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Fap2 of *Fusobacterium nucleatum* Is a Galactose-Inhibitable Adhesin Involved in Coaggregation, Cell Adhesion, and Preterm Birth

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Fusobacterium nucleatum **is a common oral anaerobe involved in periodontitis that is known to translocate and cause intrauterine infections. In the oral environment, *F. nucleatum* adheres to a large diversity of species, facilitating their colonization and creating biological bridges that stabilize the multispecies dental biofilm. Many of these interactions (called coadherences or coaggregations) are galactose sensitive. Galactose-sensitive interactions are also involved in the binding of *F. nucleatum* to host cells. Hemagglutination of some *F. nucleatum* strains is also galactose sensitive, suggesting that a single galactose-sensitive adhesin might mediate the interaction of fusobacteria with many partners and targets. In order to identify the fusobacterial galactose-sensitive adhesin, a system for transposon mutagenesis in fusobacteria was created. The mutant library was screened for hemagglutination deficiency, and three clones were isolated. All three clones were found to harbor the transposon in the gene coding for the Fap2 outer membrane autotransporter. The three *fap2* mutants failed to show galactose-inhibitable coaggregation with *Porphyromonas gingivalis* and were defective in cell binding. A *fap2* mutant also showed a 2-log reduction in murine placental colonization compared to that of the wild type. Our results suggest that Fap2 is a galactose-sensitive hemagglutinin and adhesin that is likely to play a role in the virulence of fusobacteria.**

Fusobacterium nucleatum is a non-spore-forming anaerobe (1) and is the Gram-negative species isolated most frequently from both healthy and diseased sites in the oral cavity (2, 3). Though highly associated with periodontitis, this bacterium is considered not to be a major periodontal pathogen but rather an agent hypothesized to impact the events leading to this disease (4). *F. nucleatum* is also a common isolate from extraoral infections (2, 5, 6), and recent evidence suggests that it is involved in colorectal carcinoma (7–10). Overabundance of *F. nucleatum* was observed in colorectal carcinomas and adenomas, activating Wnt signaling and oncogenes and generating a proinflammatory microenvironment conducive for colorectal neoplasia progression.

One of *F. nucleatum*'s important virulence characteristics is its ability to adhere to early and late dental plaque colonizers and to bind a variety of mammalian cells.

Coadherence (specific binding to a surface-attached bacterium) is a major attachment mechanism of dental colonizers (11–13). Coadherence not only prevents the washout of oral colonizers by the saliva and gingival crevicular fluid but also creates spatial proximity that facilitates microbial communication and metabolic synergism (14–18). *In vitro*, *F. nucleatum* can mediate coaggregation (coadherence among planktonic bacteria) between many species of dental colonizers (19, 20) and therefore has been proposed to function as a bridging organism that stabilizes the developing dental plaque (21–23). Apart from its ability to bind multiple bacterial species, *F. nucleatum* is capable of adhering to (24) and invading (25–29) various mammalian cell types, inducing the secretion of proinflammatory cytokines that contribute to the initiation and progression of periodontal diseases (29–31). *F. nucleatum* was also shown to shuttle other noninvasive bacteria into epithelial cells (32).

F. nucleatum exhibits different types of adhesions and harbors several adhesins, including an arginine-inhibitable adhesin (20, 33), a mannose-sensitive lectin involved in fusobacterial coaggregation with *Candida* species (34, 35), and Fad, which is required for cell attachment and invasion (36–38) and was recently shown to bind E-cadherin on colorectal cancer cells and promote carcinogenesis (39). FadA was also shown to be involved in *F. nucleatum* 12230 colonization of the mouse placenta (36, 39, 40).

The ability to adhere to and invade host cells is believed to play a key role in *F. nucleatum*'s oral and systemic virulence, as well as in its ability to colonize the placenta (28, 36, 38, 40). Periodontal disease is a risk factor for preterm labor (41–45), and *F. nucleatum* has been associated with preterm birth (46–50), stillbirth (51), and early-onset neonatal sepsis (52).

F. nucleatum's attachment to mammalian cells can be reversed by adding D-galactose, which also inhibits *F. nucleatum*'s ability to coaggregate with the major periodontopathogen *Porphyromonas gingivalis* and with at least nine other oral bacterial species (19, 53, 54). Spontaneous *F. nucleatum* mutants defective in galactose-sensitive coaggregation with *P. gingivalis* were also defective in attachment to a variety of mammalian cells (24, 29), suggesting that a single galactose-inhibitable adhesin plays a key role in fusobacterial virulence associated with periodontal plaque and host

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cells. Though this adhesin appears to be involved in many interactions with bacterial and host cells, it has not yet been characterized.

Genetic manipulation in fusobacteria has been hampered possibly by anaerobic growth requirements, multiple fusobacterial restriction modification systems (including several restriction enzymes with four base recognition sites) (55), and the high AT content in its genome (56). Cloning of AT-rich genomic DNA is notoriously difficult (57). While the reasons are not clear, one suggestion is that the cloned sequences act as transcriptional promoters in *Escherichia coli*.

Though two shuttle vectors have been developed for fusobacteria (58–60) and several strains have been sequenced and annotated (56, 61, 62), research on this bacterium has been hindered by the lack of genetic systems to induce random mutations.

In this study, a random insertion-inactivation mutagenesis system was created for fusobacteria. Using this system, the Fap2 autotransporter was identified as the *F. nucleatum* galactose-sensitive adhesin.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *F. nucleatum* ATCC 23726 and *P. gingivalis* PK 1924 were grown in Wilkins-Chalgren broth (Oxoid, United Kingdom) or on Columbia agar plates (Oxoid, United Kingdom) supplemented with 5% defibrinated sheep blood (Novamed, Israel). Both strains were grown in an anaerobic chamber (Bactron I-II; Shel Lab, USA) in an atmosphere of 90% N_2, 5% CO_2, and 5% H_2 at 37°C.

Escherichia coli XL-1 was grown in LB broth (Difco, USA) or on LB agar plates (Difco, USA) under aerobic conditions at 37°C.

Streptococcus sanguinis NC 02863 was grown in brain heart infusion (Difco, USA) at 37°C in an atmosphere of 5% CO_2.

All antimicrobials used in this study were purchased from Sigma-Aldrich Israel. Antibiotic concentrations (final) were as follows: ampicillin, 100 μg ml^{-1}; chloramphenicol, 30 μg ml^{-1}; and thiamphenicol, 5 μg ml^{-1}. For fusobacteria, broth was supplemented with half these concentrations.

Electroporation of *F. nucleatum*. Fusobacteria were electroporated as described previously (60). Briefly, fusobacterial cells were grown to log phase, washed three times in cold electroporation buffer (10% glycerol, 1 mM $MgCl_2$), and concentrated to an optical density at 600 nm (OD_{600}) of 6. One hundred microliters of competent cells was electroporated with 2 μl of DNA (containing 0.1 to 0.5 μg of DNA in double-distilled water) in a 0.1-cm-electrode-gap Gene Pulser cuvette (Bio-Rad), using the Micro-Pulser electroporator (Bio-Rad, USA) at settings of 2.5 kV, 200 Ω, and 25 μF. The cells were then quickly resuspended with 0.9 ml of prereduced Wilkins-Chalgren broth supplemented with 1 mM of $MgCl_2$ and incubated for 5 h at 37°C in an anaerobic chamber. Bacteria were then spread on appropriate plates and incubated for 5 days.

Creation of EZ::TnCat. A *catP* gene of clostridial origin, conferring resistance to chloramphenicol on *E. coli* and thiamphenicol on fusobacteria, was amplified by PCR (Expand high-fidelity PCR system; Roche, USA) from the pHS30 plasmid (60) using the F-CatP (GGGGAATTCTA AAACCTTGGTTGTGTTGC) and R-CatP (GGGGAATTCAACGAGTG AAAAAGTGTCCC) primers. PCR conditions were as follows: denaturation at 94°C for 2 min, followed by 10 cycles of denaturing at 94°C for 15 s, annealing at 65°C for 45 s, and elongation at 72°C for 3 min, followed by an additional 20 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 30 s, elongation at 72°C for 3 min, and a final 7-min extension at 72°C. The resulting fragment (~0.6 kbp) was cloned into EcoRI-restricted pMOD-3<R6Kγori/MCS> (Epicentre) to generate pMODCat carrying the EZ::TnCat transposon, which inserts into any target DNA (63, 64). Transposons were amplified by PCR according to the manufacturer's instructions using the F-pMOD PCR (ATTCAGGCTGCGCAACTGT) and R-pMOD PCR (GTCAGTGAGCGAGGAAGCGGAAG) primers. Transposase (Epicentre) was added to the transposons, and they were incubated in the absence of magnesium to create stable transposomes in accordance with the manufacturer's instructions.

The transposomes (2 μl) were electroporated into competent fusobacterial cells (ATCC 23726 or ATCC 10953), and clones with transposon inserts were selected on Columbia blood agar plates supplemented with 5 μg ml^{-1} of thiamphenicol.

Clones were collected and the partial library (1,200 clones, each in Wilkins-Chalgren broth supplemented with 10% [final concentration] glycerol) was stored in 96-well plates at −80°C until used.

Determination of the transposon insertion site in the fusobacterial genome. Genomic DNA was purified (GenElute; Sigma-Aldrich, Germany) from selected clones, restricted with the endonucleases ScaI, PvuII, and AhdI (New England BioLabs), which do not cleave within the transposon, and self-ligated using T4 ligase (TaKaRa, Japan), and the transposon-flanking sequences were amplified by inverse PCR (Herculase II; Agilent) using the FpMODSq (GCCAACGACTACGCACTAGCCAAC) and RpMODSq (GAGCCAATATGCGAGAACACCCGAGAA) primer pair. Whenever necessary, nested primers FNes3 (CAAGAGCTTCAGGG TTGAG) and RNes3 (ACCCGAGAAAATTCATCGATG) were used.

PCR conditions were as follows: denaturation at 95°C for 2 min, followed by 30 cycles of denaturing at 95°C for 20 s, annealing at 50°C for 30 s, and elongation at 72°C for 1.5 min and then a final 3-min extension at 72°C.

PCR products were sequenced (The Center for Genomic Technologies), and the insertion site was determined using BLAST software.

Hemagglutination assays. Fusobacterial clones were grown overnight, washed twice in phosphate-buffered saline (PBS), and brought to an OD_{600} of 1 (~10^9 CFU ml^{-1}). Sheep erythrocytes (RBCs) were washed twice in PBS and brought to a concentration of 2% (vol/vol). Fifty microliters of fusobacterial cells was mixed with 50 μl of sheep erythrocytes (2% in PBS) in round-bottom 96-well plates (Nunc, Denmark) and incubated at room temperature for 2 h. Hemagglutination was determined visually and clones were selected based on their inability to hemagglutinate RBCs.

For inhibition assays, washed bacteria were preincubated with 6 mM D-galactose (Sigma-Aldrich, Germany) or 50 mM L-arginine (Sigma-Aldrich, Germany) (final concentrations) for 30 min prior to incubation with RBCs.

Membrane protein extraction. Membrane proteins were prepared as described previously (65), with minor changes. Bacterial cells (0.5 liter) were grown overnight and washed in sodium phosphate buffer (pH 7.2) supplemented with 3 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, Germany).

A French press (8,000 lb/in^2) was used three times to disrupt the cells, and unbroken cells were removed by sedimentation at 10,000 × *g* at 4°C for 10 min. The supernatant was collected and subjected to high-speed centrifugation (150,000 × *g* at 4°C for 1 h), and the resulting pellet containing the cell walls was washed twice, resuspended in sodium phosphate buffer, and kept at −80°C until used. A sample of each pelleted membrane was boiled in SDS-PAGE sample buffer for 10 min and subjected to SDS-PAGE (4%). Coomassie blue was used to visualize protein bands.

Coaggregation assays. (i) Visual coaggregation. Bacteria were grown overnight, washed twice in coaggregation buffer (0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.15 M NaCl, and 0.02% NaN_3 dissolved in 1 mM Tris and adjusted to pH 8.0) (66), and brought to an OD_{600} of 1. Both partners (200 μl of each) were mixed in a glass test tube and incubated at room temperature for 30 min. Visual coaggregation of each mutant was evaluated and compared to wild-type (WT) coaggregation. When testing the inhibitory effect of galactose, fusobacteria were preincubated with D-galactose 60 mM (final concentration) for 15 min.

(ii) Quantitative coaggregation. As described previously (20), briefly, *F. nucleatum* and *P. gingivalis* or *S. sanguinis* were brought to an OD_{600} of 1 in coaggregation buffer and mixed (100 μl each) in round-bottom 96-well plates with or without 60 mM D-galactose (final concentration). The plates were incubated at room temperature for 30 min, and coaggregating

particles were then sedimented by centrifugation at low speed (100 × *g* for 1 min). The supernatant containing noncoaggregating cells was transferred to a flat-bottom 96-well plate, and optical density at 595 nm was measured (Genios; Tecan Systems Austria). For each mutant, percent galactose-dependent coaggregation was calculated by dividing the difference between the mutant's supernatant optical density in the presence of 60 mM D-galactose and in the absence of galactose by the difference between the optical density of the wild-type strain in the presence of 60 mM D-galactose and in the absence of galactose, as follows: (mutant without galactose − mutant with galactose)/(wild type without galactose − wild type with galactose) × 100.

Galactose-independent coaggregation was calculated by dividing the mutant's supernatant optical density by that of the wild type.

The differences between the groups were compared using the Student *t* test. A *P* value of <0.05 was considered significant.

Cell attachment assay. Bacteria were grown overnight, washed in sterile PBS, and brought to an OD_{600} of 1 in coaggregation buffer (66). The bacteria were then stained with carboxyfluorescein succinimidyl ester (CFSE) CellTrace (Molecular Probes, OR) according to the manufacturer's instructions and brought to a concentration of 3×10^6 cells ml^{-1} in coaggregation buffer. Human embryonic kidney (HEK) 293T cells (a kind gift from Avi-Hai Hovav) were grown in Dulbecco's modified Eagle medium (DMEM) (Biological Industries, Israel) supplemented with 10% fetal calf serum (FCS; Biological Industries), 2 mM L-glutamine (Biological Industries), 100 U ml^{-1} of penicillin, and 100 μg ml^{-1} of streptomycin (Biological Industries) (all final concentrations) until they reached confluence. The cells were scraped, washed once in DMEM, and brought to a concentration of 3×10^5 ml^{-1} in DMEM. The cells were then incubated with the stained bacteria at a multiplicity of infection (MOI) of 10 with gentle shaking for 30 min, followed by three washes in coaggregation buffer and resuspension in PBS supplemented with 2% FCS (final concentration).

For galactose inhibition assays, bacteria were incubated with 60 mM D-galactose (final concentration) for 30 min prior to incubation with the cells, and coaggregation buffer supplemented with 60 mM D-galactose was used to wash the cell-bacterium complex. Cell attachment was determined by flow cytometry (Accuri C6 flow cytometer; BD, USA), and data were analyzed using FlowJo 7.6.5 software (Tree Star, Ashland, OR).

***In vivo* placental colonization.** Seven- to eight-week-old outbred CF1 mice were caged together at a female-to-male ratio of 2:1, and mating was determined by the presence of a white vaginal plug. The day when the plug was detected was termed the first day of gestation. The pregnant mice were randomly distributed into study groups of six mice per group. The pregnant mice were inoculated with wild-type ATCC 23726 or with the *fap2* mutant K50 on day 15 to 17 of gestation. For the inoculation, an aliquot of 100 μl of the bacterial suspension ($4.0 \times 10^7 \sim 5.2 \times 10^7$ CFU) or sterile PBS (sham) was injected into the tail vein. After 24 h, the placentas were harvested from each pregnant mouse and homogenized under sterile conditions. Serial log dilutions were performed and plated. The plates were incubated anaerobically at 37°C for 96 h, followed by enumeration. The difference between different groups was analyzed using one-way analysis of variance (ANOVA) and the Kruskal-Wallis test. The difference between two groups was compared using the Student *t* test. A *P* value of <0.05 was considered significant. Data shown are representative of two repeated experiments.

Mass spectrometry. Coomassie-stained protein bands were excised from the denaturing gel, and mass spectrometry was carried out with Orbitrap (Thermo Finnigen). Data analysis was done using the BioWorks 3.3 package, and database searches were performed against the NCBInr database using the Mascot package (Matrix Science, England).

Statistical analyses. Unless otherwise mentioned, all data are means and standard deviations from three independent experiments performed in triplicate. The Student *t* test was used for statistical analyses; a *P* value of <0.05 was considered significant.

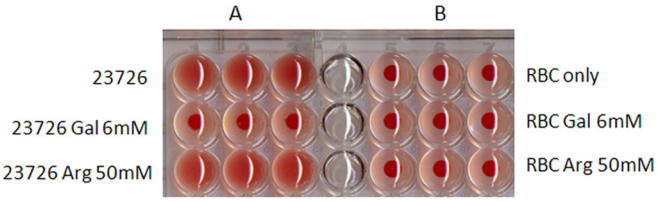

FIG 1 *F. nucleatum* ATCC 23726 hemagglutination is inhibited by D-galactose but not by L-arginine. (A) Bacteria incubated with 2% sheep red blood cells (RBC) in the absence and presence of 6 mM D-galactose (Gal) or 50 mM L-arginine (Arg). (B) Erythrocytes incubated without bacteria in the absence and presence of inhibitors. Precipitation of the red blood cells (seen as a red dot) represents a lack of hemagglutination.

RESULTS

Creation of a randomly inserted transposon mutation library in *F. nucleatum* ATCC 23726. For creation of a system for random insertional mutagenesis in fusobacteria, the *Clostridium perfringens* chloramphenicol acetyltransferase gene (*catP*) conferring resistance to thiamphenicol (a chloramphenicol analog) on fusobacteria (59) was cloned into pMOD-3<R6Kγori/MCS> (Epicentre) to generate pMODCat carrying the EZ::TnCat transposon. Transposase was added to the EZ::TnCat transposon to form transposome complexes, which were electroporated into competent fusobacterial cells. Thiamphenicol-resistant colonies were obtained at an average efficiency of 8×10^3 CFU/μg of DNA using *F. nucleatum* ATCC 23726 and at an order of magnitude lower using ATCC 10953.

Four independent clones were selected, and the transposon's location was determined by amplifying the flanking region of the transposon by PCR and BLASTing it against the genome of *F. nucleatum* ATCC 25586, the strain most similar to ATCC 23726.

Sequence analysis indicated that the transposons were inserted at unique positions in each of the four fusobacterial genomes (data not shown).

Identification of the *F. nucleatum* galactose-sensitive hemagglutinin. Hemagglutination is an attachment mechanism that is considered a virulence trait of many pathogens. Hemagglutination in some *F. nucleatum* strains, including *Fusobacterium nucleatum* subsp. *polymorphum* ATCC 10953, was found to be inhibited by arginine (67), while in others (*Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586), it is inhibited by galactose (68–70). As can be seen in Fig. 1, hemagglutination of *F. nucleatum* subsp. *nucleatum* ATCC 23726 is also galactose sensitive. In order to identify the fusobacterial galactose-sensitive adhesin, 1,200 clones with transposon inserts were screened for hemagglutination deficiency.

Three independent clones were found to be deficient in their ability to hemagglutinate sheep RBCs (Fig. 2). Analysis of the transposon location in those mutants determined that all three clones harbored the transposon in the same 11.3-kb gene, corresponding to FN1449, an autotransporter protein in *F. nucleatum* ATCC 25586 previously termed Fap2 for fusobacterial apoptosis protein (65) in ATCC 23726 (Fig. 3A).

In order to verify that the mutation affected the Fap2 protein, membrane proteins were extracted from wild-type *F. nucleatum*, from the three hemagglutination-deficient mutants (D22, K25, and K50), and from randomly selected mutants (J78 and K174) used as controls (20, 65) The same high-molecular-mass bands

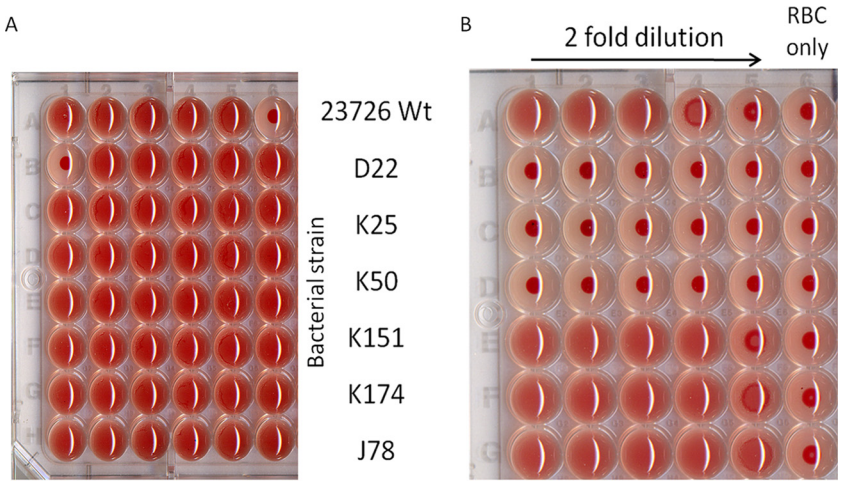

FIG 2 Isolation of *F. nucleatum* mutants deficient in hemagglutination. (A) Microtiter plate screening for library clones defective in hemagglutination. (B) Hemagglutination assay with 2-fold dilutions of the hemagglutination-deficient mutants (D22, K25, and K50) compared to wild-type *F. nucleatum* ATCC 23726 and the randomly selected transposon insertion controls: K151, K174, and J78.

were missing in all three mutants. The corresponding bands from wild-type *F. nucleatum* were sent for mass spectrometry analysis, which identified peptides belonging to the FN1449 protein of *F. nucleatum* ATCC 25586 (Fig. 3B).

The Fap2 adhesin involved in hemagglutination is also involved in coaggregation. Similar to hemagglutination, coaggregation of *F. nucleatum* ATCC 23726 with *P. gingivalis* is galactose sensitive (19, 66). All three hemagglutination-deficient mutants (but not the randomly selected J78 mutant) failed to show galactose-sensitive coaggregation with *P. gingivalis* (Fig. 4A to C). However, the mutants retained their ability to coaggregate with *Streptococcus sanguinis*; this coaggregation is mediated by the fusobacterial arginine-inhibitable RadD adhesin (20) (Fig. 4D).

Fap2-deficient mutants are impaired in cell binding. As galactose was previously demonstrated to inhibit the attachment of some *F. nucleatum* strains to mammalian cells (66, 70), we next tested whether the hemagglutination- and coaggregation-deficient clones are also deficient in attachment to nonerythrocyte mammalian cells. Wild-type *F. nucleatum* ATCC 23726, the three hemagglutination-deficient mutants, and a randomly selected control mutant, J78, were stained with carboxyfluorescein succinimidyl ester (CFSE) and incubated with HEK 293T cells. Cell attachment was determined by flow cytometry. Adherence to cells in all three *fap2* mutants was 3- to 10-fold lower than that of the wild type or the control mutant. The Fap2 mutants retained some cell binding activity (similar to the galactose-treated cells), presum-

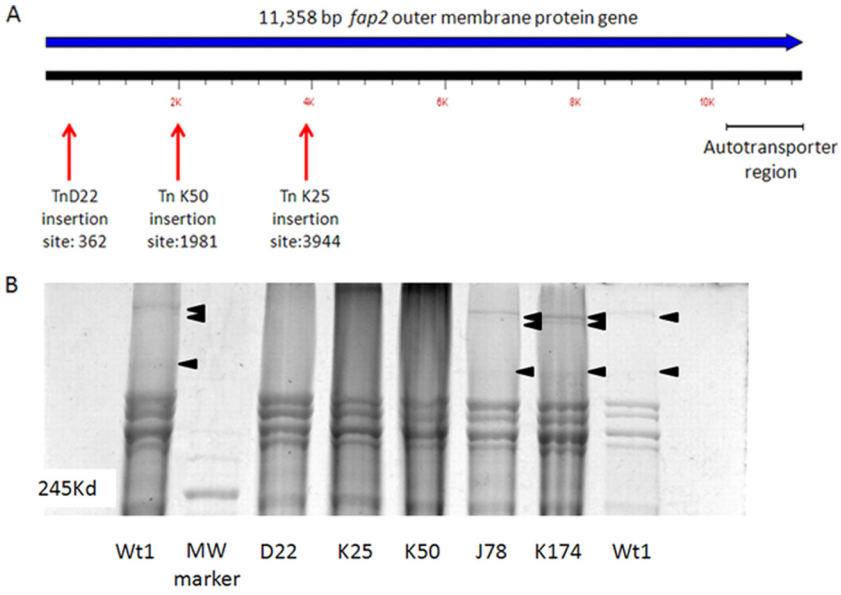

FIG 3 All three hemagglutination-deficient mutants are defective in Fap2. (A) Locations of transposon insertions in the *fap2* genes of three hemagglutination-deficient mutants. (B) SDS-PAGE demonstrating the absence of the Fap2 protein bands in membrane proteins extracted from all three hemagglutination-deficient mutants compared to the wild type and the randomly selected mutants J78 and K174. The clear band seen in the MW marker lane indicates 245 kDa.

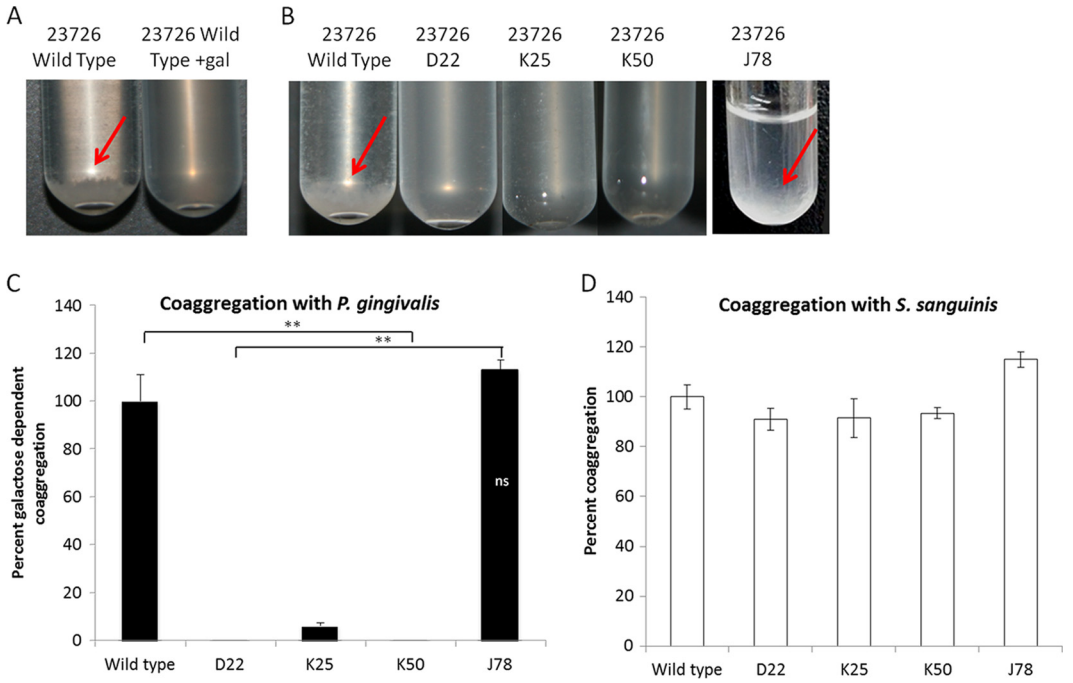

FIG 4 All three hemagglutination-deficient mutants fail to coaggregate with *P. gingivalis*. *F. nucleatum* and *P. gingivalis* were brought to an OD_{600} of 1 in coaggregation buffer mixed and incubated in a glass tube at room temperature for 30 min. Coaggregation (pellet), which is indicated with a red arrow, is absent in the presence of 60 mM D-galactose (gal) (A) and with the hemagglutinin mutants D22, K25, and K50 compared to the wild type and the randomly selected mutant J78 (B). (C and D) Coaggregation was quantified as described in Materials and Methods. All three hemagglutination-deficient mutants (but not the randomly selected mutant J78) failed to coaggregate with *P. gingivalis* but not with *S. sanguinis*. **, $P < 0.01$ compared to the wild-type control. ns, not significant.

ably due to the remaining FadA adhesin (Fig. 5). The presence of the FadA adhesin was verified in each of the tested mutants and in the wild-type strain (data not shown).

Fap2 is involved in placental colonization. Fusobacteria have been implicated in preterm births, and FadA, a fusobacterial adhesin unique to oral bacteria, was shown to be involved (36). In order to determine the role of the Fap2 galactose-sensitive adhesin in adverse pregnancy outcomes, wild-type *F. nucleatum* and the K50 hemagglutination-deficient mutant were injected into the tail veins of 7- to 8-week-old outbred CF1 female mice at day 15 to 17 of gestation. After 24 h, the placentas were harvested from the pregnant mice and homogenized. Serial dilutions were plated, and fusobacterial colonies were enumerated after 96 h of incubation. As can be seen in Fig. 6, the mutation in *fap2* reduced placental colonization by 2 orders of magnitude.

DISCUSSION

Fusobacterium nucleatum is a significant pathogen in human infections, involved in periodontitis, in a variety of systemic diseases (71–74), in colorectal carcinoma (7, 8, 10, 39, 75, 76), and in

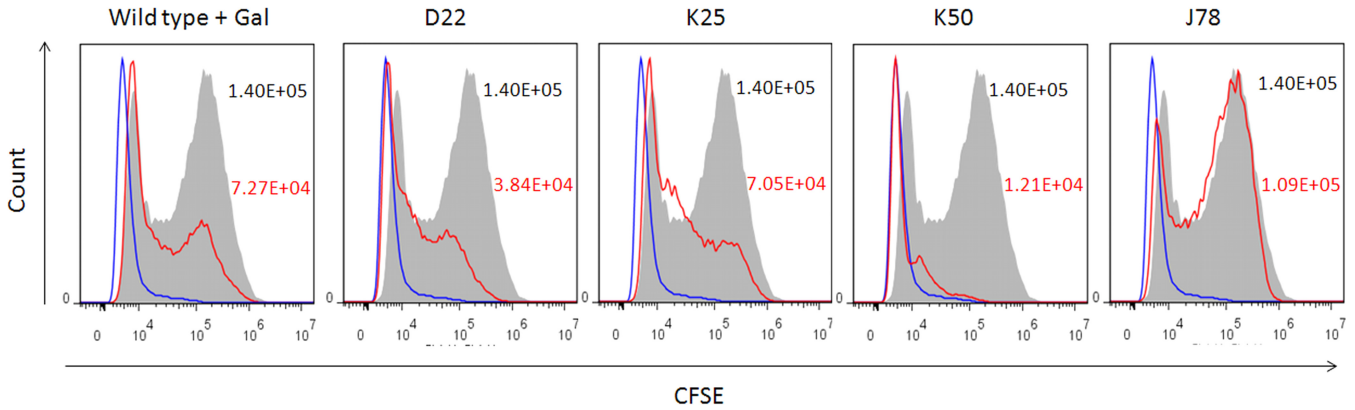

FIG 5 All three hemagglutination-deficient mutants are impaired in binding to mammalian cells. Shown are the results of fluorescence-activated cell sorter (FACS) analysis of HEK 293T cells (blue outline) incubated with carboxyfluorescein succinimidyl ester (CFSE)-labeled wild-type ATCC 23726 in the presence of D-galactose (gal) or with labeled hemagglutinin-deficient mutants (red outline) D22, K25, and K50 and J78, a randomly selected mutant used as control. Wild-type ATCC 23726 control is represented by the gray-filled histogram. Mean fluorescence intensity values are indicated for each histogram. Values for the wild type are in black, and those for the galactose-treated bacteria and the mutants are in red. Data are representative of three independent experiments.

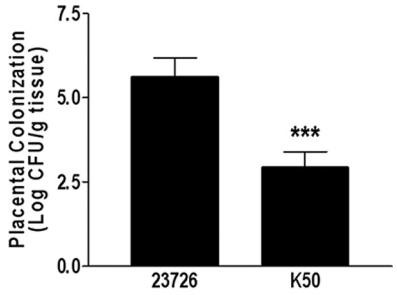

FIG 6 The Fap2 adhesin is involved in placental colonization. Wild-type *F. nucleatum* ATCC 23726 or the hemagglutination-deficient mutant K50 was injected into the tail veins of pregnant mice. After 24 h, the placentas were harvested and homogenized, and bacterial CFU were determined. ***, $P < 0.001$.

preterm births (47, 77). Its role in oral disease is of particular importance, because the oral cavity is the suspected port of entry and reservoir of this organism in systemic diseases (51). In spite of its implication in numerous diseases, little is known about how *F. nucleatum* might act to cause disease.

F. nucleatum is not known to harbor significant virulence factors. However, it is capable of inducing the secretion of proinflammatory cytokines, downregulating host immunity by inhibiting T cells (78), and inducing apoptosis in lymphocytes (79). It also expresses an IgG Fc binding protein (80) and a weak serine protease (81). It is suggested that the adherence capability is this bacterium's primary virulence trait, conferring on it the means to survive in the host.

Genetic manipulation in fusobacteria is difficult, due to their anaerobic growth conditions, the low GC content in their genome (56), and their multiple restriction systems (55). The two shuttle vectors developed for fusobacteria (58–60) and the sequencing and annotation of three strains representing subspecies *F. nucleatum* subsp. *nucleatum*, *Fusobacterium nucleatum* subsp. *vincentii*, and *F. nucleatum* subsp. *polymorphum* (56, 61, 62) have opened new avenues of research. However, though site-directed mutagenesis (40, 60, 65) and complementation assays (36) have been performed with this bacterium, research has been hindered by the lack of genetic systems to induce random insertion mutagenesis. Here we present a system for random insertion mutagenesis in *F. nucleatum*.

Transposomes electroporated into *F. nucleatum* ATCC 23726 or *F. nucleatum* ATCC 10953 (data not shown) enabled random integration of EZ::TnCat into the fusobacterial genome and the creation of the *F. nucleatum* ATCC 23726 library. Phenotypic screening of the mutant library identified three mutants that did not hemagglutinate sheep RBCs (Fig. 2).

The three isolated hemagglutination mutants were found to be defective in the same gene that codes for a fusobacterial outer membrane autotransporter (FN1449 in *F. nucleatum* ATCC 25586), previously termed *fap2* (Fig. 3). The multiple high-molecular-mass bands visualized (in agreement with the predicted Fap2 molecular mass of 389.8 kDa) are hypothesized to be due to some sort of modification such as glycosylation or truncation.

F. nucleatum ATCC 23726 is predicted to have approximately 2,100 encoded proteins, of which Fap2 is the largest one. Though only 1,200 clones were screened, three *fap2* mutants were identified. This might be explained by the large size of *fap2* (11.3 kb). Comparative analysis of the sequenced strain, *F. nucleatum* ATCC 25586, and the preliminary genome annotation of *F. nucleatum* ATCC 49256 suggested that the major protein secretion systems, such as type II, type III, and type IV, were missing (61). However, several autotransporters belonging to the type V secretion system family have been identified (13). This large and diverse superfamily of polypeptides which is produced by pathogenic Gram-negative bacteria is often associated with virulence traits such as aggregation, adherence, toxicity, biofilm formation, and invasion (82, 83).

Interestingly, one of the characteristics of autotransporter proteins is a low abundance of cysteine residues in the passenger domain, presumably to prevent disulfide bond formation of the protein while it is in the periplasm and facilitate its translocation (84). The Fap2 hemagglutinin contains over 3,000 amino acids and has no cysteine residues.

Structural analysis revealed that the predicted structure of Fap2 contains domains homologous to the Hmw1 secretion domain in *Haemophilus influenzae* (30% identity) (85), which contains a carbohydrate-dependent hemagglutination domain found in various hemagglutinins and hemolysins such as the *Bordetella pertussis* hemagglutinin (86).

Hemagglutination is a characteristic feature of oral fusobacteria (67). It has been previously shown that fusobacterial hemagglutination is mediated by a protein moiety on the bacterial cell surface (87) and that in some strains, the reaction is galactose sensitive (68). A fusobacterial hemagglutinin was previously characterized as a high-molecular-mass protein, which corresponds with our findings. A high-molecular-mass arginine-sensitive hemagglutinin was purified from *F. nucleatum* subsp. *polymorphum* ATCC 10953 and has been shown to be involved in coaggregation of streptococci (88). Similarly, Fap2 is another high-molecular-mass protein which appears to be involved in *F. nucleatum* ATCC 23726 galactose-sensitive hemagglutination and coaggregation.

Coaggregation is defined as a specific binding of two genetically distinct microorganisms (89). In the oral cavity, this interbacterial attachment mechanism serves to anchor the bacteria to a surface and withstand the salivary flow that will otherwise wash them away into the digestive tract. Coaggregation also enables bacterial communication between the coaggregating partners and facilitates a mutual metabolic relationship (14, 15, 90).

It has been shown that coaggregation is a key factor in periodontitis and that dual infection with *F. nucleatum* and *P. gingivalis* aggravates periodontal disease, which is manifested in greater alveolar bone loss (91).

So far, an arginine-inhibitable adhesin, RadD, has been identified in *F. nucleatum* (20), mediating coaggregation with streptococci and implicated in apoptosis of human lymphocytes (65).

Coaggregation with *P. gingivalis* and cell adhesion were shown by others (92) and by us (66) to be reversed in some *F. nucleatum* subspecies by adding galactose, leading to the hypothesis that the galactose-sensitive coaggregation and cell adhesion may be mediated by the same adhesin.

Hemagglutination in *F. nucleatum* ATCC 23726 is also galactose inhibitable (Fig. 1), and the three hemagglutination-deficient mutants were found to be deficient in galactose-sensitive coaggregation with *P. gingivalis* but not in arginine-inhibitable coaggregation with *S. sanguinis* (Fig. 4). They are also defective in adherence to HEK 293T cells (Fig. 5), supporting the aforementioned hypothesis that a single adhesin mediates fusobacterial attachment to host cells and galactose-sensitive coaggregation (19).

Many known pathogens express a variety of structures on their surface, which function as bacterial adhesins (93). These adhesins, such as type I fimbriae (94), YadA expressed by *Yersinia enterocolitica* (95), and the filamentous hemagglutinin expressed by *Bordetella pertussis* (96–98), have lectin-like properties and appear to act as multifactorial adhesins capable of mediating bacterium-host cell interactions, as well as promoting bacterial autoaggregation. Many microbial lectins were originally detected based on their hemagglutinating ability (99).

Fap2 of *F. nucleatum* ATCC 23726 was previously shown to be involved in induction of cell death (65). However, in light of our results, it is plausible that its involvement in apoptosis is due to its ability to enable fusobacterial adherence to host cells. In theory, an open reading frame (FN1448) downstream of the *fap2* gene could potentially be involved in the hemagglutination deficiency phenotype. However, Kaplan et al. (65) showed that an insertion mutation in *fap2* did not cause a polar effect.

Fusobacterium is prevalent in intrauterine infections, and its role in preterm birth has been documented previously (47, 50, 51, 100). Inactivation and complementation of FadA, a 12-kDa outer membrane protein, demonstrated its essential role in host cell attachment and invasion by *F. nucleatum* subsp. *polymorphum* 12230, as well as in the promotion of colorectal carcinogenesis by binding the E-cadherin receptor and inducing clathrin-mediated endocytosis and β-catenin signaling. The role of FadA in placental colonization was also demonstrated in *F. nucleatum* 12230 (36); however, its inactivation did not completely abolish placental colonization, suggesting the involvement of an additional adhesin(s).

Here (Fig. 6), we show that a Fap2 mutant (K50) of *F. nucleatum* subsp. *nucleatum* ATCC 23726 was also impaired in its ability to colonize the placenta, demonstrating for the first time that this fusobacterial subspecies can also colonize the placenta and that Fap2 is involved in placenta colonization.

This study would have greatly benefitted from a complementation mutant, which would have verified *fap2*'s role in the aforementioned interactions. However, multiple attempts to clone this gene were unsuccessful due to spontaneous deletions. These deletions seemed to be insert size dependent, because cloning of the 5′ half of *fap2* and of the 3′ half of *fap2* (~6 kbp each) was successful. However, attempts to clone one half with the other (in both high- and low-copy-number plasmids) resulted in deletions of random sizes (data not shown), in agreement with previous reports of difficulties with cloning AT-rich genomic DNA (57).

In this study, we used a transposon-based mutagenesis system in fusobacteria and created a library which enables phenotypic screening and the identification of virulence traits.

To the best of our knowledge, this is the first report of a transposon insertion mutagenesis system in fusobacteria. We have also identified Fap2 as a galactose-sensitive adhesin involved in hemagglutination, coaggregation, and adherence to mammalian cells in *F. nucleatum* subsp. *nucleatum* ATCC 23726. A better understanding of *F. nucleatum*'s interactions with periodontal bacteria and with host cells will perhaps enable us to improve control of periodontal disease and to reduce *F. nucleatum*-related systemic conditions, adverse pregnancy outcome in particular.

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