Identification and Characterization of a Novel Adhesin Unique to Oral Fusobacteria

Yiping W. Han,1,2* Akihiko Ikegami,1 Chythanya Rajanna,1 Hameem I. Kawtar,1 Yun Zhou,4 Mei Li,1 Hakimuddin T. Sojar,3 Robert J. Genco,3 Howard K. Kuramitsu,3 and Cheri X. Deng4

Departments of Biological Sciences,1 Pathology,2 and Biomedical Engineering,4 Case Western Reserve University, Cleveland, Ohio, and Department of Oral Biology, School of Dental Medicine, State University of New York at Buffalo, Buffalo, New York3

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Fusobacterium nucleatum is a gram-negative anaerobe that is prevalent in periodontal disease and infections of different parts of the body. The organism has remarkable adherence properties, binding to partners ranging from eukaryotic and prokaryotic cells to extracellular macromolecules. Understanding its adherence is important for understanding the pathogenesis of F. nucleatum. In this study, a novel adhesin, FadA (Fusobacterium adhesin A), was demonstrated to bind to the surface proteins of the oral mucosal KB cells. FadA is composed of 129 amino acid (aa) residues, including an 18-aa signal peptide, with calculated molecular masses of 13.6 kDa for the intact form and 12.6 kDa for the secreted form. It is highly conserved among F. nucleatum, Fusobacterium periodonticum, and Fusobacterium simiae, the three most closely related oral species, but is absent in the nonoral species, including Fusobacterium gongidiformans, Fusobacterium moritiferum, Fusobacterium naviforme, Fusobacterium russii, and Fusobacterium ulcerans. In addition to FadA, F. nucleatum ATCC 25586 and ATCC 49256 also encode two paralogues, FN1529 and FNV2159, each sharing 31% identity with FadA. A double-crossover fadA deletion mutant, F. nucleatum 12230-US1, was constructed by utilizing a novel sonoporation procedure. The mutant had a slightly slower growth rate, yet its binding to KB and Chinese hamster ovarian cells was reduced by 70 to 80% compared to that of the wild type, indicating that FadA plays an important role in fusobacterial colonization in the host. Furthermore, due to its uniqueness to oral Fusobacterium species, fadA may be used as a marker to detect orally related fusobacteria. F. nucleatum isolated from other parts of the body may originate from the oral cavity.

Fusobacterium nucleatum is a long filamentous, gram-negative anaerobe associated with various human diseases, including periodontal, peritonsillar, oral, facial, brain, chest, lung, abdominal, blood, and obstetrical and gynecological abscesses and infections, existing either as a mixed infection or as the dominatal, blood, and obstetrical and gynecological abscesses.

F. nucleatum is recognized as an “adhesive” organism because it binds to a variety of host mammalian cells, including epithelial and endothelial cells, polymorphonuclear leukocytes, monocytes, erythrocytes, fibroblasts, and HeLa cells, as well as salivary macromolecules, extracellular matrix proteins, and human immunoglobulin G (IgG) (2, 25, 26, 50, 59, 62, 63). It also coaggregates with a wide array of microorganisms in the oral cavity and plays an important role in plaque formation (1, 7, 20, 22, 29, 36–38, 46, 53). Identification of the adhesin molecules on F. nucleatum is thus essential for understanding its pathogenesis. It has been suggested that F. nucleatum possesses both lectin-like and non-lectin-like adhesins (44, 49, 54, 58, 60, 61). Three components, a 40- to 42-kDa major outer membrane porin protein (FomA) and 39.5-kDa and 30-kDa polypeptides, have been suggested as possible adhesins from F. nucleatum that are involved in interbacterial coaggregation (33, 34, 55). FomA was also found to bind to the human IgG Fc fragment (23). A high-molecular-mass component, ranging from 300 to 330 kDa, has been suggested as a galactose-binding agglutinin (49). However, it is unclear if any of these components are involved in F. nucleatum binding to the host cells.

F. nucleatum invades epithelial and endothelial cells in vitro, a mechanism presumably employed for its spreading into deeper tissues (25, 26). Invasion of F. nucleatum into endothelial cells was observed in vivo in the mouse placenta (25). A spontaneous mutant defective in tissue cell attachment and invasion, F. nucleatum 12230 lam, has been isolated, but the nature of its mutational change is unknown (26). The lam

* Corresponding author. Mailing address: Department of Biological Sciences, School of Dental Medicine, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106-4905. Phone: (216) 368-1995. Fax: (216) 368-0145. E-mail: ywh2@case.edu.
TABLE 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strains</th>
<th>Source of isolation or relevant characteristic</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. nucleatum</em> 12230</td>
<td>Transtracheal isolate, working strain in the lab</td>
<td>26</td>
</tr>
<tr>
<td><em>F. nucleatum</em> 12230-US1</td>
<td><em>F. nucleatum</em> 12230 ΔfadA::ermF-ermAM</td>
<td>This study</td>
</tr>
<tr>
<td><em>F. nucleatum</em> ATCC 10953</td>
<td>Inflamed gingiva; <em>F. nucleatum</em> subsp. polymorphum</td>
<td>P. E. Kolenbrander</td>
</tr>
<tr>
<td><em>F. nucleatum</em> ATCC 23726</td>
<td><em>F. nucleatum</em> subsp. nucleatum</td>
<td>P. E. Kolenbrander</td>
</tr>
<tr>
<td><em>F. nucleatum</em> ATCC 25586</td>
<td>Cervicofacial lesion; <em>F. nucleatum</em> subsp. nucleatum</td>
<td>P. E. Kolenbrander</td>
</tr>
<tr>
<td><em>F. nucleatum</em> ATCC 49576</td>
<td>Periodontal pocket; <em>F. nucleatum</em> subsp. vincentii</td>
<td>P. E. Kolenbrander</td>
</tr>
<tr>
<td><em>F. nucleatum</em> PK 1594</td>
<td>Periodontal pocket</td>
<td>P. E. Kolenbrander</td>
</tr>
<tr>
<td><em>F. nucleatum</em> DUMC1356</td>
<td>Amniotic fluid; preterm birth</td>
<td>25</td>
</tr>
<tr>
<td><em>F. nucleatum</em> DUMC2079</td>
<td>Placenta; preterm birth</td>
<td>25</td>
</tr>
<tr>
<td><em>F. nucleatum</em> DUMC2929</td>
<td>Amniotic fluid; preterm birth</td>
<td>25</td>
</tr>
<tr>
<td><em>F. nucleatum</em> DUMC3156</td>
<td>Placenta; preterm birth</td>
<td>G. B. Hill</td>
</tr>
<tr>
<td><em>F. nucleatum</em> DUMC3349</td>
<td>Placenta; preterm birth</td>
<td>25</td>
</tr>
<tr>
<td><em>F. gondii</em> DUMC CF65-1</td>
<td>Vaginal tract; bacterial vaginosis</td>
<td>25</td>
</tr>
<tr>
<td><em>F. gondii</em> DUMC CF63-1</td>
<td>Vaginal tract; bacterial vaginosis</td>
<td>25</td>
</tr>
<tr>
<td><em>F. naviforme</em> DUMC CF108-1</td>
<td>Vaginal tract; bacterial vaginosis</td>
<td>G. B. Hill</td>
</tr>
<tr>
<td><em>F. mortiferum</em> ATCC 25557</td>
<td>Maxillary abscess</td>
<td>P. E. Kolenbrander</td>
</tr>
<tr>
<td><em>F. periodonticum</em> ATCC 33693</td>
<td>Periodontitis</td>
<td>P. E. Kolenbrander</td>
</tr>
<tr>
<td><em>F. rauli</em> ATCC 25533</td>
<td>Infection in a cat</td>
<td>P. E. Kolenbrander</td>
</tr>
<tr>
<td><em>F. simiae</em> ATCC 33568</td>
<td>Monkey dental plaque</td>
<td>P. E. Kolenbrander</td>
</tr>
<tr>
<td><em>F. ulcerans</em> ATCC 49185</td>
<td>Skin ulcer</td>
<td>P. E. Kolenbrander</td>
</tr>
</tbody>
</table>

Mutant exhibited virulence similar to that of the wild type in causing fetal death in the mice (25).

*F. nucleatum* also induces an array of host cell responses. It is a strong stimulator of the production of interleukin-8 from epithelial cells, indicating its ability to induce inflammation (15, 26). It stimulates apoptosis of human peripheral white blood cells and suppresses T-cell responses (30, 56). The organism also induces production of innate antimicrobial peptides, human β-defensins, in gingival epithelial cells (40). This is presumably a mechanism to suppress the growth of competitive species.

In this study, we report the identification of a novel 13.6-kDa adhesin peptide from *F. nucleatum* involved in attachment to mammalian cells and the construction of its deletion mutant by a novel sonoporation method.

**MATERIALS AND METHODS**

**Bacterial strains, culture conditions, plasmids, and enzymes.** Bacterial strains and plasmids used in this study are listed in Table 1. All fusobacterial strains were maintained on either Trypticase soy broth (BBL) and incubated as previously described (25). The *Escherichia coli* strains were maintained in LB broth (Difco) or on LB agar (Difco) and incubated at 37°C in air. Restriction endonucleases and ligase were purchased from New England BioLabs (Beverly, MA), and *PfuUltra* high-fidelity DNA polymerase was from Stratagene (La Jolla, CA).

To construct plasmids pYH1378 and pYH1426, a 526-bp fragment, “upfadA,” and a 510-bp fragment, “downfadA,” corresponding to the upstream and downstream regions flanking the *fadA* gene, respectively, were amplified using primer sets *fadA-1f* (5′-AGGTCAAGAAGCAAAAGG-3′)-*fadA-1r* (5′-TTTTTGGATCCTCAAGCTTTAG-3′) and *fadA-2f* (5′-TTTTTGGATCCTCAAGCTTTAG-3′)-*fadA-2r* (5′-AGGTCAAGAAGCAAAAGG-3′) from pYH1378 (18) was then ligated with the “upfadA” and the “downfadA” fragments, followed by cloning into pCR2.1 (Invitrogen, Carlsbad, CA). The resulting plasmid, pYH1378, was digested with EcoRV, and a sacB gene from pRL250 (11) was inserted into the EcoRV site to generate pYH1426.

Preparation of biotinylated and nonlabeled KB surface proteins. The human oral mucosal epithelial cell line KB (ATCC CCL-17; American Type Culture Collection, Manassas, VA) was maintained in MEM medium (GibcoBRL, Rockville, MD) supplemented with 10% fetal bovine serum (Mediatech, Herndon, VA). The cultures were grown in four 75-cm² tissue culture flasks (Fisher Scientific, Pittsburgh, PA) under 5% CO₂ at 37°C to near confluence. The cells were detached from the flasks by using enzyme-free cell dissociation buffer (GibcoBRL). Following washes with sterile phosphate-buffered saline (PBS), the outer membrane components were extracted with 1% Triton X-100 (Sigma) at room temperature for 1 h, followed by centrifugation. The supernatant was transferred to a Centricon YM-3 column (Millipore, Bedford, MA) and centrifuged at 7,500 × g for 1 h. The resulting protein was dialyzed against PBS, and the concentration was determined by bicinchoninic acid (BCA) (Pierce, Rockford, IL). One hundred microliters of fractionated *F. nucleatum* component was used for each sample.

**Far-Western analysis.** A total of approximately 1 × 10⁸ to 5 × 10⁸ CFU of *F. nucleatum* 12230 or 10 μg of fractionated *F. nucleatum* component, unless
otherwise indicated, were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes (0.45-μm pore size; Millipore). The membranes were blocked with 1% bovine serum albumin (Sigma), followed by incubation with biotinylated KB surface proteins at a 1:500 dilution in TBST (50 mM Tris, pH 7.5, 0.5 M NaCl, 0.1% Tween 20) at room temperature for 1 h. The membranes were washed with TBST and incubated with avidin-horseradish peroxidase (Sigma). For controls, the membranes were incubated directly with avidin-HRP conjugate without incubation with biotinylated KB surface proteins. For competitive far-Western analysis, the membrane was preincubated with nonbiotinylated KB surface proteins in 20-fold excess at room temperature for 1 h prior to incubation with biotinylated KB surface proteins.

Preparation of "40P." One liter of freshly grown F. nucleatum 12230 culture was centrifuged, and the cell pellet was resuspended in 10 ml sterile PBS, followed by 10 min of ultrasonication in an ice-water bath with a 3-mm microtip and then twice-centrifuged supernatant was designated the cell extract, to which ammonium sulfate was added to a final concentration of 40% (wt/vol) and incubated at 4°C with agitation for >4 h. The suspension was centrifuged at 100,000 g for 2 h, and the pellet was dissolved in 10 mM Tris, pH 7.5, followed by dialysis against 10 mM Tris, pH 7.5, at 4°C. The resulting solution was designated "40P," and its protein concentration was determined by BCA.

Construction and screening of F. nucleatum 12230 cosmid library. Chromosomal DNA of F. nucleatum 12230 was purified, partially digested with Topo50I, and cloned into the EcoRI site of cosmids P.LAFR2 (19). The ligation mixture was incubated with Gigapack III XL packaging extract (Stratagene), and the cosmid phage lysate was prepared according to the manufacturer's instructions. The phage lysate was used to transfect JM109, and the cosmid clones were selected on LB plates containing 20 μg/ml tetracycline. The clones were saved in 96-well plates and stored at 80°C. Four degenerate oligonucleotide pools were designed based on the protein N-terminal sequence and the Codon Usage Data-base (Table 2). The probes were labeled with digoxigenin (DIG) by using the DIG DNA labeling and detection kit (Roche, Indianapolis, IN). They were then used to screen the F. nucleatum 12230 cosmid library by colony hybridization as described previously (21). Putative positive clones were examined by Southern blotting analysis using pool 4 probes.

**fadA** sequence analysis. The DNA sequence of the 2.4-kb fragment from pYWH401 was determined (CABI Nucleic Acid Facility, Buffalo, NY), first by sequencing the forward and reverse strands of a 200-bp PCR product obtained with primers 45C/310 and 602/881 (from the clindamycin-resistant mutants), respectively. Samples with no detectable DNA contamination were used for reverse transcription-PCR (RT-PCR). Reverse transcription was performed using SuperScript II (Invitrogen) with 1 μg DNA-free RNA and 10 pmol of the DNA dot blotting. Chromosomal DNA of F. nucleatum species was denatured by heating at 95°C for 10 min. A total of 0.5 μg denatured DNA was spotted onto an Immobilon-NY membrane (Millipore). A DIG-labeled 359-bp *fadA* fragment was used as the probe. It was amplified by PCR using primers *fadAtor* and *fadAtrev* with chromosomal DNA of F. nucleatum 12230 as the template.

**Construction of a mutant of F. nucleatum 12230 via sonosonation.** Log-phase F. nucleatum 12230 cells were washed and resuspended to a final concentration of 1 × 10^8 CFU/ml in PBS supplemented with 0.1 mM CaCl_2 and 0.1 mM MgCl_2. A total of 100 μl of the bacterial suspension was mixed with 50 μl plasmid DNA and 50 μl Opti-Gene (Perfluten protein-type A microspheres for injection, USP; Amersham, Princeton, NJ) in a 96-well plate and subjected to ultrasound (US) treatment. A custom-made regular planar piezoelectric lead-zirconate-titanate (PZT) transducer of a circular aperture with a diameter of 5.1 cm (center frequency of 0.96 MHz) was vertically directed upward to irradiate the bacteria in the 96-well plate. A signal generator (33250A; Agilent Technologies, Palo Alto, CA) controlled the duty cycle and initial amplitude of the input signal, which was amplified using a 75-W power amplifier (75A250; Amplifier Research, Souderton, PA). The amplified signal was connected to the US transducer to generate the desired US field. Pulsed US exposures at a duty cycle of 50% and a pulse repetition frequency of 1 Hz were used for a total duration of 90 s. The US beam profile was measured using a calibrated hydrophone system (HPM04/1; Precision Acoustics, United Kingdom), and the effective US output powers were calibrated using a US power meter (UPM-DT-10; Ohmic Instrument Co, Easton, MD). The acoustic pressure of US exposure was 0.5 MPa (corresponding to an initial input signal at 130 mV). Following US treatment, the suspension was plated onto Columbia blood agar plates and incubated under anaerobic conditions at 37°C for 24 h. The bacteria were then replicated onto Columbia blood agar plates containing 0.4 μg/ml clindamycin and incubated for 3 additional days. The clindamycin-resistant colonies were purified on plates before being inoculated into Columbia broth containing 0.4 μg/ml clindamycin. The genetic nature of the mutants was verified by PCR, using primers *fadAtor* and *fadAtrev*, and by Southern blot analysis, using the same 359-bp DIG-labeled *fadA* probe used for DNA dot blotting.

**Northern blot analysis.** Whole-cell F. nucleatum was boiled for 3 min in Lammli sample buffer, subjected to 15% SDS-PAGE, and blotted onto a PVDF membrane. The membrane was incubated overnight with polyclonal anti-FadA serum (unpublished results) at a 1:1,000 dilution at 4°C. After washing, the membrane was incubated with goat anti-rabbit IgG–HRP at a 1:1,000 dilution at room temperature for 1 h, followed by color development as described above.

**RT-PCR.** RNA was prepared from mid-log-phase F. nucleatum, by using the RNeasy minikit (QIAGEN, Valencia, CA), followed by treatment with RNase-free DNAse (QIAGEN). DNA contamination in the RNA samples was determined by PCR amplification of ORF2, *fadA*, and ORF3 with primers Orf2-F (5'-GGAGGGAAGATGGAGAAAGG-3') and Orf2-r (5'-TCTCTGCTATTTGCTGAGAA-3'), *fadAtor* and *fadAtrev*, and Orf3-F (5'-AAGGGTTACTTGGTGTTTGCTGAGAA-3') and Orf3-r (5'-CAATTGGAGAAACTCAAGCTTAACT-3'), respectively. Samples with no detectable DNA contamination were used for reverse transcription-PCR (RT-PCR). Reverse transcription was performed using SuperScript II (Invitrogen) with 1 μg DNA-free RNA and 10 pmol of the

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**TABLE 2. Degenerate oligonucleotide probes used to identify the putative adhesin from an F. nucleatum genomic library**

<table>
<thead>
<tr>
<th>Probe(s)</th>
<th>Sequence</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool 1</td>
<td>GCXACXGTGCTGCTGT TTAGTXGGXGAA</td>
<td>A T D A A S L V G E L Q A L D A</td>
</tr>
<tr>
<td>Pool 2</td>
<td>GCXACXGTGCTGCTGT TTAGTXGGXGAA</td>
<td>A T D A A S L V G E L Q A L D A</td>
</tr>
<tr>
<td>Pool 3</td>
<td>GCXACXGTGCTGCTGT TTAGTXGGXGAA</td>
<td>A T D A A S L V G E L Q A L D A</td>
</tr>
<tr>
<td>Pool 4</td>
<td>TTAGTXGGXGAATTACAGGCXTTTATGC</td>
<td>A T D A A S L V G E L Q A L D A</td>
</tr>
</tbody>
</table>

a Sequence identified by protein N-terminal microsequencing.

b X, A or T. The nucleotides that differ in pools 1, 2, and 3 are underlined.

c Sequence identified by protein N-terminal microsequencing.

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forward primer for each gene in a final volume of 50 µl per reaction. An aliquot of 2 µl of the RT reaction mix was then used for PCR amplification of 25 cycles (94°C for 45 s, 55°C for 30 s, and 72°C for 1 min, followed by a 7-min extension at 72°C), using both the forward and reverse primers described above. The PCR products were subjected to electrophoresis on a 1.0% agarose gel. Each experiment was repeated at least twice.

**Bacterial growth curve.** Fresh broth cultures of *F. nucleatum* were transferred into fresh medium at a 1:4 dilution. An aliquot was taken out every hour, and its optical density at 600 nm was measured using a Genesys 5 UV-visible spectrophotometer (Thermo Electron, Waltham, MA). The experiment was repeated twice.

**Tissue culture cell attachment assay.** KB cells were cultured as described above. Chinese hamster ovary (CHO) cells were maintained in F12K medium (Mediatech) supplemented with 10% fetal bovine serum. The attachment assays were carried out as previously described (26). Briefly, KB or CHO cells were seeded into 24-well trays and allowed to grow to near confluence. Immediately before the assay, the spent medium was replaced with fresh nonsupplemented medium. *F. nucleatum* strains were harvested and resuspended in PBS to a density of 5 × 10^6 cells/ml. Approximately 5 × 10^4 CFU was added into each well and incubated at 37°C under 5% CO2 for 1 h. The monolayers were then washed four times with PBS and lysed with water. *F. nucleatum* attached to the cells was enumerated on blood agar plates. Attachment values were expressed as the percentage of bacteria associated with the host cells relative to the total number of bacteria initially added.

**Nucleotide sequence accession number.** The nucleotide sequence of the 2.4-kb fragment from *F. nucleatum* 12230 containing the *fadA* gene has been deposited in the GenBank database with an assigned accession number AY850357. The accession numbers for the *FadA* sequences from other fusobacterial strains and species are as follows: DQ012969 for *F. nucleatum* ATCC 10953, DQ012970 for *F. nucleatum* ATCC 23726, DQ012971 for *F. nucleatum* ATCC 25586, DQ012972 for *F. nucleatum* ATCC 49256, DQ012973 for *F. nucleatum* ATCC 51190, DQ012974 for *F. nucleatum* DUMC1356, DQ012975 for *F. nucleatum* DUMC2079, DQ012976 for *F. nucleatum* DUMC2929, DQ012977 for *F. nucleatum* DUMC3156, DQ012978 for *F. nucleatum* DUMC3349, DQ012979 for *F. nucleatum* PK1594, DQ012980 for *F. periodonticum* ATCC 33693, and DQ012981 for *F. sinue* ATCC 33568.

**RESULTS**

**Identification of a putative adhesin molecule from *F. nucleatum* 12230.** It was shown previously that *F. nucleatum* binds to both KB cells and normal human gingival epithelial cells (26). Therefore, KB cells were used in this study for ease of manipulation. Sulfo-NHS-LC-biotin is a nonspecific biotinylating agent which does not penetrate mammalian cell membranes. Thus, upon incubation with the KB cells, it nonspecifically labeled proteins on the KB cell surface. Triton X-100 extraction of the KB cells produced a “cocktail” of biotinylated KB surface proteins. When this “cocktail” was incubated with *F. nucleatum* components immobilized on PVDF membranes, binding between *F. nucleatum* adhesins and their receptors on the KB cells could occur. When whole-cell *F. nucleatum* 12230 was tested, one dark and two light bands were identified (Fig. II, panel c). When the same components were incubated directly with avidin-HRP, the two light bands were not detected, while the dark band remained strongly visible (Fig. II, panel b). This observation indicates that the components identified by the two light bands bind KB surface proteins, while the dark band represents an *F. nucleatum* component(s) naturally bound with biotin, such as a carboxylase. The two light bands had apparent molecular masses of 40 kDa and 16 kDa. The 40-kDa component was detected only in the boiled *F. nucleatum* and not in the nonboiled sample, indicating it likely formed oligomers too large to migrate into the gel (Fig. II, panel c). The size of the monomer and its oligomerization suggest that this component could be the trimer-forming major outer membrane porin protein FomA (35). Since the 16-kDa component was more prominent, it was analyzed further.

Incubation of *F. nucleatum* 12230 cell extract with 40% (wt/vol) (NH₄)₂SO₄ resulted in the precipitation of a limited number of *F. nucleatum* components, designated “40P” (Fig. III, panel a). Far-Western blot analysis of 40P using biotinylated KB surface proteins identified one major band (Fig. III, panel c), which was not detected if the membrane was incubated only with avidin-HRP (Fig. III, panel b). The component in Fig. III, panel a, corresponding to that in Fig. III, panel c, was identified by aligning the two PVDF membranes. This component was designated “FadA,” for *Fusobacterium* adhesin A, and its N-terminal amino acid sequence was determined by protein microsequencing (ProSeq, Boxford, MA) (Table 2). Binding between FadA and the biotinylated KB surface proteins increased as the FadA quantity increased (Fig. III, panel a). Furthermore, the binding was inhibited by preincubation with nonlabeled KB surface proteins prior to incubation with the biotinylated proteins (Fig. III, panel b). These results further indicate that FadA bound specifically to a component(s) on the KB cell surface.

**Identification of the fadA gene.** A genomic library of *F. nucleatum* 12230 was constructed by cloning the bacterial chromosomal DNA into the cosmid vector pLAFR2. A total of 576 cosmids clones were saved in six 96-well plates. Asel digestion of 10 randomly picked clones showed that all were independent clones (data not shown). With a mean insert size of approximately 20 kb, and assuming that all clones were independent, this library should have covered the entire *F. nucleatum* 12230 genome four to five times. A total of four different degenerate oligonucleotide pools were used to screen the library (Table 2). Pools 1 to 3 correspond to the first 10 amino acids of the FadA N-terminal sequence. The only difference between these three pools was the codon used for the serine residue at position 6. Pool 4 corresponds to the amino acid sequence from position 7 through 16. Since the *F. nucleatum* genome consists of more than 70% AT, the pools were designed such that only the third position in selected codons carried a mixture of A and T. This design reduced the degeneracy of the oligonucleotide pools. Through repeated colony hybridization and Southern blot analyses, one true positive clone was identified and designated YWH1 (data not shown). Cosmid pYWH1 was purified and digested with different restriction endonucleases. A 6.2-kb EcoRV fragment, a 1.3-kb EcoRI fragment, and a 2.4-kb Sau3AI fragment were identified through Southern blot analysis using pool 4 oligonucleotides as probes (data not shown). A total of four ORFs were identified. The smallest ORF encodes 129 amino acids, with the first eighteen residues corresponding to a typical signal peptide, which should be absent in the secreted form. The next 16 residues perfectly matched the N-terminal peptide sequence of FadA (Table 2), indicating that the component identified by far-Western analysis was the secreted form. FadA is alanine (20%) and leucine (10%) rich. It shares no homology with any known adhesins. Secondary structure analysis performed by the Ph.D. method at the European Molecular Biology server indicated that it was
composed almost exclusively of \( \alpha \)-helix. The intact FadA had a calculated molecular mass of 13.6 kDa, while the secreted form was 12.6 kDa, smaller than the apparent molecular mass of 16 kDa identified by SDS-PAGE.

**Conservation of FadA among fusobacteria.** The presence of \( fadA \) among other species and strains of fusobacteria was examined by DNA dot blotting with 12 strains of \( F. \) nucleatum, 2 strains of \( F. \) gonidiaformans, and one strain each of \( F. \) mortiferum, \( F. \) naviforme, \( F. \) periodonticum, \( F. \) russii, \( F. \) simiae, and \( F. \) ulcerans (Fig. 2). The \( fadA \) gene appeared to exist in the three most closely related species, \( F. \) nucleatum, \( F. \) periodonticum, and \( F. \) simiae, but was absent in the other species (Fig. 2).
2). DNA sequence analysis showed that FadA was highly conserved among the fadA-positive species (Fig. 3).

Following identification of the fadA gene, the genomic sequence of F. nucleatum ATCC 25586 became available (31). A BLAST search showed that the 2.4-kb fragment containing the fadA gene was highly conserved between F. nucleatum 12230 and ATCC 25586 (data not shown). The ORF corresponding to fadA in F. nucleatum ATCC was FN0264. The three additional ORFs near fadA were identified as follows: ORF1 (FN0262), which transcribes in the opposite direction relative to fadA, encodes a formate acetyltransferase; ORF2 (FN0263), immediately upstream of fadA, encodes a peptidyl-prolyl cis-trans-isomerase; and ORF3 (FN0265) is downstream of fadA and encodes a cell division protein, FtsX, with 29% identity to the ABC transporter permease cell division protein FtsX of Listeria monocytogenes (data not shown). In addition, a paralogue (i.e., homologue on the same chromosome), FN1529, is present in F. nucleatum ATCC 25586, sharing 31% identity with FadA (Fig. 3).

More recently, the genome sequence of another strain, F. nucleatum subsp. vincentii ATCC 49256, became available (32). DNA dot blot and PCR analyses indicate the presence of fadA in this strain (Fig. 2 and 3). However, a BLAST search failed to identify fadA in the gapped genome sequence (data not shown). Instead, a FadA paralogue, FN1269, which is 31% identical to FadA and 98% identical to FN1529, was identified (Fig. 3).

Construction of a fadA deletion mutant of F. nucleatum 12230. To the best of our knowledge, currently no report is available on the construction of genetic knockout mutants of F. nucleatum. Since fadA is made up of fewer than 400 bases, it would be difficult to construct a knockout mutant by integrating a suicide plasmid containing an internal fragment of fadA. Following unsuccessful attempts to generate a correct fadA deletion mutant of F. nucleatum 12230 by either electroporation or conjugation, DNA delivery via sonoporation, i.e., transient membrane permeabilization by ultrasound, was tested. Plasmid pYH1426, which contains a homologous fragment of approximately 500 bp at either end of fadA and a 2.1-kb ermF-ermAM cassette replacing fadA, was used (Fig. 4A). The ermF-ermAM cassette confers erythromycin and clindamycin resistance (24). The plasmid also carries a 2.1-kb fragment containing a sacB gene, conferring sucrose sensitivity (11). Intact pYH1426 was mixed with F. nucleatum 12230 and Optison, followed by a 90-s (pulse repetition frequency, 1 Hz; duty cycle, 50%) ultrasonic treatment. Optison is a Food and Drug Administration-approved contrast agent consisting of albumin-coated perfluoropropane (C3F8) gas bubbles and is routinely used in ultrasound imaging for cardiac diagnosis. It has been used to facilitate sonoporation in mammalian cells (47). Under these experimental conditions, the viability of F. nucleatum 12230 was not affected; nor was there any detectable DNA damage when examined by agarose gel electrophoresis (data not shown). Ultrasonic delivery of pYH1426 into F. nucleatum 12230 produced more than 30 independent transformants, at an efficiency of approximately 0.05 transformant/μg DNA. All transformants were genetically identical double-crossover fadA deletion mutants, as determined by PCR (data not shown) and Southern blot analyses (Fig. 4B). Loss of FadA in these mutants was verified by Western blotting using anti-FadA polyclonal antibodies (Fig. 4C). One of the mutants was designated F. nucleatum 12230-US1 (Table 1).

In order to determine if insertional inactivation had any polar effects on the downstream gene ORF3, Northern blotting and RT-PCR were performed (Fig. 5). Northern blotting using fadA as a probe revealed a transcript of approximately 400 bases, indicating that fadA was transcribed monocistronically (Fig. 5A). DNA sequence analysis indicated the likely existence of a rho-independent transcription terminator immediately downstream of fadA from position 1893 to 1941 (data not shown). Transcription of both neighboring genes of fadA, the upstream ORF2 and the downstream ORF3, was unaffected in F. nucleatum 12230-US1 as indicated by RT-PCR (Fig. 5C).

Characterization of the fadA deletion mutant. F. nucleatum 12230-US1 was characterized by its growth rate, aerotolerance, and ability to bind to KB and CHO cells, each in comparison with its parental strain F. nucleatum 12230. The mutant consistently grew at a lower rate, with a doubling time in the exponential phase of approximately 6 h, compared to 5 h for the parental strain (Fig. 6). The mutant exhibited aerotolerance similar to that of the wild type (data not shown). When tested for binding to KB and CHO cells, F. nucleatum 12230-US1 was found to be severely defective. Although the percent attachment levels varied when KB or CHO cells were used, the difference between the wild type and the mutant remained consistent, with the mutant exhibiting a reduction of approximately 70 to 80% (Fig. 7). These observations indicate that FadA is nonessential for bacterial integrity but is required for its binding to host cells.
DISCUSSION

*F. nucleatum* binds to a wide variety of partners, including both eukaryotic and prokaryotic cells. Although a few putative adhesins have been suggested to be involved in interbacterial coaggregation or agglutination of red blood cells, it is unclear if they are also required for *F. nucleatum* binding to other host cells. Known for its wide-ranging adherence properties, *F. nucleatum* may possess multiple adhesins, some of which may be partner specific while others may have multiple binding substrates. In this study, a novel peptide, FadA, was identified, with the /H9251\-helix predicted as the predominant secondary structure. The secreted form of FadA has a larger apparent molecular mass than its calculated molecular mass (Fig. 1). As a putative adhesin, FadA is likely associated with the outer membrane. It is not unusual for a membrane protein to have aberrant migration on SDS-PAGE. For instance, the 40-kDa FomA protein has been reported to migrate as 37-kDa, 40-kDa, 42-kDa, and 62-kDa proteins (5, 34, 35).

Loss of FadA resulted in a 70 to 80% reduction of the organism’s ability to bind to KB and CHO cells (Fig. 7). Several possibilities exist: (i) the loss of attachment was due to the lower growth rate of *F. nucleatum* 12230-US1, (ii) the defect was due to a polar effect on the downstream gene(s), (iii) FadA serves as an accessory protein for binding, or (iv) FadA is a major adhesin directly involved in *F. nucleatum* binding to host cells. The first three possibilities are unlikely for the following reasons: (i) the incubation time during the attachment assay was 1 hour, during which the bacterial growth was minimal; (ii) Northern blot and RT-PCR analyses indicated that *fadA* was transcribed monocistronically and that transcription of ORF3 was unaffected by the mutational change in *fadA* (these observations were also supported by the detection of a putative transcription terminator immediately downstream of *fadA*), and (iii) FadA was identified by far-Western analysis as directly and specifically bound by biotinylated KB surface proteins (Fig. 1). Taken together, the most reasonable explanation would be that FadA is directly involved in binding. Further supporting this notion is that expression of FadA in *E. coli* enhanced the ability of *E. coli* to bind to mammalian cells (unpublished results). Since *F. nucleatum* 12230-US1 was de-
Effective in binding to both KB and CHO cells, it is likely that FadA binds to a receptor(s) common to both types of cells. It should be pointed out that although FadA appears to be a significant adhesin for \textit{F. nucleatum} to bind to host cells, an additional adhesin(s) exists, likely accounting for the remaining binding activities observed in \textit{F. nucleatum} 12230-US1.

Although BLAST searches failed to identify FadA in the gapped genome of \textit{F. nucleatum} ATCC 49256, DNA hybridization techniques were used to study the expression of FadA in the wild-type strain and its mutant. Figure 4 illustrates the construction of the \( \Delta \text{fadA}: \text{erm} \) mutant by double-crossover allelic exchange using the erythromycin resistance cassette \( \text{ermF-ermAM} \) inserted between bp 71 and 365 of the \textit{fadA} gene. The shaded boxes represent regions hybridizing with the probe during Southern blotting. The sizes of the fragments hybridized with the probe are indicated.

**FIG. 4.** Inactivation of the \textit{fadA} gene of \textit{F. nucleatum} 12230. A. Schematic diagram of construction of the \( \Delta \text{fadA}: \text{erm} \) mutant by double-crossover allelic exchange. The erythromycin resistance cassette \( \text{ermF-ermAM} \) was inserted between bp 71 and 365 of the \textit{fadA} gene. The shaded boxes represent regions hybridizing with the probe during Southern blotting. The sizes of the fragments hybridized with the probe are indicated. H, HindIII cleavage sites; E, EcoRI cleavage sites. B. Southern blot analysis of \textit{F. nucleatum} 12230 and \textit{F. nucleatum} 12230-US1, using a 359-bp \textit{fadA} fragment as a probe. Lanes: 1, \textit{F. nucleatum} 12230 digested with EcoRI; 2, \textit{F. nucleatum} 12230-US1 digested with EcoRI; 3, \textit{F. nucleatum} 12230 digested with HindIII; 4, \textit{F. nucleatum} 12230-US1 digested with HindIII. The DNA size markers are indicated on the left. C. Western blot analysis of \textit{F. nucleatum} 12230 and 12230-US1, using anti-FadA antibodies. Lanes: 1, protein size markers, with molecular masses shown on the left; 2, \textit{F. nucleatum} 12230; 3, \textit{F. nucleatum} 12230-US1. The arrow indicates FadA.
ization, PCR, and sequence analyses indicated that FadA is highly conserved among \textit{F. nucleatum}, \textit{F. periodonticum}, and \textit{F. simiae} yet is absent in \textit{F. mortiferum}, \textit{F. gonidiaformans}, \textit{F. naviforme}, \textit{F. russii}, and \textit{F. ulcerans} (Fig. 2 and 3). \textit{F. nucleatum}, \textit{F. periodonticum}, and \textit{F. simiae} have been reported as three closely related oral species, forming a distinct group within the genus (42, 51). The presence of FadA in these three species and its absence in others are consistent with the previously described genetic relatedness within the group. Therefore, \textit{fadA} may be used as a marker for identification of orally related fusobacteria. The conservation of \textit{fadA} in \textit{F. nucleatum} isolated from intrauterine infections and its absence in the vaginal species \textit{F. gonidiaformans} and \textit{F. naviforme} further support the hypothesis that intrauterine \textit{F. nucleatum} originates from the oral cavity rather than the vaginal tract (25). BLAST searches also identified two paralogues of FadA, FN1529 from \textit{F. nucleatum} ATCC 25586 and FNV2159 from \textit{F. nucleatum} ATCC 49256, which share 31\% identity with FadA and 98\% identity with each other. The conservation of the FadA paralogue among fusobacteria and its role in adherence are currently under investigation.

Genetic manipulation of \textit{F. nucleatum} has been difficult, presumably due, in part, to its diversified restriction endonuclease systems, which differ between strains and cleave DNA irrespective of the extent of methylation (43). Attempts to construct a \textit{fadA} deletion mutant of \textit{F. nucleatum} 12230 by either electroporation or conjugation were unfruitful. This could be attributed to one or more of the following: (i) inefficient DNA delivery by electroporation or conjugation, (ii) inefficient homologous recombination between the exogenous plasmid and the bacterial chromosome, (iii) exogenous DNA being digested by a restriction endonuclease(s) before recombination could occur, or (iv) killing of the bacteria by electroporation. DNA delivery via ultrasound has been employed with mammalian cells (4, 14, 47, 57, 64). It has been suggested that ultrasonic treatment of mammalian cells induces transient membrane permeability, allowing uptake of extracellular compounds, such as chemotherapeutic agents, genetic materials, and fluorescence markers, which normally do not permeate the cell membrane (17). Although ultrasound treatment in the presence of Optison enhances sonoporation, its mechanism is not clearly understood (47). Our results demonstrate that the same technology could also be applied to bacteria, even though the bacterial cell envelope is quite different from that of mammalian cells. Unlike electroporation, which kills the majority of the bacteria, ultrasonic treatment under the testing conditions used did not affect the viability of \textit{F. nucleatum}. By mixing \textit{F. nucleatum} 12230 with intact pYH1426, we intended to first obtain a single-crossover merodiploid construct through sonoporation and then utilize the \textit{ sacB} gene on pYH1426 to select for FadA-negative clones.
for a double-crossover mutant on sucrose medium (11). Surprisingly, all transformants obtained were fadA deletion mutants. The mechanism of this one-step double-crossover allelic exchange is unclear and is currently under investigation. It should be pointed out that, as a preliminary test, the concentrations and ratios of the bacteria, plasmid, and Optison were empirically determined and thus may be far from optimal. Additional work is needed to understand the sonoporation mechanism and to optimize its conditions.

In summary, a novel adhesin, FadA, which is unique to oral fusobacteria, was identified. It was required for F. nucleatum attachment to epithelial cells and thus may play an important role in Fusobacterium colonization in the host.

FIG. 6. Growth of F. nucleatum 12230 (solid triangles and solid line) and F. nucleatum 12230-US1 (open squares and dashed line) in Columbia broth. OD 600, optical density at 600 nm.

FIG. 7. Attachment of F. nucleatum 12230 and F. nucleatum 12230-US1 to KB (hatched bars) and CHO (open bars) cells. The levels of attachment are means and standard deviations from three separate experiments, each performed in triplicate.

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