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Host Adhesive Activities and Virulence of Novel Fimbrial Proteins of *Porphyromonas gingivalis*▽

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The fimbriae of *Porphyromonas gingivalis* mediate critical roles in host colonization and evasion of innate defenses and comprise polymerized fimbrilin (FimA) associated with quantitatively minor accessory proteins (FimCDE) of unknown function. We now show that *P. gingivalis* fimbriae lacking FimCDE fail to interact with the CXC-chemokine receptor 4 (CXCR4), and bacteria expressing FimCDE-deficient fimbriae cannot exploit CXCR4 in vivo for promoting their persistence, as the wild-type organism does. Consistent with these loss-of-function experiments, purified FimC and FimD (but not FimE) were shown to interact with CXCR4. However, significantly stronger binding was observed when a combination of all three proteins was allowed to interact with CXCR4. In addition, FimC and FimD bound to fibronectin and type 1 collagen, whereas FimE failed to interact with these matrix proteins. These data and the fact that FimE is required for the association of FimCDE with *P. gingivalis* fimbriae suggest that FimE may recruit FimC and FimD into a functional complex, rather than directly binding host proteins. Consistent with this notion, FimE was shown to bind both FimC and FimD. In summary, the FimCDE components cooperate and impart critical adhesive and virulence properties to *P. gingivalis* fimbriae.

Bacterial fimbriae (also referred to as pili) are key players in host-pathogen interactions by mediating colonization and facilitating establishment of infection (16). These filamentous appendages extend to a significant distance from the bacterial cell surface (up to several micrometers) and may thus be the first molecules to interact with the host in an attempt to initiate infection. Fimbriae function as critical colonization factors also for certain oral pathogens, including *Porphyromonas gingivalis* (11). This is a gram-negative anaerobic bacterium that is strongly associated with periodontal disease (19) and is, moreover, implicated in certain systemic inflammatory conditions, such as atherosclerosis (18). To date, two types of *P. gingivalis* fimbrial structures have been described. These comprise the so-called "major" (or "long") fimbriae encoded by the *fimA* gene (2) and the "minor" (or "short") fimbriae encoded by the *mfa1* gene (23).

The *P. gingivalis* "major" fimbriae (henceforth referred to as simply "fimbriae") have been studied in greater molecular detail, and their role in virulence was established in rodent models of periodontitis and atherosclerosis (3, 13). Although the fimbriae are traditionally recognized for their adhesive affinity for various dental or epithelial substrates and extracellular matrix proteins (1, 4, 20), they have more recently been implicated in subversion of host immune function (7, 24). For example, we have shown that the fimbriae bind the CXC-chemokine receptor 4 (CXCR4), which in turn cross talks with and suppresses the Toll-like receptor 2 (TLR2) signaling pathway, leading to enhanced *P. gingivalis* persistence in vitro and in vivo (7).

Although the fimbrilin subunit (FimA) constitutes the main structural component of *P. gingivalis* fimbriae, it is now appreciated that the native fimbrial structure contains additional protein components, encoded by genes immediately downstream of *fimA* (15, 24, 26). These accessory proteins, namely FimC, FimD, and FimE (with molecular masses of 50, 80, and 60 kDa, respectively), comprise ≤1% of the fimbrial protein content but, strikingly, appear to contribute significantly to *P. gingivalis* virulence (24, 26). Specifically, we have shown that *P. gingivalis* isogenic mutants expressing FimA fimbriae devoid of all three accessory proteins (designated DAP fimbriae) are dramatically less virulent than the parent strain in the mouse periodontitis model (24).

Since DAP fimbriae display diminished binding to certain extracellular matrix proteins (fibronectin and type I collagen) (15), the attenuated virulence of the FimCDE-deficient mutants could be attributed, at least partly, to decreased potential for colonization. However, these mutants exhibit additional differences compared to wild-type *P. gingivalis*, including increased capacity to activate transcription factor NF-κB (24). Given that increased microbial activation of NF-κB-dependent

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TABLE 1. Primers used to PCR amplify *P. gingivalis fim* genes

Primer	Gene	Direction	5′→3′ sequence[a]	Cloned gene sequence	Corresponding residues[b]
NSN244	*fimC*	Forward	CACCGGCGGAGACCTCTACAGTGG	169–1359	57–453
NSN245	*fimC*	Reverse	TTAAAGTCCTTGTTGTATGATC		
NSN247	*fimD*	Forward	CACCGAAGACATAGAGTCCGATATG	82–2010	28–670
NSN248	*fimD*	Reverse	TCATTTCTTCAGTCTTTTTGC		
NSN242	*fimE*	Forward	CACCGTAAGCGGTAGCATTGTTTC	112–1650	38–550
NSN243	*fimE*	Reverse	CTAATTCGTCACGTGAATCG		

[a] Underlined extra sequences (CACC) were added so that the coding sequences of the amplified DNA segments be inserted in frame into the pET200 vector.
[b] Corresponding amino acid sequences encoded by the cloned genes.

host defense may impede microbial persistence in the host (14), we speculated that the FimCDE-deficient mutants elicit robust host responses that contribute to their elimination (24). The concept that increased NF-κB activation may impair *P. gingivalis* persistence was demonstrated in a subsequent study by our group. Specifically, antagonistic blockade of the *P. gingivalis* fimbria-CXCR4 interaction abrogated induction of cyclic AMP (cAMP)-dependent negative regulatory signals, leading to enhanced TLR2-mediated NF-κB activation and nitric oxide production, which inhibited the persistence of the pathogen (7).

Since CXCR4 negatively regulates the innate response to wild-type *P. gingivalis* (7), and the FimCDE-deficient mutants are more immunostimulatory than the wild-type organism (24), we hypothesized that DAP fimbriae (i.e., containing FimA but lacking FimCDE) may fail to interact with CXCR4. If correct, the implication of this hypothesis would be that one or more of the FimCDE accessory molecules may interact directly with CXCR4. By the same rationale, since DAP fimbriae fail to bind fibronectin and type I collagen (15), these activities may be mediated by at least one of the accessory fimbrial components. Alternatively, DAP fimbriae may be conformationally different from wild-type fimbriae in a way that interactions with certain host molecules are negatively affected. In this paper, we addressed two major hypotheses: (i) the increased potential of DAP fimbriae for cell activation (relative to the wild type) is attributed to reduced reactivity with CXCR4, and (ii) one or more of the FimCDE components are directly involved in the interactions of *P. gingivalis* fimbriae with CXCR4 and other host proteins. Our conclusion from these studies is that the accessory FimCDE proteins endow *P. gingivalis* fimbriae with additional adhesive and virulence properties, over and above those described for the isolated FimA subunit (reviewed in references 1 and 4).

MATERIALS AND METHODS

Reagents and bacteria. Monoclonal antibodies (MAbs) to CXCR4 (clone 12G5), TLR2 (TL2.1), and isotype controls were purchased from e-Bioscience. MAbs to major histocompatibility complex (MHC) class I (W6/32) and CD14 (Tük 4) were from Abcam. Cholera toxin B subunit was from List Biological Labs and was used to label GM1 ganglioside in fluorescent resonance energy transfer (FRET). For FRET (described below), MAbs or cholera toxin B subunit were conjugated to Cy3 or Cy5 using labeling kits from Amersham Biosciences. AMD3100 and methyl-β-cyclodextrin (MCD) were from Sigma-Aldrich.

P. gingivalis ATCC 33277 and its isogenic mutant OZ5001C were grown anaerobically at 37°C in hemin- and menadione-containing GAM medium (Nissui Pharmaceutical) (24), and their fimbriae were extracted and chromatographically purified as previously described (27). The *P. gingivalis fimC*, *fimD*, and *fimE* genes were PCR amplified using chromosomal DNA as template and the oligonucleotide primers shown in Table 1. Recombinant plasmids encoding His-tagged versions of FimC, FimD, and FimE (respectively termed pSN141, pSN142, and pSN140) were constructed by cloning the corresponding amplified genes into the pET200 expression vector using the Champion pET directional TOPO expression kit (Invitrogen). The primers were designed so that the amplified gene regions would encode the mature forms of FimC, FimD, and FimE (the amino acid residue numbers are indicated in Table 1). Recombinant His-tagged FimC, FimD, and FimE were purified from cell lysates of transformed *Escherichia coli* BL21 Star(DE3) by Cu^{2+} affinity chromatography using a HiTrap chelating HP column, according to the manufacturer's instructions (GE Healthcare). When necessary, protein preparations were passed through a column of agarose-immobilized polymyxin B (Pierce) to remove residual lipopolysaccharide (LPS). Purified native or recombinant fimbrial preparations were free of any contaminating substances on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and contained negligible endotoxin (<8 endotoxin units [EU]/mg), as determined by the *Limulus* amebocyte lysate assay (BioWhittaker). Polyclonal antibodies to FimC, FimD, or FimE were raised in immunized rabbits as previously described (27). All reagents were used at optimal concentrations determined in preliminary or published studies by our laboratories (5, 7, 15, 22).

Mammalian cell culture and transfections. Monocytes were purified from human A+ buffy coats by means of centrifugation over NycoPrep1.068 (Axis-Shield) as previously described (22) and were cultured at 37°C and 5% CO_2 in RPMI 1640 (InVitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.05 mM 2-mercaptoethanol. The same medium was used to culture human monocytic THP-1 cells (ATCC TIB 202). Chinese hamster ovary (CHO)-K1 cells (ATCC CRL-9618) were maintained in Ham's F-12 nutrient mixture (InVitrogen) containing 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. CHO-K1 cells were transfected with empty vector control or human CXCR4 (pORF-hCXCR4; InVivogen) using the PolyFect transfection reagent (Qiagen) or Lipofectamine 2000 (InVitrogen) (7, 22). Cell viability was monitored using the CellTiter-Blue assay kit (Promega), and none of the treatments affected cell viability compared to that of the medium-only control.

FRET. FRET can determine sterical coassociation of fluorescently labeled receptors on the basis of measurements of nonradiative energy transfer from the excited state of a donor molecule to an appropriate acceptor (25) and was performed as previously described (5, 21). Briefly, upon stimulation of human monocytes for 10 min at 37°C with *P. gingivalis* wild-type or DAP fimbriae (1 μg/ml) or the medium-only control, the cells were labeled with a mixture of Cy3-conjugated MAb (donor) and Cy5-conjugated MAb (acceptor), as indicated in Fig. 2. The cells were washed and fixed, and energy transfer between different receptor pairs was calculated from the increase in donor fluorescence after acceptor photobleaching.

FCS. The fluorescence correlation spectroscopy (FCS) method is based on autocorrelation analysis of fluorescence intensity fluctuations, which are confocally detected when fluorescent ligands diffuse through a minute (0.2 fl) excitation volume known as the confocal volume element (17). FCS was performed using a ConfoCor instrument (Carl Zeiss) equipped with a Zeiss Neofluar 40×, numerical aperture 1.2 objective. The technique was used to obtain the diffusion time constants of free and receptor-bound fluorescein isothiocyanate (FITC)-labeled fimbriae (wild type or DAP) interacting with CXCR4-expressing CHO cells. The diffusion time constants were derived from the analysis of fluorescence

intensity fluctuations with autocorrelation functions, computed in real time by a digital signal correlator in the ConfoCor instrument, as previously described (22).

Intermolecular interactions of fimbrial proteins. Polystyrene microtiter plates (96-well Maxisorp; Nunc) were coated with 100 μl of 250 nM FimE in phosphate-buffered saline (PBS), pH 7.2, at 4°C overnight. The plates were washed with PBS containing 0.05% (vol/vol) Tween 20, and nonspecific binding sites were blocked with 5% (wt/vol) bovine serum albumin (BSA) in PBS for 1 h at room temperature. After further washing, 100 μl of 62.5 to 250 nM of FimC, FimD, or both was added and the mixture was incubated at 4°C overnight. The plates were then washed, and bound proteins were detected with specific rabbit immunoglobulin G (IgG) antibodies to FimC or FimD (at 1:64,000 or 1:32,000 dilution, respectively) followed by peroxidase-conjugated goat-anti rabbit IgG antibody. Following a final washing, tetramethylbenzidine substrate was added, the chromogenic reaction was stopped with 0.5 M H_2SO_4, and the generated optical density signal at 450 nm (OD_{450}) was read in a microplate reader. Optimal antibody dilutions were determined in preliminary experiments, in which it was also confirmed that anti-FimC and anti-FimD do not recognize the coating protein (FimE) or the reciprocal protein used as ligand (e.g., anti-FimC fails to bind FimD). Moreover, both antibodies failed to bind to plates coated with recombinant His-tagged glyceraldehyde-3-phosphate dehydrogenase, suggesting that the antibodies do not recognize the poly-histidyl tag fusion partner of the proteins used in the assay.

Binding of fimbrial proteins to host proteins. The binding of FimC, FimD, and FimE to fibronectin and type I collagen was assessed essentially as previously described for native fimbriae (15), with minor modications. Briefly, fibronectin- or type I collagen-precoated microplates (Becton Dickinson Labware) were treated with 1% (wt/vol) BSA in PBS to block nonspecific binding sites and were then incubated with 50 or 100 nM FimC, FimD, or FimE at 4°C overnight, followed by washing with PBS–0.05% Tween 20. All three proteins are His tagged, and since these assays involved adding a single fimbrial protein in the wells, detection of bound protein was carried out by using peroxidase-conjugated anti-His antibody, followed by addition of tetramethylbenzidine chromogenic substrate. The optical density signal at 450 nm was read in a Bio-Rad microplate reader.

The binding of purified fimbrial proteins of *P. gingivalis* to transfected cell lines was determined using a fluorescent cell-based assay in 96-well plates as we previously described (6, 8). Briefly, biotinylated wild-type or DAP fimbriae (1 μg/ml) or recombinantly expressed FimC, FimD, or FimE was incubated with empty vector-transfected or CXCR4-transfected CHO cells (7). Specific binding to CXCR4 was controlled by including in the assays the CXCR4-specific antagonist AMD3100. The proteins were allowed to bind for 30 min at 37°C, and after washing, bound proteins were probed with FITC-labeled streptavidin. Upon further washing, binding was determined by measuring cell-associated fluorescence on a microplate fluorescence reader (FL600; Bio-Tek Instruments) with excitation/emission wavelength settings of 485/530 nm. FimC, FimD, and FimE were used at 0.1 to 10 μg/ml in preliminary experiments and at 20 nM (1 to 1.5 μg/ml depending on molecular weight) in the presented experiments.

Intracellular cAMP induction. Levels of cAMP in activated cell extracts were measured using a cAMP enzyme immunoassay kit (Cayman) (12).

***P. gingivalis* in vivo infection.** Specific-pathogen-free BALB/cByJ mice (8 to 10 weeks old; Jackson Labs) were pretreated intraperitoneally (i.p.) with AMD3100 (25 μg in 0.1 ml PBS) or PBS alone. After 1 h, the mice were infected i.p. with *P. gingivalis* 33277 or OZ5001C (5×10^7 CFU). Peritoneal lavage was performed 20 h and 48 h postinfection. Serial 10-fold dilutions of peritoneal fluid were plated onto blood agar plates and cultured anaerobically at 37°C for enumeration of recovered peritoneal CFU. All animal procedures were approved by the Institutional Animal Care and Use Committee and performed in compliance with established federal and state policies.

Statistical analysis. Data were evaluated by analysis of variance and the Dunnett multiple-comparison test using the InStat program (GraphPad Software). Where appropriate (comparison of two groups only), unpaired two-tailed *t* tests were performed. $P < 0.05$ was taken as the level of significance. All experiments were performed at least twice, yielding consistent results.

RESULTS

Fimbriae lacking FimCDE are impaired in CXCR4 binding and induction of CXCR4-TLR2 coassociation. Using human monocytes or mouse macrophages, we have previously shown that DAP fimbria-expressing *P. gingivalis* (strain OZ5001C) is significantly more proinflammatory than the wild-type organism (strain 33277), and similar differences were seen between their respective isolated fimbriae (5, 24). However, similar to wild-type fimbriae, DAP fimbriae also depend upon CD14 and TLR2 for cell activation (5, 24). Since CXCR4 is a negative regulator of wild-type fimbria-induced CD14-TLR2-dependent signaling (7), we hypothesized that DAP fimbriae may not efficiently interact with CXCR4. We thus compared the CXCR4 binding activities of wild-type and DAP fimbriae. Transfection of CHO-K1 cells with CXCR4 (CHO-CXCR4 cells) resulted in high-level binding of wild-type fimbriae, which was abrogated by anti-CXCR4 MAb (but not by the isotype control) or by a specific CXCR4 antagonist (AMD3100) (Fig. 1). In contrast, DAP fimbriae displayed poor CXCR4-binding activity, being <30% of that seen with wild-type fimbriae ($P < 0.05$, Fig. 1).

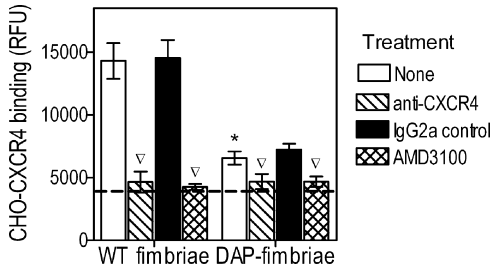

FIG. 1. Comparison of wild-type (WT) and DAP fimbriae in CXCR4 binding. CXCR4-transfected CHO-K1 cells were incubated with biotinylated WT or DAP fimbriae (1 μg/ml), with or without CXCR4 inhibitors (5 μg/ml anti-CXCR4 MAb or 1 μg/ml AMD3100). Binding was measured in relative fluorescence units (RFU) after staining with streptavidin-FITC. Background binding to empty vector-transfected cells (3,691 ± 298 RFU) is indicated by a horizontal dashed line. Data are means ± standard deviations ($n = 3$). Asterisks indicate significant ($P < 0.05$) differences between WT and DAP fimbriae. Inverted triangles show significant ($P < 0.05$) inhibition of binding by CXCR4 inhibitors.

These data were consistent with subsequent FCS analysis, a biophysical method that can determine the dynamics of ligand-receptor interactions. In FCS, receptor-bound and freely diffusing ligands are distinguished by their characteristic diffusion time constants (τ_D), since receptor-ligand complexes diffuse relatively slow in the plane of the cell membrane. We initially examined the diffusion characteristics of FITC-labeled wild-type or DAP fimbriae using control CHO cells, and the analysis in either case revealed a simple monophasic autocorrelation curve with $\tau_D = 59 \pm 1.1$ ms and $\tau_D = 58 \pm 0.5$ ms, respectively (Table 2). The existence of a single diffusion time component for either type of fimbriae indicated that neither molecule engaged in receptor interactions and, therefore, the diffusion times observed likely correspond to freely diffusing unbound molecules. In contrast, FCS measurements taken on the cell membrane of CHO-CXCR4 cells produced autocorrelation functions containing two diffusion components. In the case of wild-type fimbriae, the mean diffusion times were 66 ± 1.8 ms (τ_{D1}) and 150 ± 20 ms (τ_{D2}) (Table 2). The fast-diffusing component (τ_{D1}) is comparable to that seen with wild-type fimbriae incubated with control CHO cells and corresponds to unbound fimbriae, whereas the slow-diffusing component (τ_{D2}) is attributable to the presence of CXCR4 and corresponds to

TABLE 2. Diffusion times obtained by FCS analysis of wild-type or DAP fimbriae binding to CXCR4

Cells	Ligand	Diffusion time component	Diffusion time (ms)[a]
CHO (control)	Wild-type fimbriae	τ_D	59 ± 1.1
	DAP fimbriae	τ_D	58 ± 0.5
CHO-CXCR4	Wild-type fimbriae	τ_{D1}	66 ± 1.8
		τ_{D2}	150 ± 20*
	DAP fimbriae	τ_{D1}	62 ± 1.2
		τ_{D2}	80 ± 10

[a] FCS measurements were performed in living CHO-CXCR4 or control CHO cells using FITC-labeled wild-type or DAP fimbriae. The diffusion times were derived from the analysis of fluorescence intensity fluctuations with autocorrelation functions. Data are means ± standard deviations ($n = 6$). *, significantly ($P < 0.05$) different versus the corresponding diffusion time component (τ_{D2}) of DAP fimbriae.

bound fimbriae. FCS analysis of the binding of DAP fimbriae to CHO-CXCR4 cells also revealed two components with diffusion times of 62 ± 1.2 ms (τ_{D1}; free ligand) and 80 ± 10 ms (τ_{D2}; bound ligand) (Table 2). The observation that bound wild-type fimbriae exhibit a significantly slower diffusion time than bound DAP fimbriae ($P < 0.05$) (Table 2) suggests that the wild-type molecule interacts more strongly with CXCR4, in accordance with the results from the binding study (Fig. 1).

The instigation of an inhibitory CXCR4-TLR2 cross talk by wild-type fimbriae is dependent upon their ability to induce CXCR4-TLR2 coassociation in lipid rafts (7). We have now found that DAP fimbriae are inefficient in this activity. Indeed, FRET analysis showed that the energy transfer between TLR2 and CXCR4 in DAP fimbria-activated monocytes was significantly less than that in wild-type fimbria-activated cells ($P < 0.05$) (Fig. 2A). In contrast, wild-type and DAP fimbriae were similarly effective in inducing CD14-TLR2 coassociation (Fig. 2A). No coassociation was observed between TLR2 and MHC class I (negative control) in cells activated with either type of fimbriae (Fig. 2A). Cell pretreatment with the lipid raft-disrupting agent MCD abrogated the energy transfer between TLR2 and CXCR4 or between TLR2 and CD14 regardless of stimulus (Fig. 2A), consistent with previous observations that these interactions take place in lipid rafts (7). Therefore, the increased potential of DAP fimbriae for cell activation (5, 24) may be due to activation of TLR2 with minimal CXCR4 interference. Alternatively or additionally, it could be argued that DAP fimbriae induce lipid raft recruitment of TLR2 at higher levels than wild-type fimbriae; however, this notion was ruled out by showing that both types of fimbriae induce similar association of TLR2 with the lipid raft marker GM1 ganglioside (Fig. 2B). In short, DAP fimbriae are relatively weak in inducing TLR2-CXCR4 association and binding CXCR4 (Fig. 1 and 2 and Table 2), thereby allowing unimpeded TLR2 activation compared to wild-type fimbriae.

FimCDE-deficient *P. gingivalis* displays reduced persistence in vivo. We previously showed that wild-type *P. gingivalis* exploits CXCR4 for enhancing its persistence in a mouse i.p. infection model (7). We have now addressed whether an isogenic mutant that expresses DAP fimbriae (strain OZ5001C) displays reduced interaction with CXCR4 and persistence in vivo, in comparison with the wild-type strain, 33277. At 20 h postinfection, the recovery of the OZ5001C mutant (viable CFU counts) from the peritoneal cavity of PBS-pretreated mice was 25-fold lower than that of the wild-type strain, 33277 ($P < 0.05$) (Fig. 3A). As expected from our previous study (7), mouse pretreatment with AMD3100 resulted in dramatically reduced recovery of viable CFU counts of 33277 ($P < 0.05$) (Fig. 3A). In stark contrast, however, the recovery of viable OZ5001C CFU was not significantly affected by AMD3100 (Fig. 3A). In a second experiment, the observations were extended to 48 h postinfection, at which time the OZ5001C mutant was not recoverable in a viable state (Fig. 3B). In contrast, the 33277 wild-type strain persisted in a viable state for 48 h, although its recovery was again significantly reduced by CXCR4 blockade ($P < 0.05$) (Fig. 3B). Therefore, wild-type *P. gingivalis* (i.e., expressing FimCDE-containing fimbriae) appears to exploit CXCR4 in vivo resulting in dramatically enhanced persistence in the mouse host compared to the FimCDE-deficient strain.

Interactions of FimCDE accessory proteins with host molecules. The findings that wild-type fimbriae interact with CXCR4 considerably more efficiently than DAP fimbriae suggest that one or more of the fimbrial accessory proteins may bind CXCR4. Indeed, purified FimC and FimD exhibited low but statistically significant ($P < 0.05$) binding to CXCR4, whereas FimE failed to interact (Fig. 4). However, strong binding, comparable to that of wild-type fimbriae, was observed when FimC was allowed to interact with CHO-CXCR4 cells in

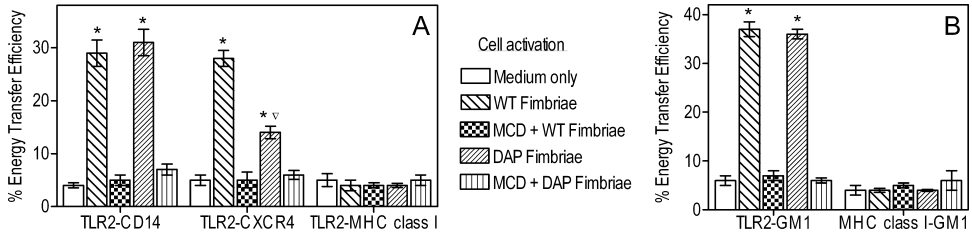

FIG. 2. Wild-type (WT) versus DAP fimbriae in inducing TLR2-CXCR4 association. Monocytes were stimulated with 1 μg/ml WT or DAP fimbriae for 10 min, with or without 10 mM MCD pretreatment. Energy transfer between TLR2 (Cy3) and CD14 (Cy5), CXCR4 (Cy5), or MHC class I (Cy5) (A) or between TLR2 (labeled with Cy3) or MHC class I (Cy3) and the lipid raft marker GM1 (Cy5) (B) was measured from the increase in donor (Cy3) fluorescence after acceptor (Cy5) photobleaching. Results are means ± standard deviations ($n = 3$). Asterisks show significant increase in energy transfer ($P < 0.05$ versus unstimulated cells). Inverted triangles show significant differences ($P < 0.05$) between WT and DAP fimbriae.

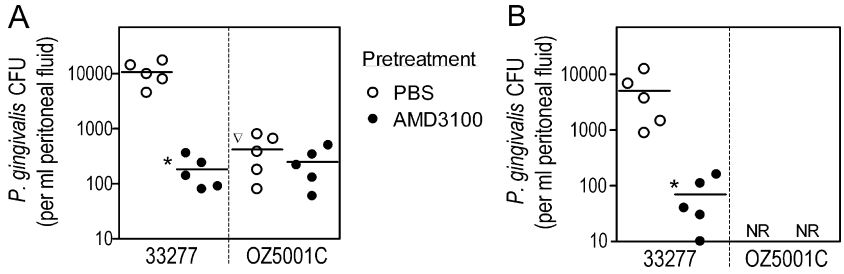

FIG. 3. Reduced virulence and lack of CXCR4 exploitation by *P. gingivalis* expressing fimbriae devoid of FimCDE. BALB/cByJ mice were i.p. pretreated with AMD3100 (25 μg in 0.1 ml PBS) or PBS alone. After 1 h, the mice were i.p. infected with 5×10^7 CFU *P. gingivalis* 33277 or OZ5001C (FimCDE-deficient isogenic mutant). Peritoneal lavage was performed 20 h (A) or 48 h (B) postinfection. Serial 10-fold dilutions of peritoneal fluid were plated for anaerobic growth and enumeration of recovered CFU. Horizontal lines show mean CFU counts. Asterisks indicate significant ($P < 0.05$) differences in *P. gingivalis* peritoneal CFU due to AMD3100 pretreatment. Inverted triangles show significant differences ($P < 0.05$) between corresponding 33277 and OZ5001C CFU. NR, not recoverable in a viable state.

the concomitant presence of both FimD and FimE ($P < 0.05$ versus binding of isolated components or DAP fimbriae) (Fig. 4). In the presence of AMD3100, the binding of FimCDE was reduced to background levels, confirming specific involvement of CXCR4 (Fig. 4). Therefore, FimCDE may cooperate for enhanced CXCR4 binding. Consistent with this notion, the combination of all three proteins, but not the individual components, induced significant CXCR4-dependent elevation of cAMP ($P < 0.05$) (Fig. 5).

In sharp contrast to wild-type fimbriae, DAP fimbriae also display negligible binding to certain matrix proteins (fibronectin and type I collagen), implying that at least one of the fimbrial accessory proteins may have a direct role (15). Although FimE was inert in terms of CXCR4 binding activity (Fig. 4), it could not be ruled out that FimE may be involved in other types of interactions. We thus determined and compared the binding activities of FimC, FimD, and FimE for fibronectin and type I collagen. FimE again failed to bind either matrix protein, although FimC and especially FimD readily bound to both fibronectin (Fig. 6A) and type I collagen (Fig. 6B). It should be noted that the lack of detection of FimE by anti-His tag MAb cannot be attributed to possible inaccessibility of the His_6 epitopes of this recombinant protein, since we have confirmed that anti-His tag MAb readily detects FimE (data not shown). These findings further support that FimC and FimD confer important adhesive functions to *P. gingivalis* fimbriae, although the precise role of FimE remained enigmatic.

Intermolecular interactions of fimbrial accessory proteins. Despite lacking direct binding activity for the host molecules tested, the presence of FimE was important for maximal CXCR4 interactions by FimCDE (Fig. 4 and 5). Moreover, FimE-deficient *P. gingivalis* produces FimA fimbriae devoid of all three accessory components (15). This suggests that FimE may recruit FimC and FimD into a functional complex. To address the notion that FimE recruits FimC and FimD, FimE was used as the coating protein and FimC and FimD as potential ligands in an enzyme-linked immunosorbent assay-based binding assay. Highly specific anti-FimC and anti-FimD antibodies (which were confirmed to lack cross-reactive activity) were used for probing bound FimC and FimD, respec-

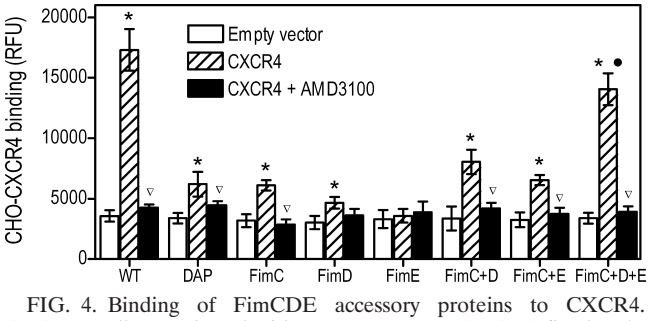

FIG. 4. Binding of FimCDE accessory proteins to CXCR4. CHO-K1 cells transfected with empty vector or CXCR4 (in the absence or presence of 1 μg/ml AMD3100) were incubated with biotinylated wild-type (WT) or DAP fimbriae (1 μg/ml) or with fimbrial accessory proteins (20 nM of total protein, corresponding to 1 to 1.5 μg/ml), alone or in the combinations indicated. Binding was measured in relative fluorescence units (RFU) after staining with streptavidin-FITC. In assaying the binding of FimC in the presence of FimD and/or FimE, only FimC was used in the biotinylated form. Results are means ± standard deviations ($n = 3$). The asterisks show significant ($P < 0.05$) binding versus empty vector-transfected cells. Inverted triangles denote significant ($P < 0.05$) inhibition of binding by AMD3100. The black circle indicates significantly ($P < 0.05$) higher FimCDE binding compared to individual fimbrial accessory proteins and DAP fimbriae.

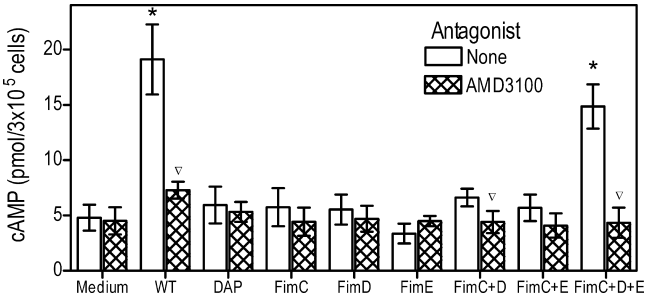

FIG. 5. Induction of CXCR4-dependent cAMP responses by fimbrial proteins. THP-1 cells were stimulated for 20 min with wild-type (WT) or DAP fimbriae (1 μg/ml) or with fimbrial accessory proteins (20 nM of total protein, corresponding to 1 to 1.5 μg/ml), alone or in the combinations indicated. The stimulation was carried out in the absence or presence of AMD3100, and the cells were assayed for induction of intracellular cAMP. Results are means ± standard deviations ($n = 3$). The asterisks indicate significant ($P < 0.05$) elevation of intracellular cAMP over basal levels (medium only). Inverted triangles show significant ($P < 0.05$) inhibition of cAMP responses by AMD3100.

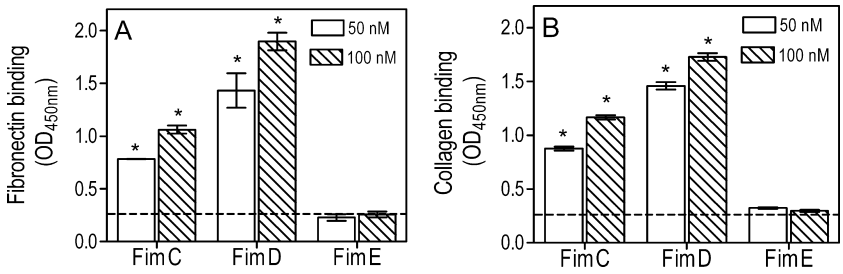

FIG. 6. Binding of fimbrial accessory proteins to extracellular matrix proteins. Binding of the indicated His-tagged fimbrial proteins to plates coated with fibronectin (A) or type I collagen (B) was determined colorimetrically after probing with anti-His tag antibody. The horizontal dashed lines indicate the average background binding of the fimbrial proteins to BSA-coated wells (OD_{450}, 0.26 ± 0.05). Data are means ± standard deviations ($n = 3$), and asterisks show significant ($P < 0.05$) binding compared to background values.

tively. We found that both FimC (Fig. 7A) and FimD (Fig. 7B) could bind immobilized FimE in a dose-dependent manner (Fig. 7). The probing antibodies showed negligible reactivity when BSA substituted for the specific ligands in the FimE-coated wells. The binding of FimC to FimE was modestly but significantly ($P < 0.05$) reduced by the concomitant presence of FimD in the assay volume (Fig. 7A), and vice versa (Fig. 7B). This might be explained by reduced epitope exposure of bound FimC to the probing anti-FimC antibodies, perhaps due to possible interactions of FimC with FimD (and vice versa). This possibility is consistent with a subsequent observation that FimD directly binds FimC. (The OD_{450} binding value was 0.36 ± 0.01 versus 0.03 for nonspecific background binding.)

DISCUSSION

The molecular basis of the multifunctional adhesive ability of *P. gingivalis* fimbriae has always been a puzzle. The results of functional mapping studies suggest that the FimA sequence contains several adhesion epitopes for distinct host molecules, although certain epitopes involve versatile structural motifs that are recognizable by more than one host protein (reviewed in reference 4). However, given that the FimA-associated accessory proteins were not included in the earlier mapping studies, it is possible that a number of activities associated with *P. gingivalis* fimbriae remained "uncharted territory." Indeed, although the fimbrial accessory proteins were first described more than 15 years ago (28), it was only recently that their function started to be elucidated. Whereas we previously obtained only indirect evidence for the biological significance of FimCDE (15, 24), we have now generated conclusive direct evidence. For example, although previous studies revealed dramatic differences between wild-type and FimCDE-deficient (DAP) fimbriae in terms of binding fibronectin or type I collagen (15), a direct role for FimCDE was only now established by showing that two of these proteins (FimC and FimD) bind to these extracellular matrix proteins. Interestingly, electron microscopic examination of *P. gingivalis* wild-type and DAP strains has revealed that DAP fimbriae are structurally distinct from wild-type fimbriae (24). However, the DAP fimbriae do not show any intrinsic defects in their potential to interact with innate receptors or to activate cells. Indeed, wild-type and DAP fimbriae interact equally well with TLR2 and CD14, although DAP fimbriae are more proinflammatory (24), owing to their relative inability to efficiently interact with CXCR4.

CXCR4 is a lipid raft-recruitable receptor that is exploited by *P. gingivalis* for inhibiting TLR2-mediated innate immune defense (7). In this paper, we have localized the CXCR4-binding activity of *P. gingivalis* fimbriae to its FimCDE accessory components. In terms of biological significance, *P. gingivalis* expressing FimCDE-deficient fimbriae is unable to exploit CXCR4 in vivo and is eliminated relatively easily by the mouse host compared to the wild-type strain. Our binding studies have revealed modest-to-weak interactions between CXCR4 and the accessory proteins in the order FimC > FimD > FimE, with the latter being completely inert. Strikingly, however, FimC in the presence of equimolar concentrations of

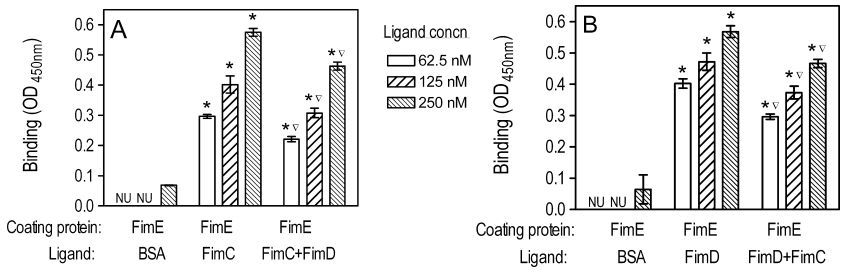

FIG. 7. Binding of FimC and FimD to immobilized FimE. Microtiter plates were coated with FimE, followed by addition of BSA (sham ligand), FimC, FimD, or FimC plus FimD. The ligands were used at the indicated concentrations, except for BSA, for which the lower concentrations were not used (NU). Binding was determined colorimetrically after probing with specific antibodies to FimC (A) or FimD (B). Background binding of FimC or FimD to BSA-coated wells was negligible, representing <9% of specific binding values. Data are means ± standard deviations ($n = 3$), and asterisks indicate significant ($P < 0.05$) differences compared to the sham ligand control. Inverted triangles show significantly ($P < 0.05$) reduced binding compared to binding when a single ligand was added in the wells.

FimD and FimE bound strongly to CXCR4, suggesting that FimCDE may cooperate to form a functional complex with enhanced binding properties over the individual molecules. These data, and those from the matrix protein binding experiments, also suggest that the likely role of FimE is not for direct interactions with host molecules but for recruitment of FimC and FimD into a functional complex associated with the FimA fimbriae. Consistent with this notion, genetic inactivation of *fimE* does not prevent the expression of *fimC* or *fimD*, but the products of these genes are released into the culture medium and are not associated with FimA fimbriae in the absence of FimE (15). Furthermore, we have shown that FimE interacts directly with FimC and FimD. The stoichiometry of this putative complex and its localization on the fimbrial structure—at the tip of the fimbrial rod and/or elsewhere—has yet to be determined. However, incorporation of quantitatively minor accessory components into other, unrelated types of fimbriae and their contribution as tip adhesive elements have previously been demonstrated for *Escherichia coli* (9, 10).

In general, it is difficult to exclude the possibility that LPS in bacterial protein samples may contribute to the results observed. However, it is unlikely that this could be the case in our study. Indeed, all three recombinant proteins (FimC, FimD, and FimE) were purified using the same protocol and similarly contained negligible endotoxic activity (<8 EU/mg protein). Most importantly, one of them (FimE) was completely inert in binding host proteins or inducing a functional cellular response. Therefore, LPS can be ruled out in terms of mediating or contributing to any of the activities observed. In addition, we noticed a synergistic effect in the binding and activation of CXCR4 when all three proteins were allowed to interact with the receptor, an effect that could not be interpreted by the additive effect of having more LPS (even at the trace amounts at which it could be detected) in the assay. On the other hand, the synergistic effect can be readily explained by cooperative interactions of the fimbrial proteins.

In a recent publication, we also showed that wild-type but not FimCDE-deficient *P. gingivalis* interacts with complement receptor 3 (CR3), a β_2 integrin with pattern recognition capacity (24). However, in contrast to our results with CXCR4 and extracellular matrix proteins, we could not demonstrate direct CR3 binding of any of the accessory proteins, tested either alone or in combinations (unpublished observations). This suggests that FimCDE-deficient fimbriae exhibit conformational differences relative to wild-type fimbriae and may thereby lack a structural pattern that is recognizable by CR3.

Our current data provide an explanation for our earlier findings that FimCDE-deficient *P. gingivalis* is relatively avirulent compared to the wild-type organism in the oral gavage model of murine periodontitis (24). The poor virulence of the FimCDE-deficient (DAP) mutants may now be attributed, at least in part, to their inability to exploit CXCR4. Indeed, the observation that the CXCR4 antagonist influences the persistence of the wild-type strain but has no effect on the FimCDE-deficient mutant examined (OZ5001C) indicates that the differential persistence of the two strains is likely attributed to different innate immune interactions, rather than possible differences in in vivo growth. In fact, both strains exhibit similar growth rates in vitro, although this cannot be reliably tested in vivo, where any potential for growth will be influenced by the host immune system. Additionally, colonization of periodontal tissues is a prerequisite for induction of bone loss and the lack of FimCDE expression may impair interactions of mutant *P. gingivalis* with matrix proteins, resulting in reduced colonization and virulence. We have previously used another FimCDE-deficient mutant (KO4) which contains a nonpolar mutation in *fimE*. This mutant expresses FimC and FimD, which cannot, however, associate with fimbriae in the absence of FimE and are thus secreted in the culture medium (15). On the other hand, OZ5001C contains an insertional inactivation of *fimC* which abrogates expression of FimCDE due to concomitant polar effects on the downstream *fimD* and *fimE* genes (15). As a consequence, KO4 and OZ5001C are phenotypically similar in that they express FimA lacking all three accessory proteins (i.e., DAP fimbriae) and display similar fimbrial structures under electron microscopic examination (24). Most importantly, both strains behave in the exact same way in adherence and phagocytic assays, susceptibility to phagocyte killing, proinflammatory activities, and in vivo virulence (24). Thus, regardless of whether expression of DAP fimbriae is due to polar or nonpolar effects, the behavior of the mutant strains is exactly the same, and consequently the strain used in the present study (OZ5001C) is a genuinely representative DAP strain.

In conclusion, we have shown that the FimCDE accessory proteins display unique properties that enrich the virulence repertoire of *P. gingivalis* fimbriae. Whereas FimC and FimD display direct binding to exploited innate immune (CXCR4) or matrix proteins (fibronectin and collagen), FimE is inert in that regard. However, FimE may act as an adaptor protein which integrates the FimCDE components into functional units of enhanced activity over that of the individual components. Thus, although the biological roles of the fimbrial accessory proteins have remained obscure until recently, the newly attributed virulence properties render FimCDE novel targets for molecular intervention against *P. gingivalis* infections.

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