Several studies using methods such as immunofluorescence and immunoperoxidase histochemistry have identified specific bacteria that invade the gingiva (4, 33, 39).

The gram-negative capnophilic coccobacillus *Actinobacillus actinomycetemcomitans*, an etiologic agent of juvenile periodontitis and other early-onset periodontal conditions, has been shown to invade gingival tissues of lesions and to localize deep in the connective tissue and along the basement membrane (4). Invasion of the gingival tissues may shelter *A. actinomycetemcomitans* from host defenses, complicate treatment aimed at its suppression, and perhaps explain the apparently episodic nature of *A. actinomycetemcomitans* infections. Furthermore, a recent study has shown that *A. actinomycetemcomitans* invades buccal epithelial cells intracellularly (37). Invaded exfoliating buccal epithelial cells might provide a protected route for bacterial transmission between oral sites within and between hosts.

A. actinomycetemcomitans invasiveness of epithelial cell lines (27, 28) and primary gingival cells (8) has been demonstrated *in vitro*. With the help of *in vitro* models, it has been established that *A. actinomycetemcomitans* invasion of epithelial cells is a multistep process requiring *de novo* protein synthesis from both cell types and involves entry, escape from the vacuole, rapid multiplication, exit from the host cell and cell-to-cell spread (28). Although host cell mechanisms of invasion have been studied intensively, the bacterial genes

required for different steps of the invasion process have yet to be identified. So far in the literature only one gene, *apaH*, encoding for a diadenosine tetraphosphatase has been related to the invasiveness of *A. actinomycetemcomitans* (38). The aim of the present study was to identify and characterize invasion-related loci from the most invasive clinical isolate studied by us, *A. actinomycetemcomitans* strain UT32 (23).

Material and methods

Bacterial strains and plasmids

A. actinomycetemcomitans strains UT32, UT12, UT26, UT41, SWD6 and SWD9 were isolated from cases of aggressive (localized) periodontitis at the University of Toronto. *A. actinomycetemcomitans* isolates UP3, UP5, UP6, UP9, UP11, UP15, UP16, UP18, UP28, UP48, UP49, UP50, UP53, UP54 and JP2 were obtained from J. DiRienzo (University of Pennsylvania). *A. actinomycetemcomitans* SUNY 465, SUNY 523 and 652, and *Haemophilus aphrophilus* ATCC 19415 were supplied by P. Fives-Taylor (University of Vermont). *A. actinomycetemcomitans* HK1651, the strain chosen for genome sequencing (www.genome.ou.edu), was provided by M. Kilian (University of Aarhus, Denmark). All strains were grown in TSB-YE medium (30 g/l tryptic soy broth, 6 g/l yeast extract, supplemented with 0.04% sodium bicarbonate and 15 g/l agar for plates) in a humidified 5% CO_2 atmosphere at 37°C. *Escherichia coli* DH5α (Life Technologies, Burlington, ON, Canada) was grown at 37°C in Luria-Bertani medium. Recombinant *E. coli* cells transformed with pUC18 (Amersham Pharmacia Biotech, Baie d'Urfé, Quebec, Canada) or pPCR-Script (Stratagene, La Jolla, CA) or their derivatives (Table 1) were cultivated in Luria-Bertani medium supplemented with 50 μg/ml ampicillin.

Immunoscreening of an *A. actinomycetemcomitans* UT32 genomic bank

A. actinomycetemcomitans UT32 genomic DNA was isolated according to standard methods and digested with *Eco*RI. Fragments greater than 2 kb were extracted from agarose gels using the freeze-thaw procedure (44), ligated to pUC18 (*Eco*RI dephosphorylated), transformed into the competent *E. coli* DH5α and selected on Luria-Bertani broth supplemented with 50 μg/ml ampicillin. *E. coli* clones were inoculated on duplicate plates and colonies from one duplicate were transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) and immunoscreened using two different polyclonal anti-*A. actinomycetemcomitans* antisera (J.J. Zambon, SUNY, Buffalo, NY and A. Progulske-Fox, University of Florida, Gainesville, FL). In brief, the nitrocellulose membranes were blocked for 1 h in skim milk (3%-TTBS, 25 mM Tris–HCL pH 8.0, 137 mM NaCl, 3 mM KCl, 0.05% Tween 20) and rinsed twice for 5 min in TTBS before incubating for 1 h with the primary polyclonal *A. actinomycetemcomitans* antisera. After two 5-min rinses in TTBS, the *E. coli* clones expressing *A. actinomycetemcomitans* antigens were detected by the addition of protein A gold (Bio-Rad Laboratories, Mississauga, ON, Canada).

DNA sequencing

Plasmid DNA from recombinant *E. coli* clones was isolated using a commercial kit (Qiagen, Chatsworth, CA). Double-stranded DNA sequencing was performed at the Hospital for Sick Children (HSC) Biotechnology Centre with a Pharmacia A.L.F. automatic sequencer using dideoxy sequencing of DNA with fluorescein-labeled primers. The Pharmacia AutoRead™ kit was used for the sequencing reactions.

Invasion assays using KB cells

KB cells, originally derived from an oral epidermoid carcinoma, have been used as a standard invasion model for *A. actinomycetemcomitans* (9, 28). The invasion assays were performed as previously reported (22, 23) except that *E. coli* cells were grown in Luria-Bertani broth to stationary phase and 200 μg/ml gentamicin was used to kill the adherent extracellular bacteria. Briefly, KB cells grown in 24-well plates were inoculated with a bacterial strain suspension of a standardized concentration and incubated with colchicine (2 mg/ml) containing medium. The suspension was later removed and a volume of gentamicin was added to eliminate any adherent, noninvasive bacteria. Finally, upon the removal of the gentamicin, the KB cells were lysed with phosphate buffered saline (PBS) containing Triton X100. Aliquots of bacterial suspensions were taken prior to the initial inoculation (A), directly after gentamicin removal (B) and after the addition of Triton X100 (C), and were plated on TSB-YE plates. A percent invasiveness was then calculated for each bacterial strain with the formula $(C - B)/A \times 100\%$. The invasion assay was performed in quadruplicate and repeated at least twice by different investigators.

Polymerase chain reaction amplification and cloning of the ORFs involved in invasion

Polymerase chain reaction (PCR) amplification was performed with the Herculase™ Enhanced Polymerase Blend (Stratagene, La Jolla, CA). The oligonucleotide primers were synthesized at the HSC Biotechnology Centre (Toronto, ON, Canada). The annealing temperature used

Table 1. pUC18 and pPCR-Script derived plasmids used in this study

Plasmids	Description
pAA321	2.579 kb *Eco*RI fragment of *A. actinomycetemcomitans* UT32 chromosomal DNA ligated to *Eco*RI site of pUC18
pAA3211	1.518 kb DNA fragment of pAa321 amplified using primers 32-18F (position 1014) and 32-19R (position 2532), and blunt-end ligated to pPCR-Script. *ApiA* transcription/translation same orientation as β-gal.
pAA3212	1.518 kb DNA fragment of pAa32-1 amplified using primers 32-18F (position 1014) and 32-19R (position 2532), and blunt-end ligated to pPCR-Script. *apiA* transcription/translation in reverse orientation from β-gal.
pAA322	5.838 kb *Eco*RI fragment of *A. actinomycetemcomitans* UT32 chromosomal DNA ligated to *Eco*RI site of pUC18
pAA3221	6.367 kb DNA fragment amplified from *A. actinomycetemcomitans* UT32 chromosomal DNA using primers 32-229F (position 422 of pAA322) and 32-228R (genome HK1651), and blunt-end ligated to pPCR-Script. *apiBC* transcription/translation in same orientation as β-gal
pAA3222	6.367 kb DNA fragment amplified from *A. actinomycetemcomitans* UT32 chromosomal DNA using primers 32-229F (position 422 of pAA322) and 32-228R (genome HK1651), and blunt-end ligated to pPCR-Script. *apiBC* transcription/translation in reverse orientation from β-gal
pHK1651	6.367 kb DNA fragment amplified from *A. actinomycetemcomitans* HK1651 chromosomal DNA using primers 32-229F (position 422 of pAA322) and 32-228R (genome HK1651), and blunt-end ligated to pPCR-Script. $apiBC_{HK1651}$ transcription/translation in same orientation as β-gal

for the following combinations were: 32-18F (5′-TAATGGCTCGTGTGGTGATAG-3′, position 1014 bp of AA321) and 32-19R (5′-AGATCGTATCGCCACCCACAT-3′, position 2532 bp of AA321), 61°C; 32-229F (5′-TAAAGAGCTGATTGAGGCG-ATGTA-3′, position 422 bp of AA322L) and 32-228R (5′-GGGGAGAAATGAGA-GGGCGAAAAT-3′, position 6789 bp of AA322L), 60°C. The amplicons were purified using the StrataPrep™ PCR Purification Kit (Stratagene) and cloned using the PCR-Script™ Amp Cloning Kit (Stratagene).

RT-PCR

Using the RNeasy kit (Qiagen), total RNA was isolated from *A. actinomycetemcomitans* UT32, *E. coli* (pAA3221) and *E. coli* (pAA3222) grown to logarithmic phase. Traces of DNA contaminants were digested by RQ1 DNAse, followed by phenol chloroform extraction and precipitation with ethanol. The Calypso™ RT-PCR system (Bio/Can Scientific, Mississauga, ON, Canada) was used in combination with primers 32-215F (5′-ATATCAAAGGCG-GAGACAGC-3′ position 4251 of AA322L) and 32-216R (5′-TGGGATTGGTTTCAT-TCACA-3′ position 4798 of AA322L) at an annealing temperature of 56°C. Absence of DNA contamination was confirmed by PCR reactions using Taq DNA polymerase (Life Technologies) without any reverse transcriptase for 35 cycles.

Southern blot analysis

A. actinomycetemcomitans genomic DNA was isolated using standard methods, digested with *Eco*RI, run in 0.9% agarose gel and submitted to alkaline transfer onto a nylon membrane (Boehringer, Mannheim, Germany) (1). DNA fragments to be labeled were obtained by PCR amplification with the enzyme Herculase™ Enhanced Polymerase Blend. Primers Aa32-18F and Aa32-19R amplified a 1518 bp fragment of pAA3211 containing the entire *apiA* gene. The commercially available primers T3 and T7, using an annealing temperature of 56°C, amplified a fragment of 6367 bp of pAA3221 containing the entire *apiBC* operon. The purified amplicons were labeled using the DIG system (Boehringer Mannheim). Hybridization was performed at 37°C in Easy-Hyb hybridization buffer (Boehringer Mannheim). The stringency washes were done as follows: twice 5 min at room temperature in 2XSSC-0.1%SDS and twice 15 min at 50°C in 0.5X SSC-0.1%SDS. Detection of hybridizing fragments was done using the CSPD® detection method (Boehringer Mannheim).

Western blot analysis

Western analysis was performed as previously described (21) with minor modifications. The protein concentration of whole bacterial cells was estimated using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). The equivalent of 3 μg of protein of *A. actinomycetemcomitans* UT32 and *E. coli* cells was loaded on SDS-PAGE gels (Bio-Rad Ready Gels [10% resolving gel Tris–HCl], Bio-Rad Laboratories) with Laemmli's buffer system (20). Polyclonal rabbit anti-*A. actinomycetemcomitans* antiserum from J. Zambon was used as primary antibody for detection of *A. actinomycetemcomitans* antigens. Goat anti-rabbit IgG (H + L) (1 : 10,000) (Jackson Immuno-Research Laboratories Inc., Mississauga, ON, Canada) was used as secondary antibody.

Insertional mutation and dot-blot screening

An internal portion of *apiA* was replaced by a 1.1 kb fragment (Sp^r) encoding the spectinomycin adenyltransferase gene of *Enterococcus faecalis*. An *apiB* isogenic mutant was similarly constructed through allelic replacement of an internal region by spectinomycin. Mutagenic *apiA* or *apiB* constructs were constructed in either pPCR-Script-Amp or pPCR-Script-Cm and electrophoresed into *A. actinomycetemcomitans,* conferring ampicillin or chloramphenicol resistance, respectively, upon integrative recombination into the chromosome (Fig. 1). A double crossover recombinant for *apiB* was achieved without multiple passages. Single crossover Campbell-type mutants were first confirmed for *apiA* by PCR amplification and *apiA* inactivation by RT-PCR. Attempts were then made to identify allelic replacement (double crossover or Campbell-type) mutants for *apiA* by successive colony passaging and dot-blot screening. PCR confirmation of allelic exchange replacement was further obtained by amplification of plasmid or spectinomycin insertions in the *apiA* and *apiB* loci.

Primers ApiAMF1F (5′-ATCTTCAAG-CCAAAACATC-3′, position 931 of AA321) and ApiAMF1R (5′-TTCGCGGCTACA-TTTTGAT-3′, position 1190 of AA321) were used at the annealing temperature of 56°C to PCR amplify a DNA fragment from *A. actinomycetemcomitans* UT32 for use as our probe. The PCR product was extracted from an agarose gel and purified as des-

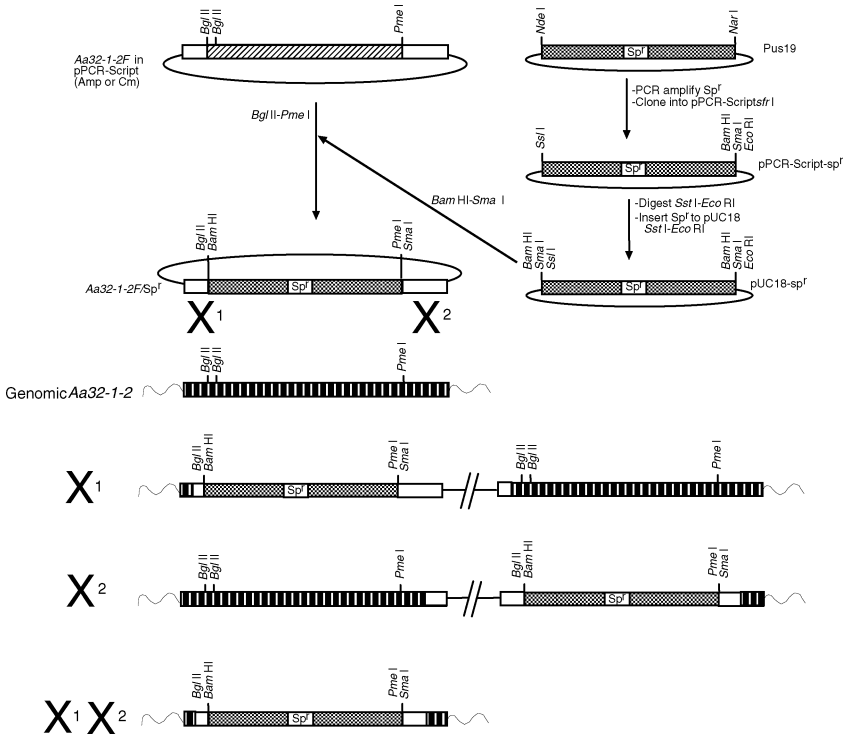

Fig. 1. Schematic model for the insertional mutagenesis of *apiA* and *apiB*. For *apiA* a single recombinant was first isolated (**X1** or **X2**) and then a double recombinant (**X1X2**) was later isolated by passaging the single recombinant multiple times. The insertional mutation of *apiB* was performed in a similar manner and resulted in a double recombinant without multiple passages.

cribed previously, and then DIG labeled using the method outlined in *The DIG System User's Guide for Filter Hybridization* (Boehringer Mannheim). Dot-blot analysis was then performed on selected colonies using CSPD® detection. A colony with a double crossover allelic replacement mutation would not produce a signal with this method. Dot blot confirmed the presence of single and double crossover mutants for the *apiA* mutant. For *apiB*, PCR amplification of the inserted spectinomycin cassette with either flanking region of mutated *apiB* confirmed the presence of a double recombinant in a selected mutant.

Outer membrane-enriched preparation

The outer membrane-enriched fractions were prepared according to the method of Barenkamp et al. (2) as modified by Haase et al. (12). The outer membrane-enriched pellet was suspended in 500 μl of distilled water (dH_2O) supplemented with 5 μl of protease inhibitor cocktail, aliquoted, stored at –70°C and analyzed by SDS-PAGE.

Binding to extracellular matrix proteins

E. coli transformants grown overnight were briefly centrifuged at 5000 × *g* for 10 min and the pellet resuspended in Tris Buffered Saline (TBS) (20 mM Tris (Sigma-Aldrich Canada Ltd., Oakville, Canada), 150 mM NaCl (Sigma), pH 7.4) to a cell density of 2 ×108 bacterial cells per ml. In parallel, the following different types of ECM proteins:

- Calf collagen type I (Chemicon, Temecula, CA),
- Chicken collagen type II (Sigma-Aldrich Canada Ltd.),
- Human collagen type II (Chemicon), III and V (Southern Biotechnology Associates, Inc., Birmingham, AL).

Fibronectin (Chemicon).

Each collagen type was adjusted to a concentration of 10 μg/ml, of which 1 ml, or 100 μl, were added, respectively, to 24- or 96-well plates and left to bind to the wells overnight at 4°C.

The unbound proteins were removed and the wells were air-dried. One milliliter, or 100 μl, of the different bacterial suspensions in TBS was then added to the 24- or 96-well plates in triplicate. The bacteria were left to interact with the immobilized proteins at 37°C for 2 h. After washing twice with TBS, the bacteria that remained bound were released by sonication (24-well plates) or strong pipetting (96-well plates), and their colony-forming units counted.

Scanning electron microscopy (SEM)

The *E. coli* transformants grown overnight were centrifuged (420 × *g*) for 5 min, washed twice with filtered deionized distilled water (ddH_2O) and resuspended in ddH_2O. The bacterial suspension was fixed by adding formaldehyde (3.7% V/V) and incubating at room temperature for 1.5 h. Dilutions of the suspension were added onto glass cover slips deposited in 24-well plates and dried overnight. The next morning, the cover slips were washed with ddH_2O, dehydrated through a series of ethanol rinses (30, 50, 70, 95 and 100%), critical-point dried with liquid CO_2 and sputter-coated with 5 nm platinum. The mounted samples were examined using a scanning electron microscope (model S-2500; Hitachi Instruments, San Jose, CA).

Nucleotide sequence accession number

The DNA sequences described here have been deposited in the GenBank library and have been assigned accession numbers AF316502 for AA321 and AF316503 for AA322L.

Results

Identification of *E. coli* clones expressing an invasion phenotype

A total of 9600 *E. coli* clones were screened for the expression of *A. actinomycetemcomitans* UT32 antigens. Fourteen *E. coli* clones that expressed *A. actinomycetemcomitans* antigens were tested for their invasion phenotype in the gentamicin protection assay. Two of the clones, *E. coli* (pAA321) and *E. coli* (pAA322) (Fig. 2), were 8.50 and 2.35 times more invasive in KB cells than the *E. coli* host (Table 2).

Table 2. Invasion of KB epithelial cells by *E. coli* transformants and *A. actinomycetemcomitans* strains

Bacterial strains	Invasion ratio[a]
E. coli transformants	
pAA321	8.50 ± 1.01
pAA322	2.35 ± 0.3
pAA3211	17.80 ± 6.74
pAA3212	2.07 ± 0.51
pAA3221	4.88 ± 1.72
pAA3222	15.65 ± 5.41
pHK1651	2.65 ± 0.86
A. actinomycetemcomitans	
UT32	384.62 ± 126.98
HK1651	1.38 ± 0.66
UT32/HK1651[b]	278.71 ± 119.36

[a]The invasion ratio = mean invasion of the *E. coli* transformants (percent) over the negative control *E. coli* (pPCR-Script) (percent) or the mean invasion of *A. actinomycetemcomitans* (percent) over the negative control *H. aphrophilus* (percent) ± the standard error.
[b]Ratio of the invasion (percent) of the two *A. actinomycetemcomitans* strains, UT32 and HK1651 ± the standard error.

Nucleotide and protein sequence analysis of AA321 and AA322

Sequence analysis of the 2.579-kb *Eco*RI fragment of AA321 revealed two ORFs, a 3′-end truncated 882 bp *parE* and 888 bp *apiA* (*Actinobacillus* putative invasin). Both ORFs are preceded by typical σ^{70} promoter sequences except for minor variations. In both cases, the TATAAT box, $^{-47}$TATAAT for *parE* and $^{-84}$TTTAAT for *apiA*, and the TTGACA sequence, $^{-114}$CTGCCA for *parE* and $^{-169}$TTGATA for *apiA*, are located further upstream from the ATG start codon than their homologs in *E. coli* (–10 and –35, respectively). *parE* encodes for a 294 aa *A. actinomycetemcomitans* protein that is highly homologous (89%) to the topoisomerase IV, subunit B

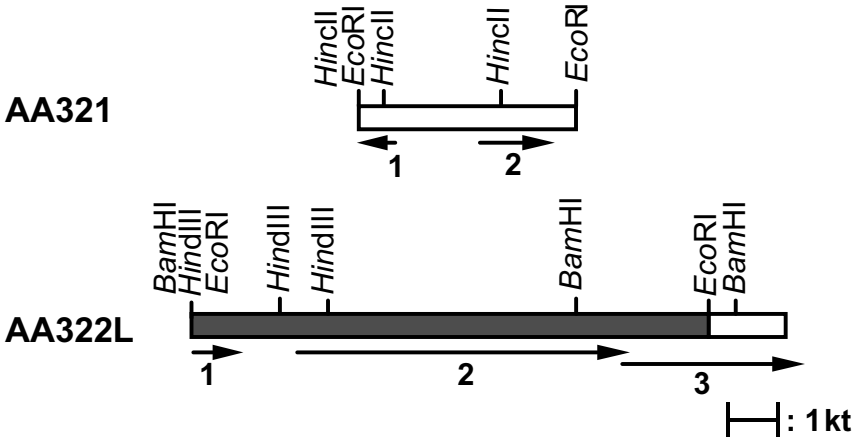

Fig. 2. Restriction map of two invasion-related *A. actinomycetemcomitans* UT32 DNA fragments. The arrows indicate the different ORFs which have been numbered for each DNA fragment. The shaded box represents the original AA322 DNA fragment containing the truncated *apiBC* operon.

(parE) (AAC23174.1) of *Haemophilus influenzae*. ApiA, a 295-amino acid protein, is 99% homologous to Omp100 (AB064943), an outer membrane protein identified in *A. actinomycetemcomitans* Y4 (18). ApiA is also homologous to members of the YadA family, which comprises surface-exposed proteins of many well known pathogens as well as free-living species (pfam.wustl.edu/hmmsearch.shtml) (15) (Fig. 3). Most of the proteins in this family have a predicted signal sequence: M_1N (R/K) followed closely by the motif Y/F-X-I/L/V-X-Y/W and then ending with I/V_{18}-A/V-V/C/A-S-E-L/F/G-A/T/S-R/K/S (14). Western blot analysis of *E. coli* expressing the 32 kDa ApiA, along with SDS-PAGE analysis of the outer membrane fraction of this clone (data not shown), revealed that it consists of a 101 kDa multimer that is stable in SDS-PAGE sample buffer even upon heating at 100°C, a characteristic of all members of the YadA family, except for DsrA (42).

Sequence analysis revealed that Aa322 was a 5833-bp *Eco*RI fragment with three potential ORFs. *FadD*, the first ORF, is truncated at its 5′-end and encodes a 149 aa *A. actinomycetemcomitans* protein that shares a similarity of 93% and 91% with the FadD long chain fatty acid CoA ligase of *Pasteurella multicoda* (AE006132.1) and *H. influenzae* (AAC21681.1), respectively. The second and third ORFs, *apiB* and *apiC* (truncated at its 3′-end), overlap by 64 bp and are possibly part of an operon. Because of the 99% similarity between the genomic sequences of *A. actinomycetemcomitans* UT32 and HK1651 (www.genome.ou.edu) in this region, we elaborated a strategy to clone the entire two-gene operon of UT32 based on PCR amplification. The upstream primer sequence, 32-229F, was derived from AA322 of UT32, while the downstream primer sequence, 32-228R, was derived from HK1651. The resulting 6367 bp amplicon, referred to as AA322L, was cloned into pPCR-Script to obtain constructs pAA3221 and pAA3222 according to their transcription/translation in relation to the β-galactosidase gene. Analysis of the operon promoter region revealed a TATA box ($^{-46}$TATTAAT) and TTGACA ($^{-78}$TCTACA) sequence closely matching typical promoter sequences, but also further upstream from the ATG than is typical for *E. coli*. Interestingly, good matches to the *E. coli* promoter sequences were also found at the 5′-end of *apiC* ($^{-65}$GATTAAT and $^{-107}$GTGAA). The transcription of *apiBC* as a single mRNA transcript was confirmed by RT-PCR analysis performed on total RNA isolated from *A. actinomycetemcomitans* UT32, *E. coli* (pAA3221) and *E. coli* (pAA3222) (data not shown) using a pair of oligonucleotide primers chosen to encompass the overlapping region of these two ORFs generating a 457 bp amplicon. BLAST searches (pfam.wustl.edu) revealed that both ApiB, a 1264 aa protein, and ApiC, a 688 aa protein, are homologous to members of the YadA family of proteins and exhibit homology to the YadA-like C-terminal region as well as the predicted N-terminal signal sequence (Fig. 3). Furthermore, aa 106 to 231, 302 to 440, 500 to 678, 723 to 745, 823 to 1029 of ApiB and aa 5 to 131, 152 to 229, 288 to 487 and 537 to 625 of ApiC represent conserved domains that are present within the YadA-related proteins (pfam.wustl.edu). Some of these conserved domains have been associated with the formation of a conserved neck region as well as a head domain consisting of degenerate 14-residue repeats (15). ApiB highest homology matches exhibited 41% similarity to Hsf of *P. multicoda* (AAK02798.1) and 40% to a putative adhesin of *E. coli* 0157:H7 and to XadA, an outer membrane protein of *Xanthomonas axonopodis* (AE012004) (www.ncbi.nlm.nih.gov). ApiC highest homology matches exhibited 47% similarity to Hsf of *P. multicoda* (AAK02798.1) and 45% to a cell surface protein of *Brucella melitensis* (AAL53054.1). ApiB also showed a considerable 42% homology to ApiC. Although undetected by Western blot analysis, a >207 kDa protein could be detected in the outer membrane-enriched fraction of *E. coli* ApiBC. This protein may correspond to a dimer formed by aggregation of ApiB (130.1 kDa) with ApiC (70.6 kDa) or an oligomer of ApiC, like the oligomerization known to occur among the YadA-like proteins (15).

Although almost identical, three nucleotide differences that lead to changes in the protein sequence could be seen when the homologous regions of $apiBC_{UT32}$ and $apiBC_{HK1651}$ were compared:

1. ^{1847}TAT, coding for an aspartic acid in UT32 corresponds to GAT, coding for a lysine in HK1651.
2. Deletion of an A (position 4101) in UT32 compared with HK1651 leads to a frameshift, premature termination of ApiB in UT32 and translation into two ORFs compared to the single long protein $ApiBC_{HK1651}$ in *A. actinomycetemcomitans* HK1651. This deletion was confirmed by sequencing an amplicon from UT32 genomic DNA.
3. ^{6593}TAA, coding for a stop codon and leading to premature termination of ApiC in UT32, corresponds to TCA in $apiBC_{HK1651}$. $apiBC_{HK1651}$ is translated into a 1966 *A. actinomycetemcomitans* protein with a 201 kDa molecular weight compared with the translation of $apiBC_{UT32}$ into two ORFs, ApiB, 130.1 kDa and ApiC, 70.6 kDa.

When tested in the gentamicin protection assay, *A. actinomycetemcomitans* UT32 was about 280 times more invasive than strain HK1651 (Table 2).

ApiA and ApiBC, invasion-related proteins

Because of the similarity of ApiA and ApiBC to other adhesins/invasins, *E. coli* transformants expressing these genes were tested for invasiveness in KB cells. The invasive ability of *E. coli* (pAA3211), *E. coli* (pAA3212), *E. coli* (pAA3221) and *E. coli* (pAA3222) was tested and compared to that of *E. coli* (pPCR-Script) in the gentamicin protection assay (Tables 1 and 2). The transformants expressing *A. actinomycetemcomitans* antigens invaded the KB cells to a greater extent than *E. coli* (pPCR-Script). *E. coli* (pAA3211) invaded the KB cells about 8 times more than *E. coli* (pAA3212), although the only difference between these two constructs is their orientation with respect to the β-gal promoter. Furthermore, *E. coli* (pAA3211) presented a very different phenotype on solidified agar medium or in broth compared with *E. coli* (pAA3212) and *E. coli* (pPCR-Script). *E. coli* (pAA3211) grew as adherent, flat, mat, rough, irregular-edged colonies, while *E. coli* (pAA3212) and *E. coli* (pPCR-Script) appeared shiny and convex, with regular edges. In broth, *E. coli* (pAA3211) grew as granular autoaggregated clumps of cells that left a clear supernatant; *E. coli* (pAA3212) and *E. coli* (pPCR-Script) growth was turbid and homogeneous. The growth characteristics both in broth and on agar medium reported for *E. coli* (pAA3211) are fairly typical of fresh *A. actinomycetemcomitans* isolates (17, 36, 43).

We used scanning electron microscopy (SEM) to detect ultrastructural differences between *E. coli* (pAA3211), *E. coli* (pAA3212) and *E. coli* (pPCR-Script) cells (Fig. 4). Although the *E. coli* (pAA3212) phenotype in broth and agar medium could not be differentiated from *E. coli* (pPCR-Script), some differences were detected by SEM. *E. coli* (pAA3212) cell length varied tremendously, from about 3 μm to more than 30 μm. Examination of one of the long cells at higher magnification revealed that although the individual cells appeared to be dividing, they did not separate, which may be explained by the formation of an

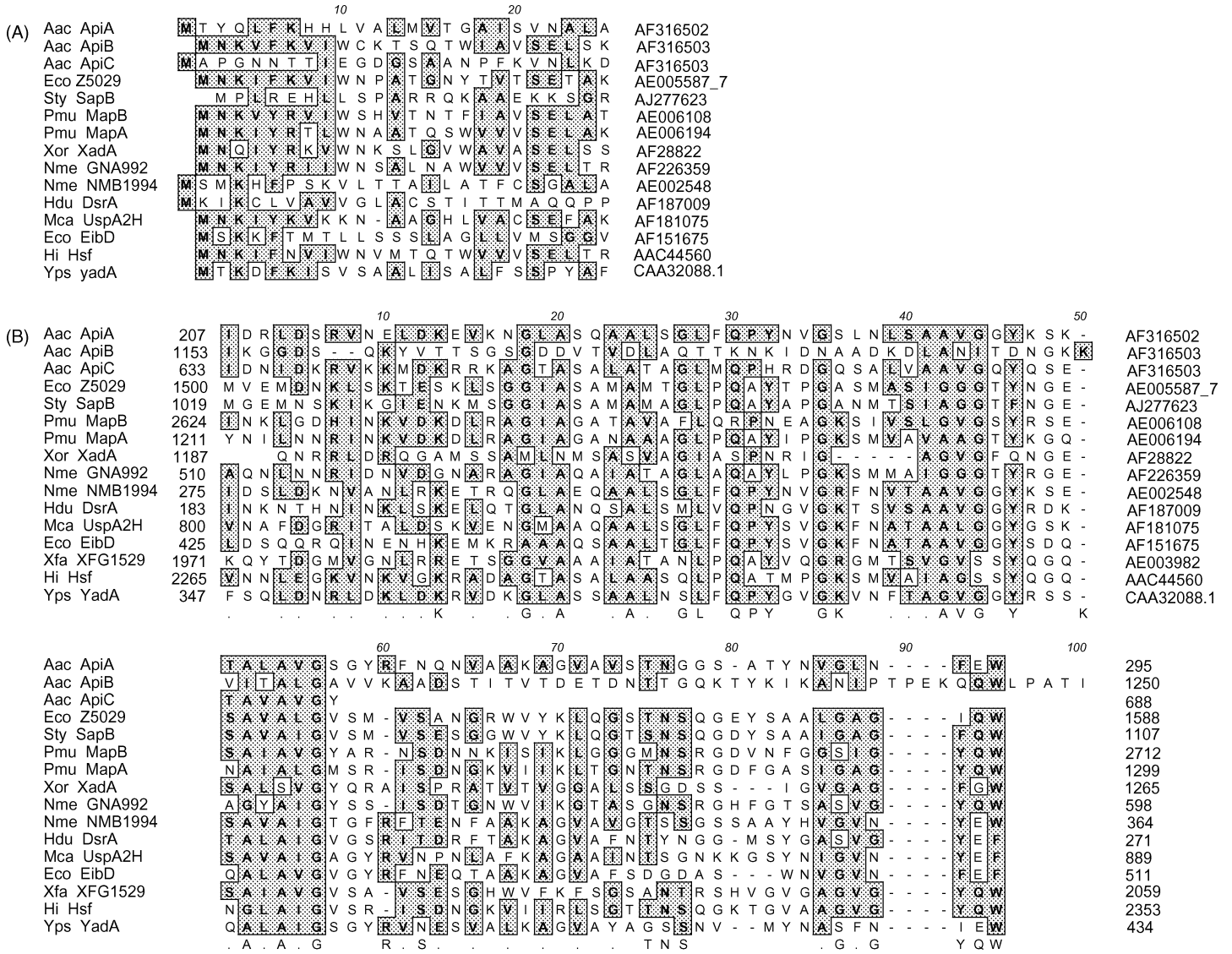

Fig. 3. Multiple sequence alignment of YadA-related proteins. The accession numbers are shown to the right of each sequence. Homologous residues are shaded and boxed. (A) Predictable signal peptide of some YadA-related proteins. (B) Conserved YadA-like C-terminal sequence of some members of the YadA family. The C-terminal sequence consists of a left-handed coiled-coil segment and a membrane anchor formed by four transmembrane β-strands. The start and end residues are numbered. Aac, *A. actinomycetemcomitans*; Eco, *E. coli*; Sty, *Salmonella typhimurium*; Pmu, *P. multicoda*; Xor, *Xanthomonas oryzae*; Nme; *Neisseria meningitidis*; Hdu, *Haemophilus ducreyi*; Mca, *Moraxella catharralis*; Hi, *H. influenzae*; Yps, *Yersinia pseudotuberculosis*.

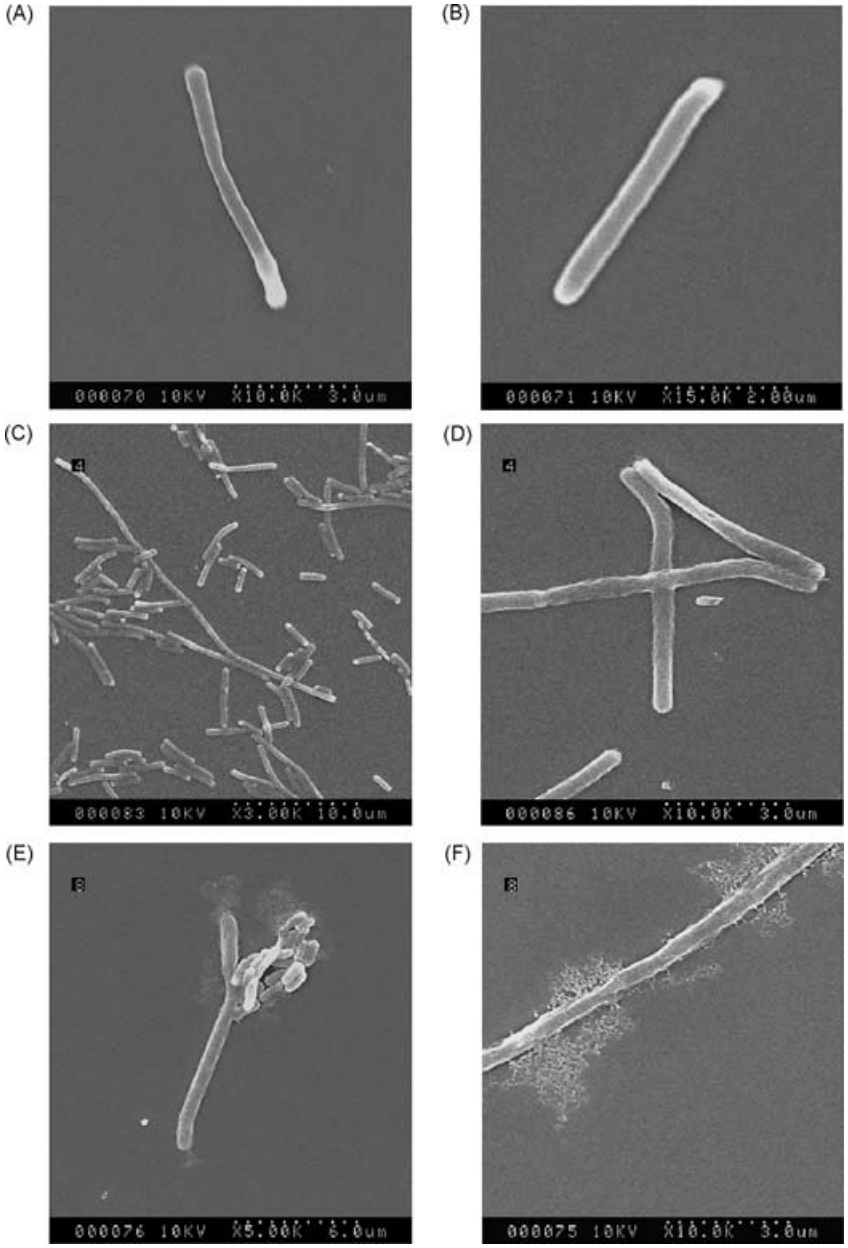

Fig. 4. Morphologic changes of *E. coli* cells expressing Aa32-1-2 as detected by scanning electron microscopy. Scanning electron photomicrographs of *E. coli* (pPCR-Script) (A, B); *E. coli* (pAA3212) (C, D); and *E. coli* (pAA3211) (E, F). Bars indicating magnification appear as dotted lines in the individual panels.

incomplete septum. Extracellular material not present in either *E. coli* (pAA3212) or *E. coli* (pPCR-Script) was visible on the extra-long cells of transformant *E. coli* (pAA3211). Similar bacterial filamentation to *E. coli* (pAA3211) and *E. coli* (pAA3212) has been reported in different bacteria in response to antimicrobial agents (5, 19) or following mutation of genes involved in the division process (6, 32, 45). A similar extracellular amorphous material, referred to as ExAmMat, often covers and connects cells of *A. actinomycetemcomitans* and the closely-related *H. aphrophilus* (16). The ExAmMat is a protein, most likely a glycoprotein, which has been shown to impart bone-resorbing activity and adhesiveness to epithelial cells (16).

One possible explanation for invasiveness and structural differences of *E. coli* (pAA3211) and *E. coli* (pAA3212) could be a difference in the level of expression of *apiA*. In pAA3211, *apiA* may be under enhanced control of the β-gal promoter, while in pAA3212, this gene may be under the control of a weaker *A. actinomycetemcomitans* promoter. To test this hypothesis the transformants were grown under IPTG-induced and non-induced conditions and the proteins compared by Western analysis (data not shown). The extra 101 kDa polypeptide possibly corresponded to an ApiA oligomer, although more abundant in *E. coli* (pAA3211) than *E. coli* (pAA3212). It was not affected by the presence of IPTG and therefore was not under the control of the β-gal promoter. Analysis by SDS-PAGE of the proteins of the outer membrane-enriched fraction of the transformants showed the extra 101 kDa protein only for *E. coli* (pAA3211), which confirmed its more abundant expression in this clone.

E. coli (pAA3222) was about three times more invasive than *E. coli* (pAA3221). Western blot analysis of whole cell lysates of *E. coli* (pAA3221) or *E. coli* (pAA3222) did not reveal any additional protein when compared with the *E. coli* host. However, analysis of the outer membrane-enriched fraction revealed an extra >207 kDa band for *E. coli* (pAA3221). Although of lighter intensity, the same extra band was also detected for *E. coli* (pAA3222) (data not shown).

Binding of *E. coli* transformants expressing invasion-related proteins to extracellular matrix proteins

A. actinomycetemcomitans can invade the gingiva and be found in contact with collagen fibers within the periodontium (3, 11, 39). Therefore, ECM proteins may serve as potential substrates for the binding and localization of *A. actinomycetemcomitans*. Mintz et al. (29) have reported that *A. actinomycetemcomitans* binds to immobilized chicken collagen type II, human collagen type I, II, III and V as well as fibronectin. In the present study, we found the following:

- *E. coli* (pAA3211) bound to immobilized collagen type II, III and V as well as fibronectin;
- *E. coli* (pAA3212) bound to chicken collagen type II;
- *E. coli* (pAa3222) bound slightly to human collagen type III;
- *E. coli* (pAA3221) did not bind any of the extracellular proteins any better than the negative control (Table 3).

Invasiveness and ECM binding of two invasion-related protein mutants, UT32ApiAm1 and UT32ApiBm1

Having demonstrated a relationship between expression of ApiA and ApiBC in *E. coli* and invasiveness, the next logical

Table 3. Binding of extracellular matrix proteins by invasive *E. coli* transformants

	Binding of *E. coli* transformants[a]			
ECM	pAA3211	pAA3212	pAA3221	pAA3222
Chicken collagen type II	9.77 ± 3.35	2.13 ± 0.93	–	2.40 ± 1.42
Human collagen type II	10.5 ± 3.18	1.45 ± 0.50	1.72 ± 1.37	–
Human collagen type III	10.71 ± 2.92	–	1.65 ± 0.93	2.07 ± 0.84
Human collagen type V	10.77 ± 3.63	–	–	–
Fibronectin	10.31 ± 2.66	–	–	1.54 ± 0.62

[a]The binding is expressed as the ratio of the mean binding of the *E. coli* transformants (percent) over the mean binding of the negative control *E. coli* (pPCR-Script) (percent) to the different ECM components ± the standard error.

step was to create *A. actinomycetemcomitans* mutants unable to express either protein. Two allelic replacement mutants were identified in our laboratory. The *apiA* allelic replacement mutant (UT32ApiAm1) was found to be 5.07 ± 1.65 times more invasive in KB cells in the gentamicin protection assay than the wild-type *A. actinomycetemcomitans* UT32 control. By contrast, the *apiB* allelic replacement mutant (UT32ApiBm1) had an invasion ratio of 0.24 ± 0.15 when compared with the wild-type control, demonstrating a fourfold decline in invasiveness.

The ECM protein-binding assay was performed with each mutant strain, the near noninvasive strain HK1651, *H. aphrophilus* and *E. coli* DH5α, and we compared their binding to that of the wild-type UT32. The results compiled in Table 4 demonstrate that *H. aphrophilus* bound less than wild-type UT32 to calf collagen type I, human collagen type II and III. Interestingly, both HK1651 and *E. coli* DH5α bound at least as readily to all collagen types as UT32. HK1651 bound to a greater extent than UT32 to human collagen type V, and *E. coli* DH5α bound to a much greater extent than UT32 to human collagen type III and fibronectin. As for the *A. actinomycetemcomitans* mutants in this assay, binding of UT32ApiAm1 to calf collagen type I, chicken collagen type II and human collagen type III and V was at least double that of the wild-type control. In contrast, UT32ApiBm1 actually had less than half the binding capacity for human collagen type II and III and for fibronectin. Thus, mutant strain UT32ApiAm1 was considerably more adherent to ECM proteins than mutant strain UT32ApiBm1, which reflected their capacity to invade epithelial cells.

Distribution and restriction fragment length polymorphism analysis of the invasion-related loci in *A. actinomycetemcomitans* isolates

As we previously reported (23), *A. actinomycetemcomitans* isolates differ in their ability to invade KB cells when tested in the gentamicin protection assays. Therefore, it was of interest to determine whether the difference in invasion might be related to the presence of *apiA* or *apiBC* or could be associated with a specific pattern of restriction fragment length polymorphism. Genomic DNA was isolated from 25 *A. actinomycetemcomitans* isolates, digested with *Eco*RI and submitted to Southern analysis using the inv-related loci as probes. All 25 isolates possessed a 2.6 kb *Eco*RI fragment with homology to *apiA* (data not shown). All *A. actinomycetemcomitans* strains also possessed DNA fragments homologous to *ApiBC*. *Eco*RI fragments of 5.8 kb and 1.5 kb hybridized to the probe for 23 of the 25 *A. actinomycetemcomitans* strains tested. Polymorphism was detected for only two strains, UT12 and 652, which exhibited, in addition to the 1.5 kb fragment, a 5.3 kb *Eco*RI fragment instead of the more widespread 5.8 kb fragment (data not shown). *A. actinomycetemcomitans* UT12 and 652 have been shown to be noninvasive or not significantly more invasive than the negative control, *H. aphrophilus* (23). It should also be noted that the restriction fragment length polymorphism analysis performed here did not allow us to detect any deletions/insertions, as was noticed for the ^{4101}A in strain UT32. Sequencing of amplicons constituting this region would have to be done to confirm the presence of such possible deletions/insertions.

Discussion

We have identified two invasion-related loci of *A. actinomycetemcomitans* UT32, *apiA* and *apiBC*, and have observed that the encoded proteins from these loci exhibit sequence similarity to members of the YadA multi-protein family. All the YadA-related proteins are homologous at their C-terminus, which comprises a membrane anchor domain formed by four amphipathic transmembrane β-strands and a short left-handed coiled-coil stalk (15). The last nine C-terminal amino acids consist of alternating hydrophobic amino acids ending in F or W and represent the targeting motif for the outer membrane of the gram-negative cell envelope. The amphipathic nature of the β-strands suggests a porin-like structure supporting oligomerization of the YadA-related proteins.

Furthermore, it was demonstrated that *E. coli* expressing ApiA or ApiBC are able to bind to ECM proteins, a feature that may aid in *A. actinomycetemcomitans* localization. The greater binding to ECM proteins by *E. coli* (pAA3211) compared with *E. coli* (pAA3212) may be explained by the increased expression of the 101 kDa protein, possibly corresponding to aggregates of ApiA monomers, as discussed earlier. Many bacteria are known to express cell surface adhesins that mediate their adhesion to ECM proteins (35). In some instances such proteins interact with only one substrate, while in other cases they interact with several collagen types and other ECM components (7, 31, 34). Therefore, only a

Table 4. Binding of extracellular matrix proteins by *H. aphrophilus* and various *A. actinomycetemcomitans* strains

	Binding of bacterial strains[a]				
ECM	*H. aphrophilus*	*E. coli* DH5α	HK1651	UT32ApiBm1	UT32ApiAm1
Calf collagen Type I	0.68 ± 0.15	–	–	–	1.80 ± 0.14
Chicken collagen Type II	–	–	–	–	2.54 ± 0.33
Human collagen Type II	–	–	–	0.69 ± 0.06	–
Human collagen Type III	0.53 ± 0.01	5.10 ± 1.16	–	0.43 ± 0.15	2.15 ± 0.06
Human collagen Type V	0.31 ± 0.12	–	1.99 ± 0.35	–	2.01 ± 0.60
Fibronectin	–	5.15 ± 1.63	–	0.465 ± 0.15	–

[a]The binding is expressed as the ratio of the mean binding of each bacterial species (percent) over the mean binding of the control *A. actinomycetemcomitans* UT32 (percent) to the different ECM components ± the standard error. A value above 1 represents a significant increase in binding; a value below 1 represents a significant decline.

few proteins, such as ApiA and ApiBC, may be responsible for the interaction of *A. actinomycetemcomitans* with the multiple ECM proteins.

It is also possible that the sequence differences between the invasion-related loci $apiBC_{UT32}$ and $apiBC_{HK1651}$ could partially explain the difference in the invasion ability of the two *A. actinomycetemcomitans* strains UT32 and HK1651. However, no difference in the invasive ability of *E. coli* (pAA3221) and *E. coli* (pHK1651) could be detected when tested in the gentamicin protection assay. It is still possible that these proteins interact differently when expressed in *A. actinomycetemcomitans* than when expressed in *E. coli*. Such findings might also provide evidence that this locus undergoes phase variation in *A. actinomycetemcomitans* by slipped-strand mispairing in this region of adenine-rich residues, which could be a way to regulate invasiveness of epithelial cells as well as antigenic variation. Indeed, localized frameshift mutations due to insertion/deletion of even a single nucleotide that lead to phase variation of important virulence factors has been reported for the capsule of *N. meningitidis* (13) and for an adhesin of *Mycoplasma hominis* (46).

It was noted that *E. coli* (pAA3211) also developed a morphology similar to freshly isolated *A. actinomycetemcomitans*. Haase et al. (12) have identified two outer membrane proteins, RcpA and RcpB, specific for the rough colony morphotype. Mintz & Fives-Taylor (30) have identified a 22 kDa cytoplasmic membrane protein, *imp*A, which, when inactivated, resulted in a change of the smooth *A. actinomycetemcomitans* cell phenotype to the intermediate phenotype as well as a change in the outer membrane protein profile. ApiA is, however, a newly identified protein that shows no sequence similarity to RcpA, RcpB (12) or ImpA (30).

Thus far, two allelic replacement mutants (UT32ApiAm1 and UT32ApiBm1) have been constructed. While UT32ApiAm1 did not produce a decrease in invasiveness and was actually five times more invasive than the wild-type control, UT32ApiBm1 demonstrated diminished epithelial cell invasion capacity. These observations parallel outcomes for the ECM binding assay, as UT32ApiAm1 bound much more readily to four of the six collagen types tested when compared with wild-type UT32, while UT32ApiBm1 affinity for ECM proteins was diminished for three of the six proteins tested. While binding by *A. actinomycetemcomitans* UT32 to a potential host cell's surface is a prerequisite for invasion, it does not imply that invasion is a phenotype required by all *A. actinomycetemcomitans* strains for colonization or survival. We noted that HK1651 and *E. coli*, two relatively non-invasive bacterial strains, were as capable of binding ECM proteins as wild-type UT32. This suggests that the genomic differences in *apiBC* exhibited by HK1651 do not affect its ability to bind. Yet, HK1651 is clearly less invasive than UT32, suggesting that a subtle difference in their invasion loci may have a crucial impact on the invasion pathway.

Substitution of the Sp^r gene for a considerable fragment of *apiB* in UT32 severely inhibited invasion; yet Sp^r substitution for *apiA* led to increased invasion and adherence to ECM proteins. Thus it appears that genetic regulation of host cell invasion by *A. actinomycetemcomitans* is complex and may involve compensatory loci. Notably, the *apiBC* locus seems to play a pivotal role in the invasion pathway, as invasion is significantly reduced when it is knocked out. In contrast, *apiA* may function in a secondary and possibly even modulatory capacity related to, but not crucial for, invasion. Although PCR confirmation indicated allelic replacement of the correct *apiA* locus, future studies by protein analysis, RT-PCR and microscopy should attempt to find the underlying reasons for the association of this knockout with the increased invasiveness phenotype.

A >207 kDa protein or complex was observed on the exterior of *E.coli* transformants that contained *apiBC* genetic fragments. This >207 kDa complex appears to be related to invasiveness. Based on its configuration it may exist as a dimer of ApiB and ApiC, or as an oligomer of ApiC. However, *E. coli* (pAA3222), in which this protein appeared less abundantly, demonstrated higher invasiveness than *E. coli* (pAA3221), in which this protein was expressed to a greater extent. It is possible that the 207 kDa component represents an inactive form of an even larger active ''adhesin/invasin'' embedded in the outer membrane, which would not have been detected by SDS-PAGE analysis because of its size and could be more abundant in *E. coli* (pAA3222). Indeed, formation of very large complexes (some in excess of 800,000 kDa) have been reported for UspA1 and UspA2, members of the YadA family (26).

The identification of *apiA* and *apiBC* and their sequence similarity to adhesins/invasins of different bacterial species suggests possible conservation in the mechanisms utilized to colonize and/or invade the epithelium. Furthermore, *A. actinomycetemcomitans*, as exemplified by *apiBC*, may use slipped-strand mispairing to control the product of this locus, which may play a role in invasiveness and possibly in recognition by the host immune system through antigenic variation. Our next aim is to continue to construct more mutants defective in the invasion-related loci and to define their role in various steps of the invasion process and in the pathogenicity of *A. actinomycetemcomitans* in an animal model.

Acknowledgements

We would like to thank Mr Robert Chernecky for technical assistance with the SEM study, Dr Leslie Laing-Gibbard for technical assistance with the invasion assays and Dr Yung-Hua Li for technical assistance with the binding assays. This study was originally supported by a start-up grant (XG 95-036) to R.P.E. from the Hospital for Sick Children Foundation, Toronto, and then a grant to G.L. from the Canadian Institutes of Health Research (CIHR) (MOP-37932).

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