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ORIGINAL ARTICLE

Characterization of *Fusobacterium nucleatum* ATCC 23726 adhesins involved in strain-specific attachment to *Porphyromonas gingivalis*

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Bacterial adherence is an essential virulence factor in pathogenesis and infection. *Fusobacterium nucleatum* has a central role in oral biofilm architecture by acting as a bridge between early Gram-positive and late Gram-negative colonizers that do not otherwise adhere to each other. In this study, we survey a key adherence interaction of *F. nucleatum* with *Porphyromonas gingivalis*, and present evidence that multiple fusobacterial adhesins have a role in the attachment of *F. nucleatum* ATCC 23726 to *P. gingivalis* in a highly strain-dependent manner. Interaction between these species displayed varying sensitivities to arginine, galactose and lactose. Arginine was found to hamper coaggregation by at least 62% and up to 89% with several *P. gingivalis* strains and galactose inhibition ranged from no inhibition up to 58% with the same *P. gingivalis* strains. Lactose consistently inhibited *F. nucleatum* interaction with these *P. gingivalis* strains ranging from 40% to 56% decrease in coaggregation. Among the adhesins involved are the previously described Fap2 and surprisingly, RadD, which was described in an earlier study for its function in attachment of *F. nucleatum* to Gram-positive species. We also provide evidence for the presence of at least one additional adhesin that is sensitive to arginine but unlike Fap2 and RadD, is not a member of the autotransporter family type of fusobacterial large outer membrane proteins. The strain-specific binding profile of multiple fusobacterial adhesins to *P. gingivalis* highlights the heterogeneity and complexity of interspecies interactions in the oral cavity.
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INTRODUCTION

Fusobacterium nucleatum is a prevalent member of the oral microbial community and considered a key organism in biofilm formation due to its ability to adhere to a large variety of microbial species.[1–3] Although present in healthy oral biofilms, this Gram-negative opportunistic pathogen is dominant in periodontal disease[4] and has been implicated in a number of invasive human infections,[5–6] acute and chronic inflammatory conditions[7–8] as well as adverse pregnancy outcomes[9–10] *F. nucleatum* pathogenicity is, in part, attributed to its function as a "bridging organism" that supports the integration of periodontal pathogens into oral biofilms.[2,11] With this unique ability to attach to both early and late colonizers, *F. nucleatum* is thought to have a central role in the ecological shift from a mostly Gram-positive to a predominately Gram-negative and thus, pathogenic, biofilm community in periodontal disease.[2]

Despite the extensive exploration of *F. nucleatum* interspecies interactions and the identification of a number of binding partners, to date, only two fusobacterial large outer membrane proteins (OMPs), RadD and Fap2, have been characterized at a molecular level for their role as adhesins in binding to a variety of Gram-positive species[12–13] and *Porphyromonas gingivalis*, respectively.[14] Both RadD and Fap2 are members of the autotransporter family of proteins,[13] which are the largest known family of virulence factors expressed by Gram-negative bacteria.[15] Autotransporters account for numerous biological functions including adhesion[16–17] cell-to-cell aggregation,[18–19] biofilm formation[20–21] and invasion.[22] It is therefore not surprising that in addition to their role in interspecies binding, fusobacterial autotransporters are multi-functionally involved in the induction of apoptosis in lymphocytes[23–24] and adherence to murine placental cells.[14]

Historically, studies have shown that interbacterial binding measured by the classical coaggregation assay often involves highly serotype or strain-specific cell-to-cell recognition.[25] For example, a panel of different *F. nucleatum* strains and several species of *Selenomonas* were found to bind only to certain subsets of oral partner species tested but no distinct group-specific pattern was observed.[2] In particular, adhesion of different *F. nucleatum* isolates to a selection of *P. gingivalis* strains varied from no interaction to very strong coaggregation phenotypes, some of which were sensitive to lactose or heat treatment, while others were not.[26] This interspecies binding variation is not limited to fusobacterial interactions but appears to be a common theme among oral bacterial species. Previous studies of different oral bacterial interactions have demonstrated that coaggregation involves highly

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specific cell-to-cell recognition of distinct isolates of a certain species and that this pattern is not generalizable to all strains of a single species or all species of a genus. Other examples of these differential binding specificities include *Actinomyces naeslundii* with different strains of *Streptococcus sanguinis* and *Streptococcus gordonii*,[25,27–29] *P. gingivalis* binding with *Veillonella, Capnocytophaga* and *Actinomyces* but not with *Streptococcus*,[30] intra- and intergenic coaggregations between *Streptococcus* and *Actinomyces*[31] among others.

Interactions between *F. nucleatum* and *P. gingivalis* are of key interest because they are frequently isolated together from several chronic immunoinflammatory diseases of the oral cavity.[32–33] While identification of Fap2 as the galactose-inhibitable adhesin of *F. nucleatum* strains ATCC 23726 for binding to *P. gingivalis* strain PK 1924 provided the first molecular identification of a fusobacterial adhesin involved in this interaction,[14] previous reports of differential binding between various strains of these species[26] as well as the finding that their coaggregation can be inhibited by carbohydrates other than galactose indicated the presence of additional adhesins. In this study, we tested the coaggregation between *F. nucleatum* strain ATCC 23726 and five strains of *P. gingivalis*, characterized the carbohydrate sensitivity of the interaction and screened the existing panel of autotransporter mutants in ATCC 23726 (ref. 13) for possible binding defects. This led to the identification of RadD as an additional strain-specific fusobacterial adhesin for interaction with *P. gingivalis* as well as the finding that further adhesins exist that do not belong to the autotransporter family of proteins despite being inhibited by the addition of arginine similar to RadD.

MATERIALS AND METHODS

Bacterial strains and culture conditions

F. nucleatum strain ATCC 23726 and its mutant derivatives defective in large outer membrane autotransporter proteins[13,24] as well as seven different *P. gingivalis* strains 4612 (ref. 34), T22 (ref. 35), MP4-504 (ref. 36), ATCC 33277 (ref. 35), 381 (ref. 37), W50 and W83 (ref. 38), were maintained on Columbia agar supplemented with 5% sheep blood or in Columbia broth (CB; Difco, Detroit, MI, USA) under anaerobic conditions (10% H_2, 10% CO_2, 80% N_2) at 37 °C. All media for *P. gingivalis* were also supplemented with hemin at 5 μg·mL^{-1} and menadione at 1 μg·mL^{-1}. Thiamphenicol at 5 μg·mL^{-1} and clindamycin at 1 μg·mL^{-1} (MP Biomedicals, Irvine, CA, USA) were used for the selection and maintenance of strains possessing the *catP* and *ermB* determinants, respectively.

Coaggregation assay

Visual. Coaggregation assays were performed in coaggregation buffer (CAB; 150 mmol·L^{-1} NaCl, 1 mmol·L^{-1} Tris, 0.1 mmol·L^{-1} $CaCl_2$, 0.1 mmol·L^{-1} $MgCl_2{\cdot}H_2O$; pH 7.5) as previously described.[13] In brief, cells were pelleted and re-suspended in CAB to a final concentration of 2×10^9 cells per mL (optical density (OD) measured at 600 nm was 2). Suspensions of strains to be examined for coaggregation were combined with an equal volume of a test strain adjusted to the same cellular concentration in CAB to a total volume of 400 μL in a reaction tube. Once the second partner strain was added, reaction mixtures were immediately vortexed for 5 s and incubated for at least 10 min prior to evaluation using a visual scoring system ranging from 0 to 4.[3] A score of 0 was assigned for no visible coaggregation and a score of 4 described complete sedimentation of strains with a clear supernatant (Supplementary Table S1).

Quantitative. Coaggregation assays were performed identical to the visual assay except that ODs of reaction mixtures were obtained spectrophotometrically immediately after the addition of second partner strain and vortexing ($OD_{t=0\ min}$). After 10 min of incubation, reaction mixtures were centrifuged at low speed (100*g* for 1 min) to pellet coaggregated cells while leaving non-aggregated bacteria in suspension. ODs of the supernatants were measured after the 10-min incubation ($OD_{t=10\ min}$) in order to quantitate coaggregation. Coaggregation test reactions were calculated as $(OD_{t=0\ min} - OD_{t=10\ min})/OD_{t=0\ min}$.[39] These values were averaged across at least three independent experiments and represented as percentages calculated relative to control reactions (reactions without the addition of a partner strain) as $(Avg_{test\ reaction}/Avg_{control\ reaction})\times100$.

Inhibition. For both visual and quantitative inhibition assays, either L-arginine, D-galactose, L-glutamic acid, D-glucose or lactose was added to the reaction tube containing only *F. nucleatum* cells to a final concentration of 100 mmol·L^{-1}. The suspension was then vortexed and incubated for 5 min prior to the addition of the coaggregation test partner. Once the partner strain was added, the reaction mixture was vortexed again and the assay was evaluated *via* the quantitative coaggregation assay as described above. The final concentration of each inhibitor per coaggregation reaction was 50 mmol·L^{-1}.

Biofilm growth

Dual species biofilms. The *F. nucleatum* and *P. gingivalis* biofilm growth protocol was modified from a previous study[40] by using 96-well collagen-coated plates (Advanced BioMatrix, San Diego, CA, USA) that were ultraviolet sterilized for 1 h prior to inoculation. For dual-species biofilms, 100 μL of CB supplemented with hemin at 5 μg·mL^{-1} and menadione at 1 μg·mL^{-1} were added into each well. *F. nucleatum* cells (50 μL containing ~5×10^7 cells) were seeded into each well and allowed to grow under anaerobic conditions (10% H_2, 10% CO_2, 80% N_2) at 37 °C for 3 h prior to the addition of *P. gingivalis* (50 μL containing ~5×10^7 cells) to allow *F. nucleatum* cells time to bind to and saturate the collagen-coated surfaces. Plates were incubated overnight under anaerobic conditions (10% H_2, 10% CO_2, 80% N_2) at 37 °C. Triplicate wells were inoculated for each experiment, which were combined for DNA extraction. At least three biological replicates were performed per condition.

Fusobacterial attachment. To ensure that each of the fusobacterial strains attached to collagen-coated wells over 3 h, after growth under anaerobic conditions (10% H_2, 10% CO_2, 80% N_2) at 37 °C, contents were removed from each well and rinsed once with 250 μL of sterile phosphate-buffered saline. Plates were inverted and dried. Afterwards, attached bacteria were fixed at room temperature for 15 min by adding 200 μL of methanol into each well. The plates were stained with a 100 μL aqueous solution of 0.5% crystal violet (Thermo Fisher Scientific, Waltham, MA, USA) for 15 min at room temperature. The plates were then carefully rinsed with Millipore water (Darmstadt, Germany) until there was no visible trace of the stain. Bound stain was dissolved by adding 160 μL of 95% ethanol. The OD of each well was measured at 570 nm and was represented as relative to a negative control wells that only contained CB (Supplementary Figure S1). At least three biological replicates were performed per *F. nucleatum* strain.

Extraction of DNA from biofilms

Prior to DNA isolation, the medium was carefully removed from the wells. Genomic DNA was isolated directly from the wells using GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, St Louis, MO, USA) according to manufacture's instructions with the modi-

fication of final elution to 30 μL. Buffers, enzymes and precipitating DNA were directly added into the wells prior to combining replicate samples and transferring the solution to the columns.

Quantitative (real-time) polymerase chain reaction

To quantify the relative proportions of each species in the respective dual-species biofilms, species-specific primer pairs were used. For *F. nucleatum* ATCC 23726 and its mutant derivatives, a portion of the *Fusobacterium*-specific *fomA* gene was amplified with Fn-F (forward) 5′-AGTTGCTCCAGCTTGGAGACCAAAT-3′ and Fn-R (reverse) 5′-AAGTTTACTTTTGTTAAAGTTTGTAATCTTCC-3′ primers. For *P. gingivalis* Pg-F (forward) 5′-AGGCAGCTTGCCATACTGCG-3′ and Pg-R (reverse) 5′-ACTGTTAGCAACTACCGATGT-3′ were chosen to amplify a portion of the *P. gingivalis* 16S *rRNA* gene. Primer pairs were tested for possible cross-reactivity with the other species. Real-time quantitative polymerase chain reaction (qPCR) was performed using an iCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA) in a total volume of 20 μL containing 10 μL of 2× iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 0.5 μmol·L^{-1} each of forward and reverse primers, 7 μL of Millipore water and 1 μL (10 ng) of template DNA. Amplification and detection were carried out in 96-well optical plates (Thermo Fisher Scientific, Waltham, MA, USA). Each PCR run was carried out with an initial incubation of 10 min at 95 °C followed by 40 cycles of denaturing at 95 °C for 15 s; annealing and elongation at 60 °C for 1 min. After the 40 cycles of amplification, an additional denaturing step was performed at 95 °C for 1 min followed by annealing and elongation at 60 °C for 1 min. A melting curve analysis was completed after each run. The DNA concentrations (ng·mL^{-1}) were calculated with standard curves obtained by 10-fold serial dilutions of bacterial genomic DNA. Three independent qPCR runs were performed with three technical replicates for each sample to assess reproducibility and inter-run variability.

Statistical analysis

Student's *t*-test was performed to determine statistical significance using Excel 2011 (Microsoft, Seattle, WA, USA).

RESULTS

Autoaggregation of bacteria and coaggregation of *F. nucleatum* ATCC 23726 with *P. gingivalis* displays a strain-specific profile

Qualitative and quantitative autoaggregation and coaggregation assays revealed a strain-dependent binding profile of *F. nucleatum* ATCC 23726 with the seven different strains of *P. gingivalis* tested in this study. Robust interactions were observed with *P. gingivalis* strains 4612 (62% ± 8% coaggregation), T22 (54% ± 8% coaggregation) binding and ATCC 33277 (65% ± 17% coaggregation; Figure 1). Binding of *P. gingivalis* strains W50 and W83 to *F. nucleatum* ATCC 23726 was notably weaker, showing only 23% ± 10% and 30% ± 22% coaggregation, respectively. *P. gingivalis* strains 381 and the clinical isolate MP-504 revealed 54% ± 2% and 73% ± 4% coaggregation, respectively, but were not chosen for further interaction studies with *F. nucleatum* due to notable autoaggregation levels. Interactions of fusobacteria with other species can be disrupted by a number of small molecules including arginine, galactose and lactose.[26] Attachment to early colonizers was described to be largely inhibitable by arginine[41] with the identification of RadD as the arginine-inhibitable adhesin for *streptococci*,[13] whereas adhesion between *F. nucleatum* and Gram-negative late colonizers including *P. gingivalis* was found to be generally sensitive to galactose.[26,42] Fap2 was recently identified as the galactose-inhibitable adhesin for this interaction.[14] Interestingly, the binding profile of *F. nucleatum* strain ATCC 23726 with the

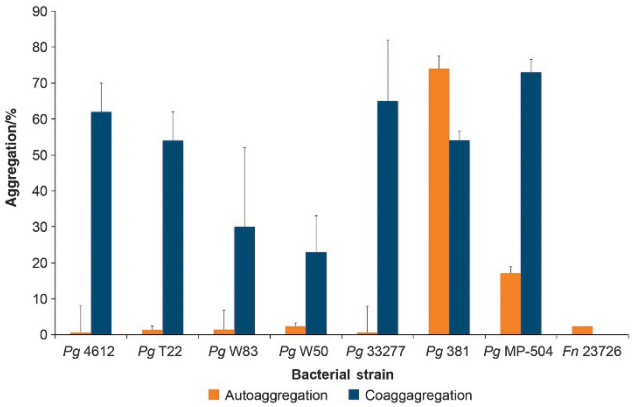

Figure 1 Quantitative autoaggregation levels of bacterial strains and coaggregation levels between *Fusobacterium nucleatum* ATCC 23726 and seven different *Porphyromonas gingivalis* strains. Data are expressed as percentage aggregation and represent the means and standard deviation of at least three independent experiments.

different *P. gingivalis* strains tested in this study exhibited a diverse inhibition profile that involved strain-dependent sensitivities to the inhibitors tested (Figure 2). Attachment to *P. gingivalis* strains 4612 and T22 was partially sensitive to arginine, galactose and lactose, whereas coaggregation with ATCC 33277 was almost completely abolished by the addition of arginine alone and partially sensitive to lactose. Coaggregation reactions in the presence of 50 mmol · L^{-1} arginine resulted in a relative decrease in coaggregation by 62% ± 2% with 4612, 70% ± 6% with T22 and 89% ± 3% with ATCC 33277 compared with the corresponding reactions with ATCC 23726 in the absence of inhibitor. Reactions between *F. nucleatum* ATCC 23726 and *P. gingivalis* strains in the presence of galactose revealed, a 58% ± 16% decrease in coaggregation with 4612 and 36% ± 18% reduction of binding with T22 compared with control reactions without inhibitor. Coaggregation in the presence of 50 mmol·L^{-1} lactose resulted in a relative decrease in coaggregation by 46% ± 13% with 4612, 56% ± 7% with T22 and 40% ± 8% with ATCC 33277 compared with the corresponding reactions with ATCC 23726 in the absence of inhibitor. Interactions between ATCC 23726 and ATCC 33277 were not affected by galactose, with coaggregation being reduced by only 12% ± 6% compared with the strains reacting in CAB. Addition of glucose or glutamic acid did not affect adhesion of any of the strain combinations tested.

Fusobacterial OMPs function as adhesins for interaction with *P. gingivalis*

In a previous study, we created gene inactivation mutants in large outer membrane autotransporter proteins (OMPs) of *F. nucleatum* ATCC 23726 and identified one of them, RadD, as an adhesin for interaction with Gram-positive *Streptococci* and *Actinomyces*.[13] Recently, an additional, one of these large OMPs, Fap2 was characterized as the adhesin for galactose-inhibitable binding of *P. gingivalis* PK 1924 to the same strain, ATCC 23726, of *F. nucleatum* used here.[14] As we observed strain-dependent coaggregation profiles in our inhibition experiments, we examined the binding of the previously generated OMP mutant panel, including the arginine-inhibitable RadD and galactose-inhibitable Fap2 to the three different *P. gingivalis* strains (4612, T22 and ATCC 33277) that exhibited significant binding in this study to the parent strain ATCC 23726 (Figure 3). Similar to the inhibition results, coaggregation between the *F. nucleatum* ATCC 23726 OMP mutant derivatives and the *P. gingivalis* strains varied

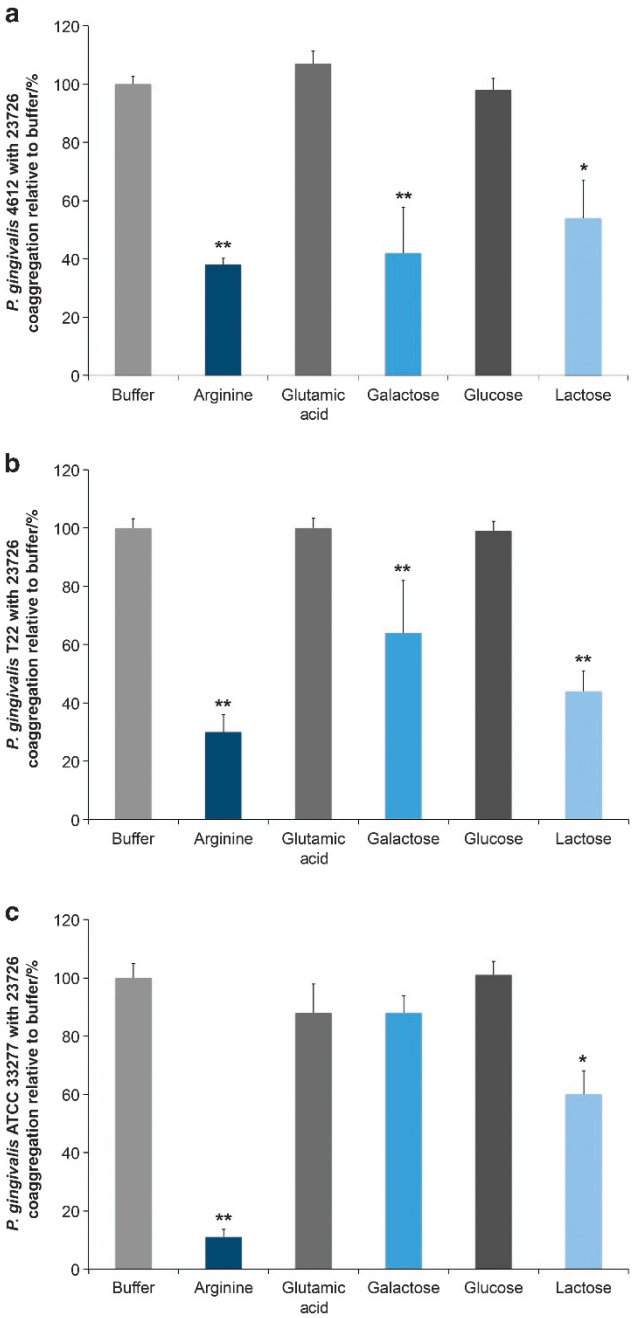

Figure 2 Quantitative inhibition of coaggregation assay between *Fusobacterium nucleatum* ATCC 23726 and *Porphyromonas gingivalis* strains in the presence of inhibitors. (**a**) *P. gingivalis* 4612 with *F. nucleatum* ATCC 23726; (**b**) *P. gingivalis* T22 with *F. nucleatum* ATCC 23726; (**c**) *P. gingivalis* ATCC 33277 with *F. nucleatum* ATCC 23726. Data are expressed as relative percentage coaggregation compared to coaggregation reaction of the partner strains in buffer set as 100% and represent the means and standard deviation of at least three independent experiments. $^{*}P<0.05$; $^{**}P<0.01$.

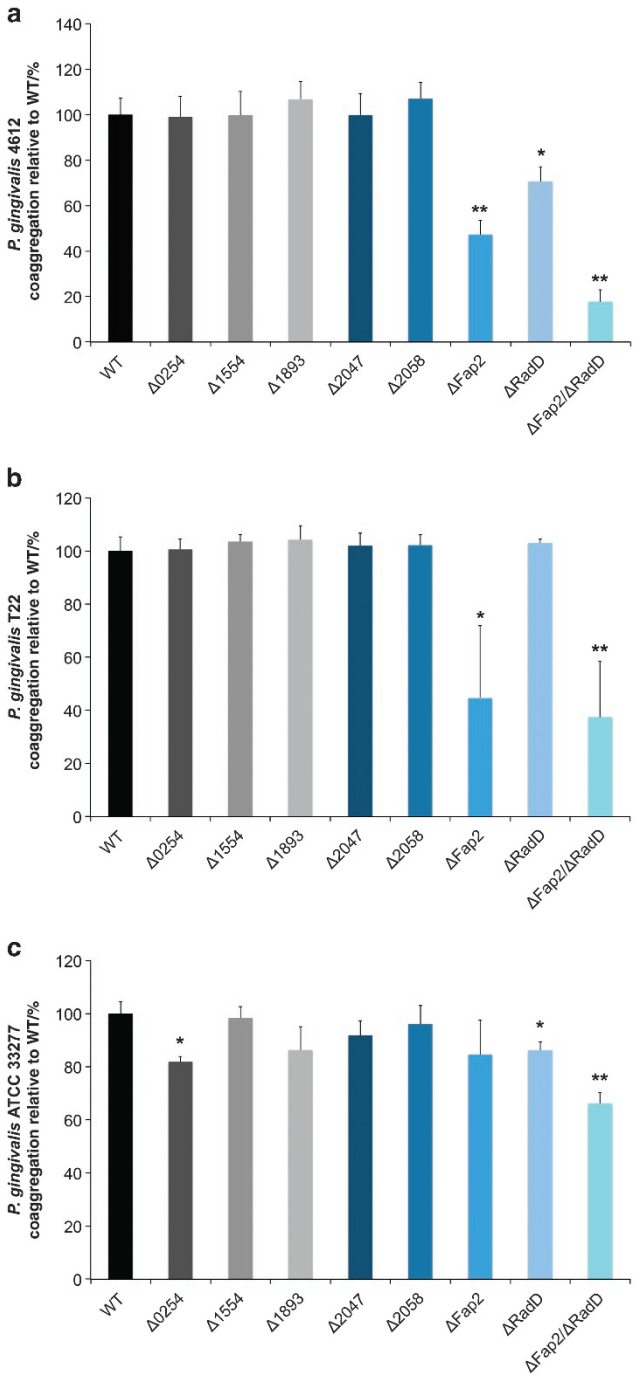

Figure 3 Quantitative coaggregation assay between *Fusobacterium nucleatum* ATCC 23726 and mutant derivatives in outer membrane proteins with *Porphyromonas gingivalis* strains. (**a**) *P. gingivalis* 4612 with *F. nucleatum* ATCC 23726; (**b**) *P. gingivalis* T22 with *F. nucleatum* ATCC 23726; (**c**) *P. gingivalis* ATCC 33277 with *F. nucleatum* ATCC 23726. Data are expressed as relative percentage coaggregation compared with coaggregation reaction of the wild type with the respective *P. gingivalis* partner strains set as 100%. Data represent the means and standard deviation of at least three independent experiments. $^{*}P<0.05$; $^{**}P<0.01$. WT, wild type.

depending on the interacting pairs. Fusobacterial interaction with *P. gingivalis* strain 4612 appears to be mediated by both Fap2 and RadD, with the average relative decrease for the individual mutants (Fap2 mutant, 53% ± 6% and RadD mutant, 29% ± 6%) adding up to the decrease seen for the Fap2/RadD double mutant (82% ± 5%). Among the OMP mutants tested here, only lack of Fap2 resulted in a reduction of the interaction between *F. nucleatum* ATCC 23726 and *P. gingivalis* T22 with a decrease of 55% ± 27% relative to coaggregation with the wild-type parent strain. Binding to *P. gingivalis* 33277 displayed only a slight decrease with several of the OMP mutants tested. However, with the exception of the decrease (34% ± 4%)

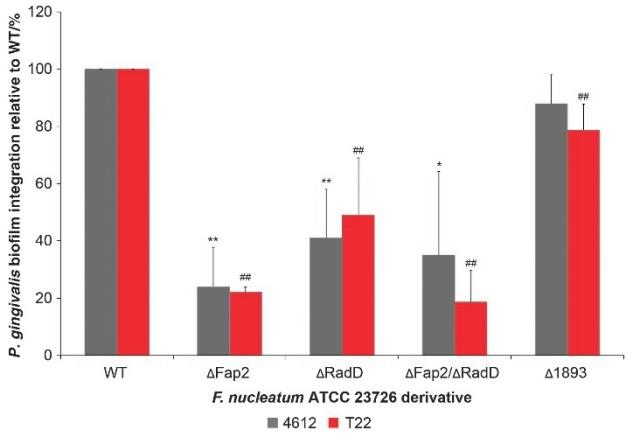

Figure 4 ***Porphyromonas gingivalis*** **integration in dual-species biofilms with** ***Fusobacterium nucleatum*** **ATCC 23726 ΔFap2, ΔRadD and ΔFap2/ΔRadD outer membrane proteins mutant derivatives.** Biofilm integration is given as a percentage relative to biofilm integration measured with WT *F. nucleatum* ATCC 23726. At least three independent experiments were performed per strain combination. Data represent the means and standard deviation of at least three independent experiments. $^{*}P<0.05$; $^{**}P<0.01$; $^{\#\#}P<0.01$. WT, wild type.

observed with the Fap2/RadD double mutant, none of the apparent reductions were significant.

Biofilm integration

Previous studies have shown that the same strains of bacteria grown under biofilms conditions reveal different gene expression and transcriptomic patterns when compared with their planktonically grown counterparts.[43–45] As our coaggregation experiments are typically performed with planktonically grown cells, we wanted to confirm that the differences seen in coaggregation patterns with the *F. nucleatum* OMP mutants is relevant for the integration of *P. gingivalis* strains 4612 and T22 into pre-existing fusobacterial biofilms. *P. gingivalis* strain 33277 was not included in the biofilm studies because coaggregation reactions with *F. nucleatum* OMP mutants did not show significant involvement of these adhesins in this interaction.

Our dual-species biofilm studies were conducted with *P. gingivalis* 4612 and T22 and revealed significant reduction in *P. gingivalis* 4612 integration into a biofilm when grown with *F. nucleatum* derivatives lacking Fap2 (24% ± 14% integration), RadD (41% ± 17% integration), or the Fap2/RadD double mutant (35% ± 29% integration) when compared with biofilms containing wild-type *F. nucleatum*. *P. gingivalis* T22 exhibited a similar pattern of biofilm integration as 4612 with significantly decreased integration when grown with Fap2 (22% ± 2% integration), RadD (49% ± 20% integration) or the Fap2/RadD double mutant (19% ± 11% integration; Figure 4). Biofilm growth with the *F. nucleatum* ATCC 23726 mutant derivative carrying a deletion in OMP FN1893 served as a control to ensure that decreased *P. gingivalis* integration in the dual-species biofilms were mutation specific and not the result of a general biofilm phenotype effect caused by lack of an OMP.

DISCUSSION

In this study, we provide evidence that in addition to the previously described Fap2-mediated interaction between *F. nucleatum* ATCC 23726 and *P. gingivalis* strain PK 1924,[14] multiple adhesins have a role in the attachment of this fusobacterial strain with *P. gingivalis* in a highly strain-dependent manner. The notion of isolate-specific interaction is not new and has been observed for a number of different species pairs including *F. nucleatum* with a variety of *Selemonas*, *Streptococcus* and *Actinomyces* species as well as different isolates of *P. gingivalis* and other oral bacteria.[2,26,46] Other studies describe similar phenomena of strain-specific interactions for *Actinomyces* species with different *Streptococci*[25,28] as well as a number of additional oral bacterial species.[29–30,47] In addition to Fap2, several adhesins involved in some of these interactions have been identified including SspA/B in the binding of *S. gordonii* with *P. gingivalis*[48] and the fusobacterial RadD in the interaction between *F. nucleatum* and *Streptococcal* species as well as *Actinomyces*.[13] However, most studies have only investigated individual strains for each of the species involved and comprehensive studies including multiple isolates are still lacking. Considering the previously observed strain-dependent variations as well as the observation that the inhibition profiles of interactions between species can depend on isolates or serotypes tested, the presence of additional adhesins would be expected.

Quantitative coaggregation studies between *F. nucleatum* ATCC 23726 and different strains of *P. gingivalis* revealed robust coaggregation with strains, 4612, T22 and ATCC 33277, but a relatively weak interaction with strains W50 and W83 (Figure 1). This differential affinity between *F. nucleatum* and the periodontal pathogen *P. gingivalis* could have a role in virulence of oral biofilms. W50 and W83 are highly pathogenic strains of *P. gingivalis*,[49–50] and the type of *F. nucleatum* strain already present in the oral biofilm could determine whether more or less virulent variants of *P. gingivalis* integrate into the biofilm. For example, W83 has been shown to associate with *F. nucleatum* in oral epithelial cell invasion[51] and *F. nucleatum* clinical isolate TDC100 enhanced invasion significantly more than *F. nucleatum* ATCC 25586. Thus, understanding which adhesins are involved in interactions with more virulent pathogens could shed light on the molecular mechanisms of pathogenic biofilm formation.

Similar to previous observations for binding of *F. nucleatum* to *Streptococci*,[52] the interactions between *F. nucleatum* ATCC 23726 and at least two of the three strongly binding strains of *P. gingivalis* is multimodal, as substantial levels of coaggregation remained even in the presence of inhibitors (Figure 2). Consistent with a strain-dependent multi-modal interaction, binding of *F. nucleatum* to *P. gingivalis* was mediated through adhesins sensitive to arginine, galactose and lactose at varying degrees. This was especially interesting because historically, fusobacterial adherence to the predominantly Gram-negative late oral colonizers, including *P. gingivalis*, has largely been associated with galactose-inhibitable interactions, while coaggregation with Gram-positive early colonizing species are suggested to be mediated by arginine-inhibitable interactions.[26] In contrast to the binding characterized between *F. nucleatum* ATCC 23726 and *P. gingivalis* strain PK 1924,[14] which followed this paradigm, the attachment of the same fusobacterial strain to the *P. gingivalis* derivatives tested in this study was only partially sensitive to the addition of galactose (4612 and T22) or not at all (ATCC 33277). Surprisingly, arginine had a stronger effect on the coaggregation with all three *P. gingivalis* strains with the binding to ATCC 33277 being almost completely abolished by the addition of this amino acid (Figure 2c). Lactose-inhibition was observable in coaggregation with all three *P. gingivalis* strains used in this study and were consistent with previous findings that suggest lactose-inhibitable coaggregations may be a common form of interaction among changing populations of bacteria in the shift from health to a state of severe periodontal disease.[2,26]

Screening of the autotransporter large OMP mutant collection from a previous study,[13] revealed that in addition to Fap2, RadD functions as one of the adhesins mediating the binding between *F. nucleatum* ATCC 23726 and *P. gingivalis* 4612 (Figure 3a). This was unexpected because RadD was previously identified as a major arginine-inhibitable adhesin for interactions of *F. nucleatum* with several Gram-positive species.[12–13] Among the OMP mutant collection, only lack of Fap2 resulted in a partial reduction (~50%) of coaggregation with *P. gingivalis* strain T22 indicating that the additional adhesin that contributes to the interaction between these strains constitutes a different type of cell surface feature. This unidentified adhesin is likely to provide the arginine-inhibitable feature of the interaction, as Fap2-mediated adhesion to *P. gingivalis* has been described previously as galactose-inhibitable.[14] Both RadD and Fap2 are multifunctional OMPs that were previously characterized for their role in induction of cell death in human lymphocytes.[24] RadD has additional functions as the above-mentioned arginine-inhibitable adhesin for attachment to Gram-positive early colonizers[12–13] and the newly discovered role as one of the adhesins for attachment to *P. gingivalis* strain T22. Multi-functionality has been described for other large OMPs including TolC of *Escherichia coli*,[53] OprF for *Pseudomonas*[54] and the major OMP of *Campylobacter jejuni*.[55] The arginine-inhibitable adhesin that largely mediates the coaggregation of *F. nucleatum* ATCC 23726 with *P. gingivalis* ATCC 33277, which could also contribute the arginine-sensitive attachment to T22 has yet to be identified in *F. nucleatum*.

Our results for integration of *P. gingivalis* 4612 and T22 into fusobacterial biofilms formed by *F. nucleatum* ATCC 23726 wild-type and its mutant derivative lacking Fap2, RadD or both as well as FN1893 confirmed the importance of Fap2 and RadD for attachment of 4612 to ATCC 23726 (Figure 4). Under biofilm growth conditions RadD may also have a role in binding between *F. nucleatum* ATCC 23726 and *P. gingivalis* T22. This additional function of RadD was not apparent in the interaction with T22 and could be due to differential expression of adhesins in this strain of *P. gingivalis* under biofilm growth conditions. Adhesins are critical virulence factors whose expression are regulated and coordinated to ensure that the necessary adhesin is expressed at the right time.[56–57] When bacterial species have integrated into existing biofilms, significant changes come about compared with their planktonic counterparts including gene expression patterns and physiological properties.[45,58]

In summary, we report that interspecies interactions between *F. nucleatum* and *P. gingivalis* involve a number of different fusobacterial adhesins. The previously characterized Fap2 (Coppenhagen-Glazer, 2015) appears to be a more prominent adhesin that takes part in the attachment to three of the four *P. gingivalis* strains investigated so far for their attachment to *F. nucleatum* on a molecular level. The arginine-inibitable adhesin RadD, which we originally identified as a major adhesin for interaction with streptococci and other early colonizers[12–13] contributes to the attachment to at least one strain of *P. gingivalis* and possibly a second one under biofilm growth conditions. In addition, we provide evidence for the presence of at least one additional adhesin that is sensitive to arginine and is not a member of the autotransporter family type of fusobacterial large OMPs. These findings are consistent with earlier observations of different coaggregation groups for *F. nucleatum* with *P. gingivalis* that vary in their sensitivity to a variety of inhibitors.[26,59] Because certain strain–strain interactions could be more pathogenic than others, we believe that an improved understanding of the array of adhesins involved in interspecies attachment will continue to clarify the role of *F. nucleatum* in health and disease.[39]

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