FADA FROM \textit{FUSOBACTERIUM NUCLEATUM} UTILIZES BOTH SECRETED AND NON-SECRETED FORMS FOR FUNCTIONAL OLIGOMERIZATION FOR ATTACHMENT AND INVASION OF HOST CELLS*

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Running title: characterization of the FadA adhesin/invasin from \textit{F. nucleatum}

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\textit{Fusobacterium nucleatum} is a Gram-negative anaerobe associated with various human infections including periodontal diseases and preterm birth. A novel FadA adhesin was recently identified for host-cell binding. It consists of 129 amino-acid (aa) residues, with an 18-aa signal peptide. Expression of FadA in \textit{Escherichia coli} enhanced bacterial binding to host epithelial and endothelial cells. In both \textit{E. coli} and \textit{F. nucleatum}, FadA exists in two forms, the intact pre-FadA and the secreted/mature FadA (mFadA), with pre-FadA anchored in the inner membrane and mFadA secreted outside the bacteria. Pre-FadA and mFadA formed high molecular weight (M.W.) complexes. When each form was purified to a single species, mFadA was soluble at neutral pH while pre-FadA was insoluble. Pre-FadA became soluble when mixed with mFadA or under acidic pH. When fluorescent-labeled mFadA alone was added to the epithelial cells, no binding was detected. However, when mixed with non-labeled pre-FadA, binding and invasion of mFadA into epithelial cells was observed. FadA is a unique bacterial adhesin/invasin in that it utilizes its own two forms for both structural and functional purposes. The pre-fadA-mFadA complex is likely anchored in the inner membrane and protrudes through the outer membrane. Internalization of the pre-FadA-mFadA ensures invasion of the bacteria into the host cells.

\textit{Fusobacterium nucleatum} is a gram-negative anaerobe associated with various human infections. It is ubiquitous to the oral cavity and is implicated in periodontal diseases (1). The organism coaggregates with microbial species in the oral cavity, playing a critical role in periodontal plaque formation (2). It is also isolated from infections and abscesses of other parts of the body and is one of the most prevalent species in human intrauterine infections (3-5). \textit{F. nucleatum} may translocate from the oral cavity to different sites in the body haematogenously and cause localized abscess or infection (6). The organism binds to and invades different types of host cells. Attachment and invasion of epithelial and endothelial cells by \textit{F. nucleatum} was observed \textit{in vitro}, which elicited pro-inflammatory responses (7,8). Attachment and invasion of endothelial cells was observed \textit{in vivo} in infected mouse placentas, leading to bacterial colonization in the placenta and resulting in adverse pregnancy outcomes (7).

It has been postulated that \textit{F. nucleatum} may possess lectin-like and nonlectin-like adhesins for binding to various partners (9-12). Different \textit{F. nucleatum} strains may bind to the same partner via different adhesins (9). The same adhesin(s) may also be involved in binding to different partners (12). Several putative adhesin molecules have been suggested for \textit{F. nucleatum} for involvement in binding to other microbial species or human IgG (13-17). However, none has been characterized, and it was not known if they were involved in bacterial binding to the host cells.

A novel adhesin, FadA, from \textit{F. nucleatum} 12230, was recently identified to be involved in attachment to host epithelial cells (18). The \textit{fadA} gene was highly conserved among oral fusobacterial species, including \textit{F. nucleatum}, \textit{F. periodonticum} and \textit{F. simiae}, but was absent from the non-oral fusobacteria. FadA consists of 129 aa residues, with the first 18 encoding a typical signal peptide (MKKFLLLAVLAVSASAFA) (18). It was not known, however, if the signal peptide was
cleaved during secretion in *F. nucleatum*. Based on the amino-acid sequence, FadA appeared to be predominantly α-helical. Using a novel gene-disruption technique, sonoporation, we constructed the first double cross-over allelic exchange mutant of *F. nucleatum*, US1, carrying a deletion of fadA. Binding of US1 to the oral mucosal cell KB and Chinese hamster ovarian (CHO) cells was each reduced by 70-80% compared with the wild-type strain. Therefore, FadA was involved in binding to epithelial cells (18). In the current study, the FadA adhesin was expressed in *Escherichia coli* as a His•Tag fusion protein, which was purified and characterized. The recombinant FadA not only attached to but also invaded the host cells as a heterogenous complex. The formation of such a complex required both the intact and secreted forms of FadA and the complex is likely anchored in the inner membrane and protrudes through the outer-membrane.

**Experimental Procedures**

**Bacterial strains, plasmids, and culture conditions**- The bacterial strains and plasmids used for this study are listed in Table 1. All *F. nucleatum* strains were propagated as described (19). All *E. coli* strains were maintained in LB Broth (DIFCO) or on LB Agar (DIFCO) and incubated at 37°C in air. Plasmid pYWH417-6 was constructed as following. The fadA gene from *F. nucleatum* 12230 was amplified from pYWH401 using primers "fadAtop-NdeI", 5’GGAATTCCATATGAAAAAATTTTTATTATCAGAG3’, and "fadAbottom-XhoI-His•Tag", 5’CCGCTCGAGGTTACCAGCTCTTAAAGCCTG3’, using Vent DNA polymerase (New England BioLabs, Ipswich, MA). The PCR fragments contained Nde I and Xho I sites (underlined) at their 5’ ends, respectively. Following endonuclease digestion and purification using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), the fragment was cloned into pET21(b) (Table 1) at the Nde I and Xho I sites, followed by transformation into competent *E. coli* DH5α (Gibco BRL, Rockville, MD).

**DNA sequencing analysis**- Plasmids were purified from DH5α using Wizard® Plus Midipreps DNA Purification System (Promega, Madison, WI) and their DNA sequences analyzed at the Molecular Biotechnology Core (Lerner Research Institute, Cleveland, OH) using the T7 terminator and promoter primers. Once the constructs were verified, the plasmids were transformed into BL21(DE3) for protein expression.

**Expression of the recombinant proteins**- *E. coli* BL21(DE3) carrying either pET21(b), pYWH417-6, or the mutant plasmids were grown to OD$_{600}$ of 0.5. The cultures were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma, St. Louis, MO) at a final concentration of 0.1 mM for 2-3 hours unless otherwise specified.

**Tissue cell attachment assay**- CHO (ATCC CRL10154, ATCC), human umbilical vein endothelial cells (HUVEC; ATCC CRL-1730), oral mucosal cell line KB (ATCC CCL-17, known to be contaminated with HeLa markers) and primary cultures of human gingival epithelial cells (HGEC) were all maintained as previously described (7,8,18). Immortalized human oral keratinocytes OKF6/Tert cells were obtained from Dr. J. Reinwald (Harvard University, Boston, MA) and were grown as previously described (20). The attachment assays were carried out as previously described (8,18). Briefly, cells were seeded into 24-well trays and allowed to grow to near confluence. The bacteria were then added to the monolayers at a multiplicity of infection of 50–150:1. For inhibitory attachment assays, purified mFadA or reconstituted pre-FadA-mFadA complex was added to the monolayers prior to the addition of bacteria. Following one-hour incubation at 37°C under 5% CO$_2$, the monolayers were washed with phosphate-buffered saline (PBS, Sigma) and lysed with water. It was shown previously that water lysis under the test conditions did not affect bacterial viability (8,18). Serial dilutions were plated onto agar plate, followed by incubation under appropriate conditions to allow for growth of the total cell-associated bacteria (7,8). The bacterial colonies were enumerated and the levels of attachment were expressed as the percentage of bacteria recovered following cell lysis relative to the total number of bacteria initially added. Each experiment was performed in triplicate.

**Purification of recombinant Fad A mixture under denaturing conditions**- The bacterial pellet (about 1 gram) from 250 ml of IPTG-induced culture was collected by centrifugation at 3000×g for 10min, resuspended in 5 ml of buffer A (50 mM NaH$_2$PO$_4$, 0.3 M NaCl, 8 M Urea, pH8.0) and...
incubated at room temperature for 1 hr. Clear lysate was collected by centrifugation (7000×g, 20 min) and mixed with 1 ml of TALON® cobalt resin (BD Clontech, Mountain View, CA) for 1 hr at room temperature. The mixture was transferred to a small column. After all the solution was removed, the resin was washed with 6 ml of buffer A, and eluted with 4 ml of buffer B (50 mM NaH₂PO₄, 0.3 M NaCl, 8 M urea, pH 5.0). The eluted sample (4 ml) was dialyzed extensively against 10 mM Tris-HCl, pH 7.4 at 4ºC, in a dialysis tubing with M.W. cut-off of 6-8,000 Da. The protein concentration was determined using the BCA kit (Pierce, Rockford, IL).

Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) - Protein bands from SDS-PAGE were excised and eluted from the gel slices. The gel-purified proteins were dialyzed against PBS and analyzed by MALDI-TOF MS. The mass spectrometer (Biflex III, Bruker Daltonics, Billerica, MA) was used at an acceleration voltage of 20 kV operated in the linear mode with delayed extraction and a pulsed nitrogen laser source (λ = 337 nm). One microliter of the mixture was applied onto the laser target probe and was air-dried before being introduced into the mass spectrometer. A total of 200-300 spectra were obtained for each sample. The spectra were averaged and analyzed using the Xacq software provided by the manufacturer. The instrument was calibrated using a mixture of standard proteins. A mass accuracy of 0.02-0.08% was routinely obtained.

Production of polyclonal antibodies against FadA - Two 12-week-old male New Zealand white rabbits were each injected intradermally with 500 µg mFadA in Freund’s complete adjuvant (Sigma), followed by three weekly boosts three weeks later, by injecting 500 µg FadA in Freund’s incomplete adjuvant each time. Antisera were collected one week after the final boost.

Production of monoclonal antibodies against FadA - The mouse anti-FadA monoclonal antibody (mAb) 5G11-3G8 was produced at the Hybridoma Core (Lerner Research Institute) following standard procedures. Briefly, the hybridomas secreting mAb were derived from the BALB/c mice immunized with recombinant mFadA. Antibodies of the desired specificity were identified by their specific binding to FadA in ELISA, followed by Western blotting analysis using purified FadA proteins and Fusobacterium nucleatum 12230. One of the hybridoma clones was designated as 5G11-3G8. The mAb from this clone was obtained from the serum-free culture, followed by purification using the protein G column, and stored at a final concentration of 4 mg/ml.

Western blotting analysis - Bacteria culture supernatant, cell pellets, or fractions were loaded onto 12% SDS-PAGE gels. After electrophoresis, the bacterial components were transferred to Immobilon-P PVDF membranes (Millipore, Bedford, MA). Expression of the FadA-His•Tag fusion protein was detected using either INDIA™ HisProbe-HRP (1:1,000 dilution, Pierce), or rabbit anti-FadA polyclonal antibodies (1:1,000 dilution), or mAb 5G11-3G8 (1:20,000 dilution), and HRP-conjugated goat-anti-rabbit IgG (1:1,000 dilution, Sigma), or HRP-conjugated goat-anti-mouse IgG (1:2,500 dilution, Pierce), followed by chemiluminescence using SuperSignal West Dura Extended Duration Substrate (Pierce) or HRP color development reagent (Bio-Rad).

Gel filtration column chromatography - Approximately 1.8 mg of protein in 1 ml of elution buffer (20 mM Tris-Cl, 0.1 M NaCl, pH 7.5) was applied to a Sephacryl S-300 column (1.6×60 cm, Amersham Biosciences, Piscataway, NJ), connected and operated by a BioLogic LP system (Bio-Rad). The protein was eluted at a flow rate of 0.5 ml/min at 4ºC, fractions collected at 2 or 2.5 ml/tube and the absorption at 280 nm recorded. A standard curve was generated using blue dextran (2,000 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.3 kDa), all purchased from Sigma.

Bacterial fractionation - Bacteria were collected by centrifugation, washed twice with PBS, and resuspended in 20 mM Tris-HCl, 0.1 mM PMSF, pH 7.4. All samples were kept at 4ºC unless otherwise stated. Bacteria were disrupted by sonication in an ice-water bath using a model 60 Sonic Dismembrator (Fisher Scientific) (15 seconds with 45 seconds interval, 20 cycles) with an output power of 18 watts for a total of 5 min.
The lysate was centrifuged at 4°C at 3000×g for 15 min and again at 7000×g for 10 min to remove unbroken cells. The supernatant was then subjected to ultracentrifugation at 100,000×g for 1 hr at 15°C producing the clear supernatant, i.e., cell extract. The pellet was washed with ice-cold 20 mM Tris, 0.1 mM PMSF, pH 7.4, resuspended in the same buffer containing 0.8% N-lauroylsarcosine (Sigma) and incubated at 30°C for 30 min. After ultracentrifuge at 100,000×g for 1 hr at 15°C, the clear supernatant was saved as the inner-membrane fraction. The procedure was repeated once and the pellet was resuspended in 20 mM Tris, 0.1 mM PMSF, pH 7.4, and was saved as the outer-membrane fraction. The protein concentrations were measured by BCA. To detect mFadA in the culture supernatant of E. coli, 20 ml of the culture supernatant was filtrated through a Nalgene 0.2 µm syringe filter, followed by 80% ammonium sulfate precipitation in the presence of 1 mM PMSF at 4°C for 16 hr. Sedimented protein was obtained by centrifugation at 10,000×g at 4°C for 5 min. The proteins were dissolved in 1 ml of 20 mM Tris-HCl buffer, pH 8.0, followed by washing with 20 mM Tris-HCl buffer contained 80% ammonium sulfate and dialyzed against 10 mM Tris-HCl buffer at 4°C for 16 hr.

Purification of mFadA- The bacterial pellet from 3 liters of IPTG-induced culture was collected by centrifugation and resuspended in 300 ml PBS. The suspension was incubated at 60 °C for 30 min, followed by centrifugation at 3000×g for 10 min. The bacterial cell pellet was saved for purification of pre-FadA (see below). The clear supernatant was mixed with 6 ml of TALON® Cobalt resin in 50 mM NaH₂PO₄, 0.3 M NaCl, pH 8.0. After transferring to a column and washing, mFadA was eluted with 20 ml washing buffer containing 0.15 M imidazol. The eluate was suspended in 8 M urea pH 4.0 and applied to the High S cationic exchange column (Bio-Rad) pre-equilibrated with buffer C (50 mM acetic acid, 0.3 M NaCl, 8 M urea) at a flow rate of 0.5 ml/min. The column was then washed with 50 ml buffer C to collect mFadA. The eluted sample was dialyzed against 50 mM phosphate buffer, pH 8.0, at 4°C for 16 hrs. The protein concentration was determined by BCA.

Fluorescent labeling of proteins- Purified mFadA or cytochrome c were labeled with Alexa fluor 488 (Molecular Probes, Eugene, OR) according to manufacture’s instructions. Briefly, purified mFadA was mixed with Alexa fluor 488 stock solution at a molar ratio of 5:1 and incubated at room temperature in dark for 1 hr. The labeled protein was purified by D-Salt Dextran Desalting Column (Pierce), and concentrated by Microcon YM-3 (Millipore, Bedford, MA).

Reconstitution of pre-FadA-mFadA complex- Purified mFadA was mixed with purified and acid-dissolved pre-FadA at a molar ratio of 5:1 by slowly adding pre-FadA into mFadA, followed by incubation at 4°C for at least 2 hrs. The mixture was dialyzed against 10 mM Tris-Cl, 0.1 M NaCl, pH 7.4, with M.W. cut-off of 6-8,000 Da. For the epithelial cell binding assays, fluorescent-labeled mFadA was used for the complex reconstitution (below).

Binding of fluorescent-labeled recombinant FadA to OKF6/Tert cells- Immortalized human oral keratinocytes OKF6/Tert cells were inoculated into a Lab-Tek II chamber slide system (Nalge Nunc International co., Naperville, IL) and grown until 50% confluent. Following pre-incubation in culture medium containing 5% BSA for 1 hr, the fluorescent-labeled proteins were added and incubated overnight at 37°C under 5% CO₂. The cells were washed 4 times with Hank’s buffer. The actin and cell nuclei were sequentially stained.
with 20 µM Alexa fluor 568 Phaloidine (Molecular Probe) at 37°C for 15 min and 10 µg/ml of DAPI (Molecular Probe) at room temperature for 5 min, respectively. For the competition assays, non-labeled pre-FadA-mFadA complex (mFadA: pre-FadA ratio of 5:1) in 20 fold excess or mFadA alone in 20-fold excess was added to the monolayers 8 hours prior to the addition of the fluorescent-labeled FadA complex. The fluorescence was observed under an Olympus IX71 microscope using emission filter BA420 and excitation filter BP330-385 for DAPI (exposure time: 2.5 ms), emission filter BA515IF and excitation filter BP460-490 for Alexa fluor 488 (exposure time: 250 ms), and emission filter BA590 and excitation filter BP510-550 for Alexa fluor 568 (exposure time: 500 ms). The images were captured using an Olympus DP70 camera operated with DP controller software version 1.2.1.108.

### RESULTS

The fadA gene was amplified from pYWH401 by PCR, cloned into the expression vector pET21(b), and transformed into E. coli DH5α (Table 1). The recombinant plasmid carried a fusion gene expressing FadA with eight additional residues, Leu-Glu-His6, at the carboxyl end. Through DNA sequencing analysis, a wild-type FadA fusion construct was identified and designated as pYWH417-6 (Table 1). A mutant construct was also identified and designated as pYWH417-2, with a Glu-to-Lys substitution at position 26 in the mature form of FadA (mFadA E26K). This mutation was presumably introduced during PCR amplification (Table 1). All three plasmids, pYWH417-2, pYWH417-6, and pET21(b), were transformed into E. coli BL21(DE3). Expression of the recombinant fusion proteins was tested with different amounts of IPTG and induction times, followed by Western blotting analysis using INDIATM HisProbe-HRP specific for His•Tag. An optimal amount of FadA-His•Tag fusion protein was detected from BL21(DE3)/pYWH417-6 following a 2-hr induction with 0.1 mM IPTG (Fig. 1A). Under all conditions tested, no His•Tag fusion was detected from BL21(DE3)/pET21(b) (Fig. 1A). The apparent M.W. of the recombinant fusion protein was between 16-17 kDa, higher than the calculated M.W. of 15.7 kDa for the intact or 13.7 kDa for the mature (secreted) protein (Fig. 1A).

The stability of the expressed recombinant proteins was also examined. As shown in Fig. 1B, wild-type FadA-His•Tag fusion was consistently induced even after 5 hours of incubation with 0.1 mM IPTG. The expressed protein appeared to be rather stable, with no detectable degradation following one hour incubation on ice. In contrast, expression of FadA E26K-His•Tag fusion was much weaker and unstable (Fig. 1B). Moderate expression was detected following 0.5 hr of induction with either 0.1 mM or 1 mM IPTG. However, the protein level quickly decreased with time. It was only weakly detected after 1 hr of induction and was not detected at all after 2 hours, indicating degradation of the expressed protein. Thus BL21(DE3)/pYWH417-2 was no longer used in the subsequent studies. For each strain tested, an equal amount of colony-forming units (cfu) was loaded onto each lane, as determined by OD600 and verified by SDS-PAGE (data not shown).

BL21(DE3) carrying pET21(b) or pYWH417-6 were tested for attachment to host epithelial and endothelial cells following IPTG induction. BL21(DE3)/pET21(b) demonstrated varying background levels of attachment to
HGEC, KB, CHO and HUVEC cells (Fig. 2). For each cell type, the level of attachment was consistently enhanced 3-4 fold when BL21(DE3)/pYWH417-6 was tested (Fig. 2). Thus, the expressed FadA-His•Tag fusion protein was functional and facilitated E. coli attachment to different host cells.

The recombinant FadA-His•Tag fusion protein was purified from E. coli BL21(DE3)/pYWH417-6 following IPTG induction using the cobalt column under denaturing conditions. When examined by 15% SDS-PAGE, a major upper band and a minor lower band were detected in the cobalt column eluate, with the upper band migrating near 16 KD and the lower band migrating faster (Fig. 3A). Each band was gel purified and subjected to N-terminal peptide sequencing (Lerner Research Institute) and mass spectrometry analysis. The first 5 aa residues of the upper band were ATDAA, matching those immediately following the putative 18-aa signal peptide. Thus, the upper band is presumably the secreted/mature form of FadA (mFadA). Surprisingly, the first 5 aa residues of the lower band were MKKFL, matching those of the intact form of FadA (pre-FadA). The average molecular mass was 13,641 Da for the upper band and 15,509 Da for the lower band, as determined from the detection of the singly and doubly protonated molecules by MALDI-TOF MS (Fig. 3B and 3C). These results were consistent with the calculated molecular mass of the presumed mFadA (ca. 13,650 Da) and pre-FadA (ca. 15,513 Da), respectively. The noise level of the mass spectra was low, indicating the two bands did not cross-contaminate. Since the outcome was the opposite of what was expected, with the smaller component migrating slower than the larger component on SDS-PAGE, the experiments were repeated to ensure that no mislabeling of samples occurred. Therefore, despite their anomalous migration on SDS-PAGE, two forms of FadA expressed as intact (pre-FadA) and secreted (mFadA) species were correctly assigned following N-terminal protein sequencing and mass spectrometry.

Rabbit polyclonal anti-mFadA antibodies were raised and used to detect the presence of FadA in various F. nucleatum strains. FadA was present in all seven wild-type strains, F. nucleatum 12230, F. nucleatum ATCC10953, F. nucleatum ATCC25586, F. nucleatum ATCC23726, F. nucleatum ATCC49256, F. nucleatum ATCC51190, and F. nucleatum PK 1594, but was absent in F. nucleatum 12230-US1, the fadA-deletion mutant constructed previously in our laboratory (Fig. 4). These results agreed with the previous report on the conservation of fadA among oral fusobacteria and confirmed that the fadA genes were actively transcribed in different strains (18). In all seven strains, an upper and a lower band were detected, although their respective intensities varied from strain to strain. In F. nucleatum ATCC23726, the lower band was more prominent than the upper band, whereas in F. nucleatum ATCC51190, the former was hardly visible (Fig. 4). This could be due to differential expression of FadA in different strains, or due to the varying specificity of the polyclonal antibodies for each FadA variant. Nonetheless, the results suggested co-existence of mFadA and pre-FadA in F. nucleatum.

Localization of mFadA and pre-FadA in E. coli BL21(DE3) and F. nucleatum, respectively, was assessed by bacterial fractionation. E. coli BL21(DE3) carrying either pYWH417-6 or pET21(b), and F. nucleatum strains 12230 and US1 were each sonicated, followed by centrifugation. The clear supernatants were saved as cell extracts. The pellets were treated with 0.8% N-lauroylsarcosine, followed again by centrifugation. The supernatants were saved as the inner-membrane fractions and the pellets as the outer-membrane fractions. The recombinant mFadA and pre-FadA mixture purified with the cobalt column was included as positive controls. mFadA was detected in the cell extracts of both E. coli and F. nucleatum, but non-associated with either the inner or outer-membrane (Fig. 5A and 5B). Therefore, it was likely secreted, or released from the bacteria upon sonication. Indeed, mFadA, but not pre-FadA, was detected in the culture supernatant of E. coli BL21(DE3)/pYWH417-6, and the amount of mFadA secreted into media increased with the duration of IPTG induction (Fig. 5C). Pre-FadA was associated with the inner membrane and non-secreted (Fig. 5A and 5B). No FadA was detected in the negative controls of E. coli BL21(DE3)/pET21(b) or F. nucleatum 12230-US1. Interestingly, although pre-FadA existed in lower quantities than mFadA, as indicated by Coomassie blue stain, it reacted more strongly.
with the anti-FadA antibodies on Western blots (compare lanes 1 of Fig. 5A and 5B).

In order to delineate the association between the two forms of FadA, the mFadA-and-pre-FadA mixture purified using the cobalt column (Fig. 3A) was subjected to Sephacryl S-300 size-exclusion column chromatography, with a fractionation range of 10-1500 kDa. The standard size positions were established using blue dextran (2,000 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa), and the elution fractions were collected at 2 ml/tube. The blue dextran came out in the void volume, in fraction 23. When a total of 1.8 mg of the FadA mixture was applied to the column, FadA was eluted starting in fraction 19 through 52, as determined in chromatogram and by SDS-PAGE (Fig. 6A and 6B). These results indicated that FadA formed oligomers with continuously varying sizes, from larger than 2,000 kDa to smaller oligomers. While mFadA was detected in all fractions 19-52, pre-FadA was only present in the early fractions, whose quantity in relation to mFadA decreased with the size of the complex. Therefore, pre-FadA was likely involved in formation of high M.W. FadA oligomers. A 30 kDa component often co-purified with FadA and was eluted in fraction 39 (Fig. 6B), non-associated with the high M. W. oligomers.

To assess their respective functions, pre-FadA and mFadA were separately purified (Fig. 7A and 7B). To extract mFadA, IPTG-induced E. coli BL21(DE3)/pYWH417-6 was incubated with PBS at 60°C for 30 min. mFadA in the supernatant of the hot-PBS extract was purified using the cobalt column. In order to eliminate the trace amount of pre-FadA in the preparation, high S cation-exchange column chromatography was employed utilizing the charge difference between pre-FadA and mFadA, as the signal peptide contained two positively-charged lysine residues. mFadA came out in the washes while pre-FadA was retained on the column (see below). To purify pre-FadA, the mFadA-and-pre-FadA mixture was first purified from BL21(DE3)/pYWH417-6 cell pellet using cobalt column. The eluate was then applied to the High S column to separate these two proteins. The column was washed extensively with buffer C until all mFadA was eluted. Pre-FadA was then eluted with buffer D. The eluted pre-FadA was applied to 13% preparative SDS-PAGE and was gel-purified. Silver staining of SDS-PAGE showed that pre-FadA and mFadA were each purified to a single species (Fig. 7A and 7B). Purified mFadA was soluble in 50 mM phosphate buffer, pH 8.0. In contrast, purified pre-FadA was only soluble under acidic conditions (pH 2.6-3.0). Under neutral conditions, it was insoluble and only became soluble when mixed with mFadA at a molar ratio of mFadA : pre-FadA > 3:1 (data not shown).

Purified mFadA was labeled with Alexa fluor 488 and tested for binding to immortalized human oral epithelial OKF6/Tert cells. As a control, cytochrome c, whose M.W. (12.3 kDa) is close to that of FadA, was also labeled with Alexa fluor 488 and tested in parallel. No binding by either cytochrome c or mFadA was detected by epifluorescent microscopy following overnight incubation (Fig. 8). When non-labeled pre-FadA was mixed with Alexa fluor 488-labeled mFadA at a molar ratio of 5:1 (mFadA: pre-FadA), formation of high M.W. complexes was detected by gel filtration column chromatography (data not shown), and binding of mFadA was observed (Fig. 8A and 8B). Association of the non-labeled pre-FadA with OKF6/Tert cells was detected by Western blotting analysis (Fig. 8C). Confocal microscopy revealed that mFadA was internalized in the OKF6/Tert cells (Fig. 9). It is possible that the non-labeled pre-FadA was also internalized. The fluorescent binding was inhibited by the non-labeled pre-FadA-mFadA complex in 20-fold excess, but not by mFadA alone, strongly suggesting specific binding by the complex (Fig. 8A and 8B). Binding by pre-FadA alone was not testable due to its insolubility under neutral pH.

To assess the importance of FadA in mediating F. nucleatum binding to the host cells, competitive attachment assays were performed. While attachment of F. nucleatum 12230 to OKF6/Tert cells was not inhibited by mFadA alone, it decreased >80% in the presence of 0.5 or 1 µg/µl of re-constituted pre-FadA-mFadA (1:5 molar ratio) complex (Fig. 10). Thus, the pre-FadA-mFadA complex played a significant role in the attachment of F. nucleatum 12230 to the host cells. The threshold for inhibition was between 0.25-0.5 µg/µl of the reconstituted complex. Since the precise size of the complex was unclear, the molar concentration of the inhibitory threshold could not be determined.
DISCUSSION

To the best of our knowledge, this is the first functional expression of a fusobacterial adhein in *E. coli*. The expression vector pET21(b) was chosen so that the His•Tag fusion was conjugated to the carboxyl-terminal of FadA and would not be lost due to secretion. FadA was stably expressed in *F. nucleatum* (data not shown). Its expression in *E. coli* also appeared to be stable, making the subsequent characterization feasible. The recombinant FadA enhanced the ability of *E. coli* to bind to HGEC, KB, CHO and HUVEC cells, each by 3-4 fold. Thus, a similar mechanism may be involved in FadA binding to these different cells. The FadA receptor may exist on all these different cell types. The 3-4 fold increase was consistent with the decrease in binding of the *fadA*-deletion mutant US1, when compared to the wild-type *F. nucleatum*.

Two forms of recombinant FadA were identified: the intact pre-FadA and the secreted mFadA. Surprisingly, pre-FadA migrated faster than mFadA on SDS-PAGE. The calculated M.W. of both components were consistent with those determined by mass spectrometry. Thus, the aberrant migration was not due to post-translational modification. One possible explanation is that the hydrophobic signal peptide bound more SDS, enabling pre-FadA to migrate faster than predicted in the gel. Alternatively, the shape of pre-FadA and mFadA may differ, resulting in aberrant migration. The mobility of mFadA and pre-FadA in relation to the protein size markers also varied when different SDS-PAGE or running buffers were used (compare lanes 1 in Fig. 5). Furthermore, it was noticed that using INDIATM HisProbe-HRP, only one FadA species was detected. However, both species were detected using rabbit polyclonal anti-FadA antibodies, with pre-FadA reacting more strongly than mFadA.

Identification of pre-FadA and mFadA by amino-terminal protein sequencing and mass spectrometry also confirmed that the first 18 aa of the intact protein indeed encoded a signal peptide. The Sec, Type IV, and Type V secretion pathways have recently been found to be conserved in the genomes of *F. nucleatum* ATCC 25586 and ATCC 49256 (21). Therefore, it is likely that FadA in fusobacteria was secreted in a similar fashion as in *E. coli*. The co-existence of pre-FadA and mFadA in *E. coli* was not an artifact of over-expression because two similar forms were also identified in different strains of *F. nucleatum*, although their relative quantities and ratios varied. Furthermore, the location of these two components in *E. coli* and *F. nucleatum* were consistent, with pre-FadA associated with the inner membrane and mFadA easily dissociated from the bacteria by either sonication or hot PBS extraction. The location of these two forms makes sense because pre-FadA is not secreted while mFadA is. Secretion of mFadA into the culture medium by *E. coli* BL21(DE3)/pYWH417-6 increased with the duration of IPTG induction. The native pre-FadA and mFadA each migrated faster than their recombinant counterparts. This is probably due to the lack of the His•Tag in the native proteins. Based on the size, it is unlikely that post-translational modification occurred to the native FadA in fusobacteria.

When pre-FadA and mFadA were co-purified, they formed oligomers with continuously varying sizes. The ratio of pre-FadA to mFadA decreased with the size of the oligomer. Pre-FadA was absent in the smaller oligomers, suggesting it was only required for the formation of high M.W. oligomers. Pre-FadA and mFadA were each purified to a single species, following extensive procedures. Binding to epithelial cells was not detected with mFadA alone. However, when the same amount of fluorescent-labeled mFadA was mixed with pre-FadA at a ratio of 5:1, binding of mFadA was observed. The binding was unevenly distributed in the monolayer, consistent with what was previously observed with *F. nucleatum* binding to the epithelial cells (8). This could be due to heterogeneous expression of the FadA receptor among the cells. The fluorescent binding was inhibited by the non-labeled pre-FadA-mFadA complex but not by mFadA alone. These results suggest that the co-existence of pre-FadA and mFadA served not only a structural but also a functional purpose.

Although pre-FadA is non-exposed on the bacterial surface, it plays an important role in FadA function. The peptide was insoluble under physiological pH, and only became soluble at acidic pH's or when mixed with mFadA. This is
consistent with the observation that pre-FadA was associated with the inner membranes. The insolubility of pre-FadA was presumably due to the high hydrophobicity of the signal peptide. In the presence of mFadA, oligomerization of pre-FadA with mFadA may prevent pre-FadA from auto-aggregation. It is possible that the signal peptide is inserted in the inner membrane, serving as an anchor for the complex. Based on the results, we would like to propose the following model: due to its α-helical nature and low M.W. of the monomers, FadA may form a filamentous structure similar to some other adhesins such as pili. The mFadA monomers are added at the base of the filament as they are secreted. As the complex builds, it extends through the outer membrane. Extension of the filament ceases when the non-secreted pre-FadA is added at the base, with its signal peptide inserted in the inner membrane. The different ratios of pre-FadA and mFadA observed in different F. nucleatum strains may indicate different lengths of the FadA filaments. Extension and retraction of filamentous bacterial appendages was first observed in type IV pili of Pseudomonas aureginosa (22). The difference is that a large number of genes have been identified to be involved in the type IV pili assembly and function (23). Our model does not rule out the possibility of additional components to be involved in FadA function. One such candidate is the 30 kDa component which was often co-purified with FadA. The identity of the 30 kDa component, as well as the validity of the model, is currently under investigation. The hydrophobicity of pre-FadA may cause aggregation of the individual filaments, forming high M.W. bundles, which may be the active form of FadA. Results of the inhibitory/competitive attachment assays indicate that FadA plays a significant role in mediating F. nucleatum attachment to host cells. It is possible to develop antagonist(s) to reduce binding of F. nucleatum to host cells based on the FadA structure. It should be pointed out, however, residual binding by F. nucleatum 12230 was observed in the presence of pre-FadA-mFadA complex, suggesting that additional adhesin(s) may also exist on F. nucleatum.

The current study also revealed that although FadA was first identified as an adhesin (18), it appeared to be involved in invasion as well. Upon overnight incubation of the epithelial cells with non-labeled pre-FadA and Alexa fluor 488-labeled mFadA, fluorescence was detected inside the cells. This internalization was unlikely due to release of Alexa fluor 488 from the complex because it was inhibited by non-labeled pre-FadA-mFadA complex. Detection of fluorescence throughout the thickness of a cell suggests a significant amount of protein was internalized. Due to its insolubility, pre-FadA was not labeled. The association of pre-FadA with OKF6/Tert cells was readily detected by Western blotting analysis. Thus, it is reasonable to speculate that pre-FadA was internalized into the host cells as part of the complex. Internalization of the pre-FadA-mFadA complex, rather than mFadA alone, is an efficient “quality control” mechanism. Since the complex is anchored in the bacterial membranes, its internalization inevitably leads to the invasion of the bacteria. In contrast, mFadA can be dissociated from the bacteria, thus, internalization of mFadA may not facilitate the bacterial invasion. The mechanism of FadA-mediated invasion is being further tested. Our recent success in crystallization of mFadA will in no doubt facilitate understanding of the FadA structure and function (24).

In summary, our results indicate E. coli as a suitable host for functional expression of F. nucleatum proteins. FadA is a uniquely “self-sufficient” adhesin and invasin in that it utilizes its own two forms for anchoring, functional oligomerization, and attachment and invasion of the host cells.

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**FOOTNOTES**

§M. X., M. Y., and M. L. contributed equally to this work.
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| Strain or plasmid | Relevant characteristic | Source or reference |
|-------------------|-------------------------|---------------------|
| **Strains**       |                         |                     |
| *F. nucleatum* 12230 | Transtracheal isolate, working strain in the lab | (8,18) |
| *F. nucleatum* 12230-US1 | fadA-deletion mutant     | (18) |
| *E. coli* DH5α       | endA1 hsdR17 (rK-) glnV44 thi-1 recA1 gyrA (NaF) relA1 Δ(lacIZYA-argF) U169 deoR (Φ80dlacA (lacZ)M15), used for transformation | GIBCO BRL |
| *E. coli* BL21(DE3)  | F’ompT hsdS <sub>B</sub> (r<sub>B</sub>-m<sub>B</sub>)gal decm (DE3), carrying RNA polymerase under the P<sub>lacUV5</sub> promoter on λ DE3, used for protein expression | Novagen |
| **Plasmids**       |                         |                     |
| pET21(b)           | Protein expression vector carrying lacI, amp’, and the following: T7lac promoter-Nde I-T7•Tag-polylinker-Xho I-| Novagen |
|                    | Hi•Tag-Bpu1102 I-T7 terminator |                     |
| pYWH401            | pCR2.1 carrying a 2.4-kb fragment containing fadA from *F. nucleatum* 12230 | (18) |
| pYWH417-6          | pET21(b) with fadA inserted in the Nde I and Xho I sites, creating a fusion protein of wild-type FadA-Leu-Glu-6xHis | This study |
| pYWH417-2          | Same as above except the Glu26Lys mutation in FadA | This study |
FIGURE LEGENDS

Fig. 1. Detection of FadA-His-tag fusion proteins by Western blotting analysis using INDIA™ HisProbe-HRP. A. E. coli BL21(DE3) carrying either the cloning vector pET21(b) or the fadA-containing pYWH417-6, as indicated above the lanes, were grown to OD_{600} 0.5, followed by induction with IPTG at a final concentration of either 0.01 mM or 0.1 mM, for 1 or 2 hours, as indicated below the lanes. B. BL21(DE3)/pYWH417-6 was induced with 0.1 mM IPTG for up to 5 hours and BL21(DE3)/pYWH417-2 was induced with 0.1 mM or 1 mM IPTG for up to 3 hours. Following induction, BL21(DE3)/pYWH417-6 was collected immediately (0 hr) or incubated on ice for 1 hr before collection. BL21(DE3)/pYWH417-2 was collected immediately (0 hr), as indicated at the bottom of the gels. M, protein molecular size markers, as indicated on the left of the gels.

Fig. 2. Attachment of E. coli BL21(DE3) carrying either pET21(b) (open bars) or the fadA-containing pYWH417-6 (shaded bars) to human gingival epithelial cells (HGEC), oral mucosal cell line KB cells, Chinese hamster ovarian (CHO) cells, and human umbilical vein endothelial cells (HUVEC). Standard deviations are shown above the bars.

Fig. 3. Identification of recombinant pre-FadA and mFadA in E. coli (A, B, C) and F. nucleatum (D). A. Coomassie blue staining following 15% SDS-PAGE. Lane 1, whole cell lysate of E. coli BL21(DE3)/pYWH417-6 following IPTG induction; lane 2, flow-through of the lysate from the cobalt column; lane 3, eluate from the cobalt column. Each lane contained approximately 5 µg of total protein. M, protein size markers as indicated on the left. The arrows on the right indicate FadA. B. MALDI-TOF MS of the gel-purified FadA upper-band (mFadA, as indicated by arrow in A). C. MALDI-TOF MS of the gel-purified FadA lower-band (pre-FadA, as indicated by arrow in A). The major signals corresponding to the singly ([M+H]^+) and doubly [M+2H]^{2+} protonated molecules were indicated in the spectra.

Fig. 4. Identification of two forms of FadA in F. nucleatum strains. Coomassie blue staining (upper panel) and Western blotting using anti-FadA poly-clonal antibodies (lower panel) of different F. nucleatum strains following 12% SDS-PAGE. Lane 1, F. nucleatum 12230; lane 2, F. nucleatum ATCC10953; lane 3, F. nucleatum ATCC25586; lane 4, F. nucleatum ATCC23726; lane 5, F. nucleatum ATCC49256; lane 6, F. nucleatum ATCC51190; lane 7, F. nucleatum PK 1594; lane 8, F. nucleatum 12230-US1. Each lane contained approximately 7.5 µg of total protein. The protein sizes are indicated on the left of the gels.

Fig. 5. Localization of mFadA and pre-FadA through bacterial fractionation. A. and B. E. coli BL21(DE3) carrying pYWH417-6 or pET21(b), F. nucleatum 12230 and F. nucleatum 12230-US1 were fractionated, followed by 15% (E. coli) or 12% (F. nucleatum) SDS-PAGE analysis. The gels were either stained with Coomassie blue (A) or analyzed by Western blotting using anti-FadA poly-clonal antibodies (B). Lanes 1, pre-FadA and mFadA mixture purified under denaturing conditions as controls; lanes 2, bacterial whole-cell components; lanes 3, cells extracts; lanes 4, inner-membrane fractions; lanes 5, outer membrane fractions. Each lane contained approximately 5 µg of total protein. The bacterial strains are indicated on top of panel A. Fn, F. nucleatum. The protein size markers are indicated on the left of the gels. C. Western blotting analysis of E. coli BL21(DE3)/pYWH417-6 culture supernatant (lanes 1, 3, 5) or cell pellet (lanes 2, 4, 6) prior to IPTG induction (lanes 1 and 2), at 1 hour (lanes 3 and 4) or 2 hours (lanes 5 and 6) after IPTG induction, using mAb 5G11-3G8 as the primary antibodies. An aliquot of 12 µl of supernatant concentrated by 20 folds (1, 3, and 5) or a total of 5 µg proteins (2, 4, and 6) was loaded onto each lane. Arrows indicate mFadA and pre-FadA, respectively.

Fig. 6. A. Sephacryl S-300 size-exclusion column chromatography of the pre-FadA-and-mFadA mixture purified under denaturing conditions. A total of 1.8 mg of the pre-FadA-and-mFadA mixture was applied
to Sephacryl S-300 column. The elution profile is shown as UV absorption at 280 nm. The fractions were collected at 2 ml/tube. The elution positions of the protein standards were indicated by arrows. B. 15% SDS-PAGE of selected fractions, as indicated below the lanes, followed by silver-staining. Each lane contained 0.3 µg of total protein. M, the protein size markers as indicated on the left of the gel.

**Fig. 7.** Purification of mFadA (A) and pre-FadA (B). Approximately 1 µg protein was loaded into each lane, followed by 13% SDS-PAGE and silver staining. A. Lane 1, whole cell *E. coli* BL21(DE3)/pYWH417-6 following IPTG induction; lane 2, supernatant following hot PBS extraction; lane 3, eluate from the cobalt column; lane 4, washes from the High S cation exchange column. B. Lane 1, whole cell *E. coli* BL21(DE3)/pYWH417-6 following IPTG induction; lane 2, cell pellet following hot PBS extraction; lane 3, cells after urea-solubilization; lane 4, eluate from the cobalt column; lane 5, eluate from the High S cation exchange column; lane 6, pre-FadA purified from preparative SDS-PAGE.

**Fig. 8.** Detection of FadA binding to human oral epithelial cells. A and B. Epifluorescent microscopy analysis. The proteins were added to the OKF6/Tert cells and incubated overnight. i. control well with no additions; ii. Alexa fluor 488 (green)-labeled cytochrome c (0.05 µg/µl); iii. Alexa fluor 488-labeled mFadA alone (0.05 µg/µl); iv. FadA complex consisting of Alexa fluor 488-labeled mFadA (0.05 µg/µl) and non-labeled pre-FadA (0.01 µg/µl) (molar ratio 5:1); v. competition assay with pre-incubation of non-labeled mFadA in 20-fold excess 8 hours prior to the addition of fluorescent-labeled pre-FadA-mFadA complex; and vi. competition assay with pre-incubation of non-labeled reconstituted pre-FadA-mFadA complex in 20-fold excess prior to the addition of fluorescent-labeled pre-FadA-mFadA complex. In all experiments, pre-FadA was not labeled. The cell nuclei were stained with DAPI (blue) and actin stained with Alexa fluor 568 phalloidin (red). Panel A shows only Alexa fluor 488. In panel B, the cell nuclei (blue) and actin (red) are shown in overlay. C. Western blotting analysis of OKF6/Tert cells following lysis with 1% SDS, using mAb 5G11-3G8 as the primary antibodies. Lane 1, OKF6/Tert cells alone; lane 2, OKF6/Tert cells incubated with 1 µg/µl mFadA; lane 3, OKF6/Tert incubated with 1 µg/µl reconstituted pre-FadA-mFadA complex. An aliquot of 10 µg total proteins were loaded onto each lane. mFadA and pre-FadA were indicated by arrows on the left of the gel.

**Fig. 9.** Confocal microscopy analysis of internalization of Alexa fluor 488-labeled pre-FadA-mFadA complex into the OKF6/Tert cells. The experiment was performed as described in Fig 8. The epithelial cell actins were stained with Alexa flour 568 phalloidin (red), and nuclei stained with Draq5 (blue), respectively. The sections shown are the z series, starting from the top through the bottom of the monolayer at 0.98 µm intervals.

**Fig. 10.** Attachment of *F. nucleatum* 12230 to OKF6/Tert cells (solid black column), in the presence of 0.05, 0.25, 0.5, 1 µg/µl of mFadA (grey columns) or reconstituted pre-FadA-mFadA (1:5 molar ratio) complex (checker-board columns). The fadA deletion mutant of *F. nucleatum* 12230, US1, was included as a control (stippled column). The standard deviations are shown above the columns.
Figure 1

A

| IPTG (mM) | 0.1 | 0.01 | 0.1 | 0.01 | 0.1 | 0.01 | 0.1 | 0.01 |
|-----------|-----|------|-----|------|-----|------|-----|------|
| Induction time (hr) | 1 | 1 | 2 | 2 | 1 | 1 | 2 | 2 |

M

22KD

16KD

B

| IPTG (mM) | 0.1 |
|-----------|-----|
| Induction time (hr) | 1 | 1 | 2 | 2 | 3 | 3 | 4 | 4 | 5 |
| Post-induction incubation time (hr) | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |

M

17KD

pYWH417-6

pYWH417-2

0.1

0.1

1

0
Figure 2
Figure 6

A

![Graph showing OD 280 vs. Fraction Number with markers for 2,000 KD, 66 KD, 29 KD, and 12.4 KD](image)

B

![Image showing protein bands with markers for 36 KD, 22 KD, and 16 KD](image)
Figure 8

A

B

v

vi

C

1 2 3

mFadA

pre-FadA
Figure 9
Figure 10

The figure shows a bar graph illustrating the percentage of bacteria attached to cells under different conditions. The x-axis represents the concentration of protein (mFadA alone and pre-FadA-mFadA (1:5) at 0.05, 0.25, 0.5, and 1 μg/μl), while the y-axis shows the percentage of bacteria attached to cells. The graph compares the attachment of bacteria (Fn and US1) under these conditions.
FadA from fusobacterium nucleatum utilizes both secreted and non-secreted forms for functional oligomerization for attachment and invasion of host cells
Minghua Xu, Mitsunori Yamada, Mei Li, Hongqi Liu, Shu G. Chen and Yiping W. Han

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