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Streptococcus mutans SpaP Binds to RadD of *Fusobacterium nucleatum* ssp *polymorphum*

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Summary

Adhesin-mediated bacterial interspecies interactions are important elements in oral biofilm formation. They often occur on a species-specific level, which could determine health- or disease association of a biofilm community. Among the key players involved in these processes are the ubiquitous fusobacteria that have been recognized for their ability to interact with numerous different binding partners. Fusobacterial interactions with *Streptococcus mutans*, an important oral cariogenic pathogen, have previously been described but most studies focused on binding to non-mutans streptococci and specific cognate adhesin pairs remain to be identified. Here, we demonstrated differential binding of oral fusobacteria to *S. mutans*. Screening of existing mutant derivatives indicated SpaP as the major *S. mutans* adhesin specific for binding to *Fusobacterium nucleatum* ssp. *polymorphum* but none of the other oral fusobacteria tested. We inactivated RadD, a known adhesin of *F. nucleatum* ssp. *nucleatum* for interaction with a number of gram-positive species, in *F. nucleatum* ssp. *polymorphum* and used a *Lactococcus lactis* heterologous SpaP expression system to demonstrate SpaP interaction with RadD of *F. nucleatum* ssp. *polymorphum*. This is a novel function for SpaP, which has mainly been characterized as adhesin for binding to host proteins including salivary glycoproteins. In conclusion, we describe an additional role for SpaP as adhesin in interspecies adherence with RadD-SpaP as the interacting adhesin pair for binding between *S. mutans* and *F. nucleatum* ssp. *polymorphum*. Furthermore, *S. mutans* attachment to oral fusobacteria appears to involve species- and subspecies-dependent adhesin interactions.

Keywords

Streptococcus mutans; *Fusobacterium*; adhesin; SpaP; RadD

INTRODUCTION

The bacterial species of the human oral cavity depend on their ability to attach to surfaces or each other for colonization and persistence in this nutritious ecological niche. Consequently, the proteins involved in adherence are important components enabling microorganisms to

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form and reside in complex oral biofilms, in which distinct groups of bacteria perform specific functions (Kolenbrander *et al.* 2010; Rickard *et al.* 2003). While microbial interactions within these biofilms trigger important physiological changes in partner species that influence many properties including virulence features, physical attachment via specific adhesins is key for successful initiation of surface colonization and biofilm integration (Guo *et al.* 2014; Wright *et al.* 2013).

Oral representatives of the ubiquitous fusobacteria have been noted for their binding to a diverse array of microbial species and are considered to be important for biofilm formation and architecture (Kolenbrander *et al.* 1993; Kolenbrander *et al.* 2010; Rickard *et al.* 2003). Fusobacteria enable their own integration into biofilms by adhering to surface-attached early colonizers such as streptococci and actinomyces. Moreover, fusobacteria recruit other bacterial species including important periodontal pathogens that cannot directly attach to surfaces or early colonizers. This feature can promote microbial community shifts and impacts polymicrobial pathogenesis.

Cultivable oral fusobacteria are predominantly comprised of the species *F. periodonticum* and *F. nucleatum* (Potts *et al.* 1983). While *F. periodonticum* contains a single species, *F. nucleatum* includes five subspecies *nucleatum*, *polymorphum*, *fusiforme, animalis,* and *vincentii* (Bolstad *et al.* 1996). Consolidation of the subspecies *fusiforme* and *vincentii* into one group was recently proposed based on their phylogenetic similarities (Kook *et al.* 2013). Fusobacteria not only thrive in subgingival biofilms (Aruni *et al.* 2015), they are present in supragingival plaque (Haffajee *et al.* 2008), predominant in early childhood caries (Corby *et al.* 2005), dentinal and root caries lesions (Lima *et al.* 2011), ecological niches that are also dominated by oral streptococci including the cariogenic species *Streptococcus mutans*. Streptococci are the most prevalent early colonizers and comprise the primary binding partner for recruitment of fusobacteria into oral biofilms (Kolenbrander *et al.* 2010; Rickard *et al.* 2003). While the streptococcal adhesin for interaction with fusobacteria remains to be identified, we previously characterized RadD as a major fusobacterial adhesin in *F. nucleatum* ssp *nucleatum* for the well-established physical attachment to *S. sanguinis* and *S. gordonii* (Kaplan *et al.* 2009). The binding of *F. nucleatum* to *S. mutans*, however, has been demonstrated (Bradshaw *et al.* 1998; Falkler *et al.* 1981) but largely remains to be investigated.

Surface fibrils have been implicated in the interaction of streptococcal species with host proteins, eukaryotic cells and other species including with *F. nucleatum* (Handley *et al.* 1985). Corncob formation of *S. cristatus* with *Corynebacterium matruchotii* as well as *F. nucleatum* was impaired in a mutant strain lacking long fibrils that were suggested to be encoded by the Sortase A (SrtA) recognition consensus containing SrpA protein. Other (SrtA) dependent cell wall-anchored proteins such as SspA/SspB of *S. gordonii* have been implicated in the interactions between oral streptococci and other oral species including *Porphyromonas gingivalis*, actinomyces and *Candida* (Back *et al.* 2015; Brooks *et al.* 1997; Daep *et al.* 2006; Demuth *et al.* 1996; Egland *et al.* 2001; Jakubovics *et al.* 2005). The SspA/SspB homolog of *S. mutans*, SpaP, has been demonstrated to bind to host salivary proteins (Demuth *et al.* 1990; Lee *et al.* 1989), host matrix proteins including type I collagen, fibronectin, laminin, or keratin as well as serum components such as fibrinogen (Beg *et al.*

2002; Busscher *et al.* 2008; Kelemen *et al.* 2004; Kishimoto *et al.* 1989; Petersen *et al.* 2002; Sciotti *et al.* 1997; Soell *et al.* 2010). Recognition of certain actinomyces strains was reported when SpaP is expressed in *S. gordonii* in conjunction with CshA, a protein typically not present in *S. mutans* (Jakubovics *et al.* 2005).

In this study, we characterized the adhesion of oral fusobacterial species to *S. mutans* and identified SpaP as the major adhesin of *S. mutans* that directly interacts with the adhesin RadD of the *F. nucleatum* subspecies *polymorphum* but none of the other oral fusobacteria tested.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

All strains used in this study are listed in Table 1 and were grown as previously described (Jakubovics *et al.* 2005; Kaplan *et al.* 2009; Levesque *et al.* 2005; Zhu *et al.* 2009). In brief, fusobacteria were grown at 37°C on Columbia agar plates supplemented with 5 % sheep blood or in Columbia broth (CB) (Difco, Detroit, MI) under anaerobic conditions (5 % CO_2, 5 % H_2, 90 % N_2). *S. mutans* wildtype and mutant strains were grown in Todd Hewitt (TH) broth (BD Difco, Detroit, MI) at 37°C in the presence of 5% CO_2. The medium was supplemented with 15 μg/mL of erythromycin for growth of the respective *srtA*-, *fruA*-, *wapA*-, and *wapE*-deficient UA140 and UA159 derivatives. The *gbpC*- and *spaP*-deficient *S. mutans* strains were cultured in TH medium supplemented with spectinomycin (500 μg/mL). *Lactococcus lactis* MG1363 containing vector pUB1000 and the corresponding plasmids expressing *spaP* genes from different strains of *S. mutans* (Jakubovics *et al.* 2005) were grown statically in M17 medium supplemented with 0.5% (w/v) glucose and 5 μg/mL of erythromycin at 30°C.

Strain Construction

S. mutans—Genomic DNA was prepared from *S. mutans* UA159 *srtA*-, *fruA*-, *wapA*-, and *wapE*-defective strains (Levesque *et al.* 2005) and *S. mutans* UA159 strain lacking *gbpC* (Zhu *et al.* 2009) using the MasterPure™ DNA purification kit (Epicentre, Madison, WI, USA). Between 1 and 5 μg of this genomic DNA was directly transformed into *S. mutans* strain UA140 via competence-stimulating peptide (CSP)-induced natural transformation to generate the corresponding mutant derivatives in this background. Transformants were selected on TH agar containing 15 μg/mL erythromycin (*srtA*-, *fruA*-, *wapA*-, and *wapE*) or 500 μg/mL spectinomycin (*gbpC*) and confirmed via PCR and DNA sequencing. To generate an insertional gene inactivation mutant derivative of *S. mutans* UA140 in *spaP*, an internal 1035-bp fragment (nucleotide 66 to nucleotide 1100) was amplified with primer pair SPAP-F (CGCGGATCCTCTAGGAACAGTAGCAGCAGTCT) containing a *BamHI* site (underlined) and SPAP-R (CTAGTCTAGATAAGTCGCCTTAGCATTCTCATT) containing a *XbaI* site (underlined) using *Pfu* polymerase (Stratagene) with standard amplification protocols and inserted into suicide vector pFW5 (Podbielski *et al.* 1996). The resulting plasmid pFW5-*spaP* was confirmed by restriction analysis, PCR amplification and DNA sequencing prior to transformation into *S. mutans* UA140 as described above. Transformants

were selected on TH agar containing 500 μg/mL spectinomycin and confirmed by PCR, sequencing and Western Blot (Supplemental Fig. 1).

***F. nucleatum* ssp. *polymorphum*—**A Δ*radD* mutant derivative of *F. nucleatum* ssp. *polymorphum* ATCC10953 was constructed by inactivating the *radD* encoding gene (FNP_1046) via single homologous recombination as described earlier (Kaplan *et al.* 2009). In brief, a 1032 bp internal gene fragment (nucleotide 4931 to nucleotide 5962) was amplified using the primer pair RADD-F (GCGGCTGAATTCCTGGAACAGGAATGTATTTAACAGGTAACAGC) and RADD-R (GCGGAGGGATCCCATTAGCTGCTTTATTATATCCAGATTTTGTATAAATACC) appended with *EcoRI* and *BamHI*, respectively, from genomic DNA of *F. nucleatum* ssp. *polymorphum* ATCC10953 and sub-cloned into the pJET1.2/blunt vector. The resulting plasmid pBS24 was digested with *EcoRI*/*BamHI*, ligated into *EcoRI*/*BamHI* digested pHS31 vector (Kaplan *et al.* 2009) and transformed into *Escherichia coli*. After confirmation of the integration plasmid by restriction analysis and sequencing, the plasmid DNA was electroporated into *F. nucleatum* ssp. *polymorphum* ATCC10953 and plated on selective medium containing 5μg/ml thiamphenicol. The insertional mutant was confirmed via PCR, sequencing.

Coaggregation Assay

Interspecies coaggregation was performed in modified coaggregation buffer (CAB) and quantified as described (Kaplan *et al.* 2009). For measuring coaggregation in the presence of saliva, saliva was collected from several volunteers, pooled and treated according to published procedures (Kitada *et al.* 2012). Briefly, the pooled saliva was clarified by centrifugation at 2100xg for 10 min. The supernatant was then removed and filtered through 0.22um (Millipore) filters. The filter-sterilized saliva was added to a final concentration of 50% to the individual coaggregation partners prior to combining the strains. The coaggregation index (C.I.), representing the coaggregation efficiency between two species, was calculated as follows: $((OD600_{(A)}+OD600_{(B)})/2\text{-}OD600_{(A+B)})/(OD600_{(A)}+OD600_{(B)}/2)$. $OD600_{(A)}$ and $OD600_{(B)}$ represent the optical density of each individual species at 600nm, and $OD600_{(A+B)}$ represents the optical density of the mixture supernatant after 10 min incubation or as indicated if different from the standard procedure.

Biofilm integration assay

Biofilm Growth—Overnight cultures of *S. mutans* were diluted into TH medium containing 25% saliva, 0.5% mannose and 0.5% sucrose to a concentration of 2×10^5 cells/ml and 500 ul each of this suspension was inoculated into wells of a 48 well culture plate prior to incubation under anaerobic conditions (10% H2, 10% CO2, 80% N2) at 37°C for 18 hrs to allow for biofilm formation. The medium was removed and the wells were washed for three times with 1X PBS. Overnight cultures of *F. nucleatum* ssp *polymorphum* were adjusted to 5×10^8 cells in fresh CB containing 50% saliva and 50 ul were added into each well and incubated under anaerobic conditions for 4 hrs prior to harvesting and DNA extraction. Triplicate wells were inoculated for each experiment, which were combined for DNA extraction. At least three biological replicates were performed per condition.

Crystal violet Assay—Biofilm formation of *S. mutans* was evaluated via Crystal Violet (CV) staining according to published procedures (Zmantar *et al.* 2010). In brief, supernatants were removed from each well and rinsed once with 250 μl of sterile phosphate-buffered saline (PBS). Plates were inverted and dried. Next, 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 FisherScientific, Waltham, MA) for 15 min at room temperature. The plates were then carefully rinsed with Millipore water until there was no visible trace of the stain. Bound stain was dissolved by adding 160 μl of 95% ethanol. The optical density (OD) of each well was measured at 570 nm and was represented as relative to negative control wells that only contained TH.

Extraction of DNA from Biofilms—Prior to DNA isolation, the supernatant was carefully removed from each well and the wells were rinsed twice with 1XPBS. Genomic DNA was isolated directly from biofilm cells attached to the wells using QIAamp DNA Micro kit (Qiagen) according to manufacturer's instructions with modification of the final elution to 30 μl. Lysis buffer was directly added to the wells, biofilms were scraped off and added directly into a 0.5 ml screw cap microtube containing 0.1mm silica beads. The samples were treated with bead beating for 30 sec three times at 1 minute intervals. After centrifugation of the samples at 13000xg for 5 min, the supernatant was transferred to a fresh tube and incubated with Proteinase K for 1 hr at 56°C. The samples were treated according to manufacturer's instructions.

Quantitative (Real-Time) Polymerase Chain Reaction (qPCR)—Relative proportions of *F. nucleatum* ssp *polymorphum* integrated into *S. mutans* biofilms were determined by quantitative assessment of DNA with species-specific primer pairs similar to our published procedures (Park *et al.* 2016). Gene specific primer pairs gtfBF 5′ GCCTACAGCTCAGAGATGCTATTC and gtfB R 5′ GCCATACACCACTCATGAATTGA 3′ were used to amplify the *S. mutans*-specific *gtfB* gene, while *fomA* of *F. nucleatum* was amplified with primer pair fomA-F 5′ GTTGCTCCAGCTTGGAGACCAAAT and fomA-R 5′AAGTTTACTTTTGTTAAAGTTTGTAATCTTCC (Park *et al.* 2016) for specific detection of *Fusobacterium nucleatum.* Amplification and signal detection by qPCR on iCycler Thermal Cycler (Bio-Rad, Hercules, CA) was performed in a total volume of 20 μl containing 2 μl of 10× iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), 1ul of 0.5 μM each of forward and reverse primers, 7 μl of Millipore water and 1 μl (10ng) of template in 96-well optical plates (Thermo Fisher Scientific, Waltham, MA). 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 sec; 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 tenfold serial dilutions of bacterial genomic DNA. All standards were run in duplicate to generate a standard curve to determine the efficiency of each primer set. Three independent qPCR runs were performed with three technical replicates for each sample to assess reproducibility and inter-run variability. Relative ratios of the tested species to each other were calculated as previously described (Park *et al.* 2016).

Western Blotting

Whole cell lysates of *S. mutans* UA140 and *ΔspaP* UA140 (1×10^7 cells) midlog phase cultures were obtained by boiling the samples in 1× non-reducing Laemmli buffer (without BME), which were resolved on 4–12% precast gradient gels (NuPAGE™ Novex™). The proteins on the gel were transferred to a nitrocellulose membrane (BIO-RAD) using the Trans-Blot Semi-Dry Electrophoretic Transfer cell (BIO-RAD) at 15V for 1 hr. The blot was then processed according to standard protocols. Monoclonal antibody $4–10A_{8C}$ was used at a 1:100 dilution (Brady *et al.* 1992). Anti-mouse HRP conjugated secondary antibody was detected by using a SuperSignal West Dura Extended Duration Substrate kit (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions.

Statistical Analysis

Statistical significance ($p<0.05$) of differences was evaluated via one-way analysis of variance (ANOVA) with *post hoc* Tukey's test.

RESULTS

S. mutans UA140 Adheres Differentially to Fusobacteria

All experiments in this study were performed with *S. mutans* UA140, since this strain exhibited noticeably better binding to fusobacteria than the *S. mutans* reference strain UA159 (Supplemental Fig. 2). *S. mutans* UA140 adhered to *F. periodonticum* and three of the four *F. nucleatum* subspecies tested (Fig. 1). *F. nucleatum* ssp. *polymorphum* (C.I. = 78±9) and *F. nucleatum* ssp. *animalis* (C.I. = 88±12) exhibited the highest extent of co-aggregation with *S. mutans*, followed by *F. periodonticum* (C.I. = 66±6) and *F. nucleatum* ssp. *nucleatum* (C.I. = 52±9). The *F. nucleatum* subspecies *vincentii* did not display substantial coaggregation with *S. mutans* (C.I. = 26±6) and was therefore not further included in our analysis.

SpaP Acts as *S. mutans* Adhesin for Binding to *F. nucleatum* ssp. *polymorphum*

Many surface proteins in gram-positive microorganism including *S. mutans* depend on sortase A (SrtA) for proper cell wall anchoring and surface display (Marraffini *et al.* 2006; Paterson *et al.* 2004). To investigate if SrtA-dependent proteins play a role in adherence between *S. mutans* and fusobacteria, a *srtA* mutant of *S. mutans* was generated (see Material and Methods for details) and examined for binding to the fusobacterial species and subspecies tested in this study (Fig. 2). *F. nucleatum* ssp. *polymorphum* demonstrated a significant reduction in coaggregation with the *srtA* derivative of *S. mutans* UA140 (C.I. = 18±5; $p<0.01$), while none of the other fusobacteria exhibited any notable difference in adherence. To further investigate which one of the known SrtA-dependent proteins played a role in this process, we generated *spaP-, fruA-, wapA-, wapE-,* and *gbpC*-deficient *S. mutans* derivatives of UA140 for testing of coaggregation with *F. nucleatum* ssp. *polymorphum*. Among these mutant strains, only the *spaP*-deficient strain displayed significantly reduced coaggregation compared to wildtype (Fig. 3). To further confirm SpaP as the *S. mutans* adhesin mediating binding to *F. nucleatum* ssp. *polymorphum*, we employed a previously established system in which streptococcal membrane proteins

including SpaP derived from three different strains of *S. mutans* (*S. mutans* NG8, Ingbritt 162 and Guy's) are functionally displayed on the surface of *L. lactis* (Jakubovics *et al.* 2005). While *F. nucleatum* ssp. *polymorphum* recognized all three SpaP proteins from the different *S. mutans* strains, none of the other oral fusobacterial species did (Fig. 4). Comparison of the SpaP sequences expressed in the *L. lactis* system with those of *S. mutans* UA140 and UA159 revealed that SpaP of UA140 is most similar to SpaP of NG8, while SpaP of UA159 is more closely related to those expressed by *S. mutans* Ingbritt 162 and Guy's (Supplemental Fig. 3). Specifically, SpaP of NG8 and SpaP of UA140 are both lacking alanine-753, which is present in the other SpaP sequences included in the comparison, and contain a five amino acid long insertion after position 797 that is absent in the SpaP proteins encoded by strains UA 159, Ingbritt 162 and Guy's. Furthermore, the *spaP* gene of Ingbritt 162 contains a frameshift mutation that results in a truncation of the protein after position 1214 (Jakubovics *et al.* 2005). This SpaP derivative is still predominantly associated with the membrane despite missing the cell wall anchorage region and part of the C-region.

F. nucleatum ssp. *polymorphum* RadD Recognizes *S. mutans* SpaP

Since we previously found that RadD is a major adhesin for binding of *F. nucleatum* ssp. *nucleatum* to a variety of Gram-positive oral species (Kaplan *et al.* 2009), we generated a *ΔradD* derivative of *F. nucleatum* ssp. *polymorphum* (See Materials and Methods for details) to investigate if this large outer membrane protein is involved in mediating the interaction of this fusobacterial subspecies with *S. mutans*. We found that binding of this *ΔradD* mutant strain to *S. mutans* was completely abolished compared to the interaction displayed by the parent strain (Fig. 5a). The *ΔradD* derivative of *F. nucleatum* ssp. *nucleatum* was also defective in binding to *S. mutans* (Fig. 5a), even though this subspecies of *F. nucleatum* does not recognize SpaP as cognate adhesin (Fig. 4). We then tested attachment of the different SpaP variants expressing *L. lactis* strains used above (Fig. 4) with the *ΔradD* derivative of *F. nucleatum* ssp. *polymorphum* and confirmed that RadD was essential for effective binding to SpaP (Fig. 5b).

Since SpaP has been well established to bind to the salivary agglutinin complex consisting primarily of the high molecular weight scavenger protein gp340 (Ericson *et al.* 1983; Kishimoto *et al.* 1989; Oho *et al.* 1998), we tested if the presence of saliva altered the co-aggregation properties of *S. mutans* and *F. nucleatum* ssp *polymorphum* wildtype and mutant strains (Fig. 6A). Confirming the specificity of the interaction identified here, when SpaP as well as RadD were eliminated co-aggregation of *S. mutans* and *F. nucleatum* ssp *polymorphum* was significantly decreased in both the presence and absence of saliva. The impact of eliminating only a single binding partner was not as apparent in the presence of saliva and may reflect additional molecular interactions involving both salivary and/or bacterial constituents. Although deletion of *spaP* did not significantly decrease the ability of *S. mutans* alone to produce biofilms in the presence of sucrose and saliva (Fig. 6B), incorporation of *F. nucleatum* ssp *polymorphum* into similarly grown *S. mutans* biofilms was significantly decreased when both SpaP and RadD were eliminated (Fig. 6C). Consistent with the co-aggregation results in the presence of saliva, this effect was less apparent when only one of the two binding partners was missing.

DISCUSSION

Fusobacteria establish themselves as prevalent members of oral biofilms via their ability to bind to a large variety of early-colonizing species that attach directly to oral surfaces (Kolenbrander *et al.* 1993; Kolenbrander *et al.* 2010). While research has highlighted the interaction between *F. nucleatum* and non-mutans streptococci (Kaplan *et al.* 2009; Kolenbrander *et al.* 1993), co-adherence with mutans streptococci such as the cariogenic *S. mutans*, has been reported (Bradshaw *et al.* 1998; Falkler *et al.* 1981) but largely remains to be investigated. Here, we evaluated the interaction of *F. periodonticum* and different subspecies of *F. nucleatum* with *S. mutans* and identified RadD and SpaP as the cognate adhesin pair for the specific interaction between *F. nucleatum* ssp. *polymorphum* and *S. mutans*.

Interspecies adherence between oral bacteria is often species- or even strain-dependent (Kolenbrander *et al.* 1993) and the differential binding of the fusobacteria tested in this study to *S. mutans* (Fig. 1) is consistent with previous observations of variability in the attachment of *F. nucleatum* strains to different isolates of *S. mutans* (Falkler *et al.* 1981). Species-specificity is not limited to interbacterial interactions but was also observed for the attachment of *F. nucleatum* subspecies to eukaryotic cells (Xie *et al.* 1991). Furthermore, certain subspecies of *F. nucleatum* colonize distinct ecological niches of the oral cavity (Bolstad *et al.* 1996; Eren *et al.* 2014) and their relative abundance is correlated with health and disease (Uzel *et al.* 2011). Specific interspecies recognition plays an important role in the sophisticated oral biofilm structure, architecture and communication (Guo *et al.* 2014; Kolenbrander *et al.* 2010; Rickard *et al.* 2003). Fusobacteria significantly contribute to this important aspect of oral microbial community formation by connecting early colonizing species with the many pathogenic members containing late colonizers.

Our results suggest that the large outer membrane protein RadD, which we previously identified as the major adhesin for binding of *F. nucleatum* ssp. *nucleatum* to a number of early-colonizing Gram-positive species (Kaplan *et al.* 2009), also functions as adhesin for the interaction with *S. mutans* for at least two subspecies of *F. nucleatum* (Fig. 5A). RadD of *F. periodonticum* and *F. nucleatum* ssp. *animalis* were not tested, because only *F. nucleatum* ssp. *nucleatum* and *F. nucleatum* ssp. *polymorphum* can currently be genetically manipulated. Interestingly, however, only RadD of *F. nucleatum* ssp. *polymorphum* recognizes *S. mutans* SpaP as its cognate counterpart. Despite containing RadD homologues (Supplemental Fig. 4AC) and our finding that the *ΔradD* derivative of *F. nucleatum* ssp. *nucleatum* is deficient in interaction with *S. mutans* (Fig. 5A), none of the other fusobacterial species and subspecies tested bind to *S. mutans* via SpaP (Figs. 3 and 4) or any other SrtA-dependent surface protein (Fig. 2). Thus, in contrast to *F. nucleatum* ssp. *polymorphum*, the RadD-mediated interaction between *S. mutans* and *F. nucleatum* ssp. *nucleatum* appears to involve a binding partner that is not linked to the cell surface by SrtA. This disparity in RadD-specificity could be due to the extensive variability in the N-terminal part of the RadD protein between the *F. nucleatum* subspecies (Supplemental Fig. 4A). Further detailed RadD sequence comparison indicates that a SpaP-binding motif might be located in the highly variable regions between amino acids 285 to 625 or 1716 and 1811, which contain a number of residues that are uniquely present in *F. nucleatum* ssp.

polymorphum (Supplemental Fig. 4B). Alternatively, observed sequence variations might affect the formation or accessibility of a conformational-depending binding site.

SpaP, the cognate *S. mutans* adhesin for *F. nucleatum* ssp. *polymorphum* (Fig. 3) is a SrtA-dependent surface protein of the Antigen I/II family, whose members are highly conserved among most oral streptococci and function as multiligand-binding proteins in eukaryotic cell and protein recognition, surface binding and attachment to other microorganisms (Nobbs *et al.* 2009). Previous studies suggest an important role for SpaP in disease development: Lack of SpaP decreased *S. mutans* cariogenicity in a gnotobiotic rat model (Crowley *et al.* 1999) and a close association between childhood caries prevalence and a high proportion of SpaP in *S. mutans* was reported (Duran-Contreras *et al.* 2011). SpaP has been shown to recognize a number of host ligand proteins, which include salivary proteins, extracellular matrix (ECM) proteins, and certain serum components (Beg *et al.* 2002; Brady *et al.* 2010; Busscher *et al.* 2008; Kelemen *et al.* 2004; Love *et al.* 1997; Nakai *et al.* 1993; Petersen *et al.* 2002; Sciotti *et al.* 1997; Soell *et al.* 2010; Switalski *et al.* 1993). Specifically, the N-terminal alanine-rich region (A-region) as well as parts of the variable region (V-region) mediate binding to the ECM proteins type I collagen, fibronectin, keratin and laminin, while distinct parts of the A-region and the C-terminal region are involved in binding of salivary glycoproteins such as the salivary agglutinin among others.

RadD of *F. nucleatum* ssp *polymorphum* appears to be recognized by parts of SpaP that are not involved in interaction with salivary glycoproteins, since addition of saliva did not interfere with the ability of *S. mutans* to bind to this fusobacterial subspecies between planktonic as well as biofilm cells (Fig. 6). Furthermore, the C-terminally truncated SpaP of *S. mutans* Ingbritt 162 that is lacking the binding site for salivary agglutinin, still binds to *F. nucleatum* ssp *polymorphum* (Figs. 4 and 5). Similarly, lack of the alanine-753 and a five amino acid insertion after position 797 in the SpaP proteins encoded by strains NG8 and UA140, do not seem to be important for RadD recognition, since SpaP of NG8 displays binding comparable to SpaP of Ingritt 162 and Guy's in the *L. lactis* display system (Fig. 4 and Supplemental Fig. 3).

While SpaP was also found to participate in binding to certain actinomyces when expressed in *S. gordonii*, this interaction requires the presence of CshA, a protein typically not present in *S. mutans* (Jakubovics *et al.* 2005). In contrast, SspA and SspB, the SpaP homologues in *S. gordonii*, have been identified as adhesins for a variety of interspecies interactions including *Actinomyces naeslundii* (Egland *et al.* 2001; Jakubovics *et al.* 2005), *Porphyromonas gingivalis* (Brooks *et al.* 1997; Daep *et al.* 2006) and *Candida albicans* (Demuth *et al.* 1996). Distinct binding motifs that are localized in different parts of the molecule mediate adherence to the various species: Adhesion to *A. naeslundii* involves the A-region, which participates in formation of the fibrillar stalk, and binding to *P. gingivalis* has been attributed to the globular domain forming the C-terminal region. Demuth and coworkers (Demuth *et al.* 2001) demonstrated that the corresponding region in SpaP is unable to mediate binding to *P. gingivalis*, suggesting the importance of species-specific sequences for the recognition of interacting partner species. While our comparison of available RadD sequences suggest a potential role of certain highly variable N-terminal regions in SpaP recognition, further studies are needed to confirm this possibility and to

explore if a linear motif of SpaP mediates its selective recognition of *F. nucleatum* ssp. *polymorphum*, or whether the interaction is dependent on a conformational determinant

Our finding, that effective binding of *F. nucleatum* ssp. *polymorphum* to *S. mutans* is mediated by the interaction of the adhesin pair RadD-SpaP adds further functionality and potential virulence attributes to these two multifactorial proteins. Interaction between these two species could broaden their options for integration into the supragingival microbial communities. Since few studies examine oral fusobacterial distribution on a subspecies level (Eren *et al.* 2014; Uzel *et al.* 2011), we can only speculate about the possible biological relevance of the apparent interaction between fusobacteria and *S. mutans*. Stable colonization of the oral cavity by fusobacteria has been demonstrated prior to the eruption of teeth (Kononen 1999), while *S. mutans* is generally considered to require tooth surfaces for efficient establishment, even though it has been detected in edentulous infants prior to tooth eruption (Berkowitz 2006). The ability of *S. mutans* to attach to fusobacteria including *F. nucleatum* ssp. *polymorphum* could thus constitute an additional opportunity for oral biofilm integration of cariogenic *S. mutans*. In contrast, attachment of *F. nucleatum* ssp. *polymorphum* to *S. mutans* in the supragingival plaque could be beneficial, since this fusobacterial subspecies was found to have acid-neutralizing abilities (Takahashi *et al.* 1997).

In summary, most of the predominant oral fusobacterial species and subspecies are able to adhere to the early colonizer *S. mutans* albeit at varying degrees. Furthermore, we identified SpaP of *S. mutans* as a specific adhesin for recognition of the *F. nucleatum* ssp. *polymorphum* adhesin RadD. This is the first time a cognate adhesin pair was identified for attachment of a fusobacterial species to another organism. While *S. mutans* does not interact with a large number of other bacterial species (Wang *et al.* 2011), it is an interesting phenomenon that the ubiquitous fusobacteria, which are known to bind to numerous other microorganisms recognize at least two different surface structures on *S. mutans* for attachment. This could possibly expand their own ability as well as that of *S. mutans* to effectively colonize available oral surfaces.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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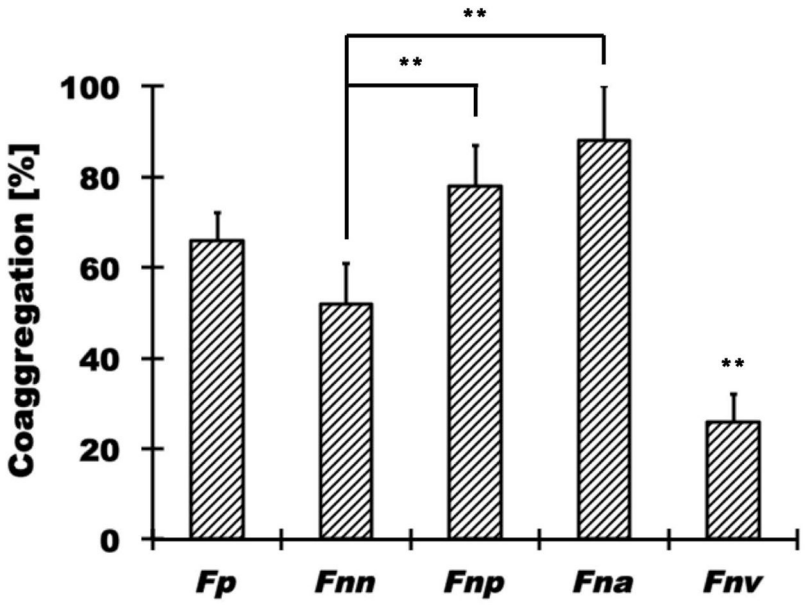

Fig. 1. Quantitative coaggregation assays between *S. mutans* and different oral fusobacteria

The coaggregation of *F. periodonticum* [*Fp*] and several *F. nucleatum* subspecies (ssp. *nucleatum* [*Fnn*], ssp. *polymorphum* [*Fnp*], ssp. *animalis* [*Fna*] and ssp. *vincentii* [*Fnv*]) with *S. mutans* was evaluated in a quantitative coaggregation assay as described in Materials and Methods. *Fnv* coaggregation with *S. mutans* was significantly different (** $p<0.01$) from all other fusobacteria tested, while *Fnn* was significantly different from *Fnp*, *Fna* and *Fnv* but not *Fp*. Values represent the average of at least three independent experiments, and the error bars correspond to the standard deviations.

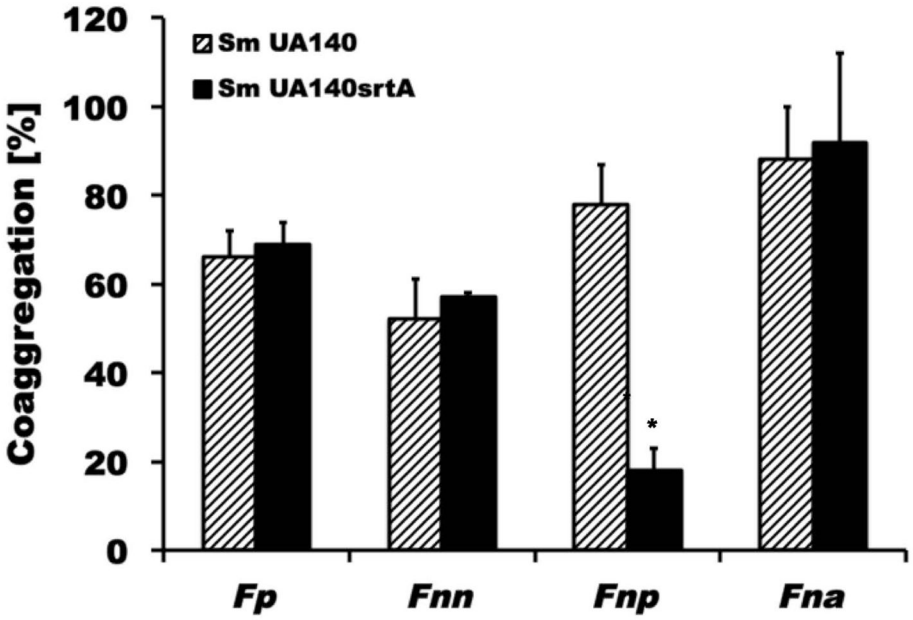

Fig. 2. Quantitative coaggregation assays between *S. mutans* derivatives defective in *srtA* with oral fusobacteria

Coaggregation of different oral fusobacteria with wildtype *S. mutans* UA140 (diagonally striped bars) and the corresponding *srtA* mutant derivative (solid black bars). *Fnp* coaggregation with the *srtA* derivative of *S. mutans* was significantly different (* $p<0.05$) from the interaction with the wildtype parent. Values represent the average of at least three independent experiments, and the error bars correspond to the standard deviations.

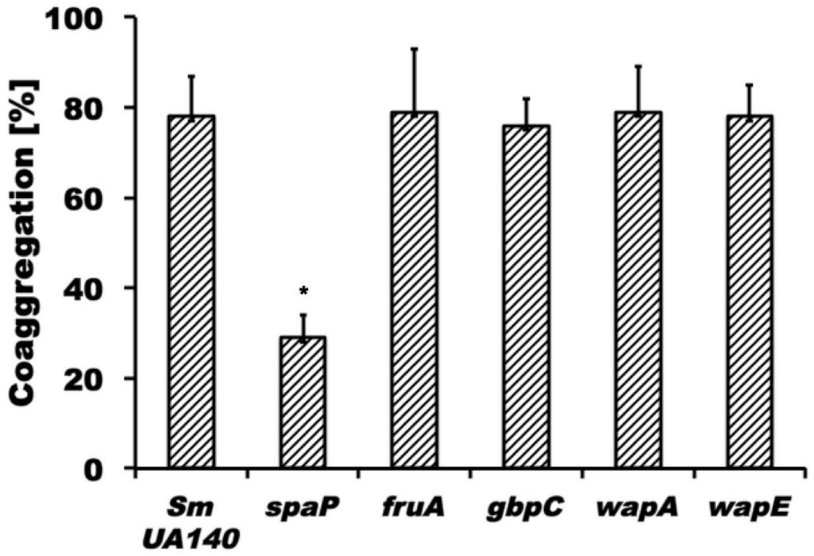

Fig. 3. Quantitative coaggregation assays between *S. mutans* derivatives defective in *srtA* dependent surface proteins with *F. nucleatum* ssp. *polymorphum*

Coaggregation of *F. nucleatum* ssp. *polymorphum* and mutant derivatives of *S. mutans* UA140 defective in *spaP*, *gbpC*, *wapA*, *wapE* or *fruA*. *Fnp* coaggregation with the *spaP* derivative of *S. mutans* was significantly different (* $p<0.05$) from the interaction with the wildtype parent and the other mutant derivatives tested. Values represent the average of at least three independent experiments, and the error bars correspond to the standard deviations.

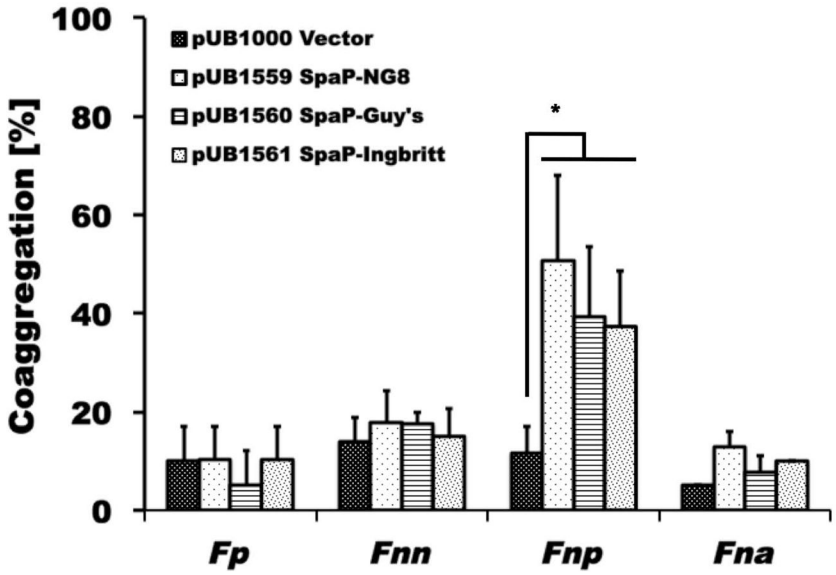

Fig. 4. Quantitative coaggregation of SpaP-producing *L. lactis* strains with different fusobacteria

Quantitative coaggregation of *Fp*, *Fnn*, *Fnp*, and *Fna* with *L. lactis*/pUB1000 (vector – black dotted bars), *L. lactis*/pUB1559 (producing SpaP of *S. mutans* NG8 – white dotted bars), *L. lactis*/pUB1660 (producing SpaP of *S. mutans* Guy's – striped bars), and *L. lactis*/pUB1660 (producing SpaP of *S. mutans* Ingbritt 162 – irregularly dotted bars). Only binding of *Fnp* to the SpaP derivatives expressing *L. lactis* strains was significantly higher (* $p<0.05$) than all other interactions tested. Values represent the average of at least three independent experiments, and the error bars correspond to the standard deviations.

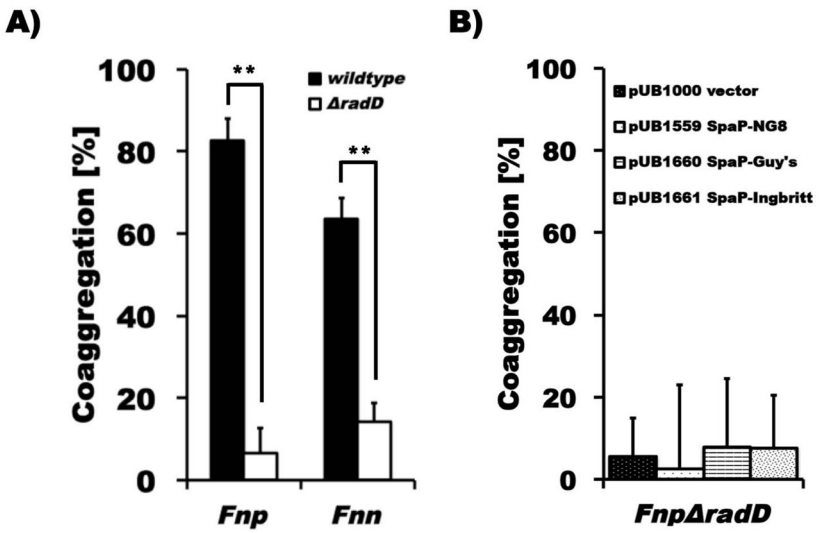

Fig. 5. Quantitative coaggregation *F. nucleatum* ssp. *polymorphum* and *F. nucleatum* ssp. *nucleatum*Δ*radD* derivative with *S. mutans* and SpaP-producing *L. lactis* strains

A) Quantitative coaggregation of *Fnp* (black bars) and *Fnn* (white bars) wildtype and the respective *ΔradD* mutant derivatives, which bind significantly less (** $p<0.01$) to wildtype *S. mutans* strain UA140, and B) *FnpΔradD* with the same SpaP-producing *L. lactis* strains tested in Fig. 4. Values represent the average of at least three independent experiments, and the error bars correspond to the standard deviations.

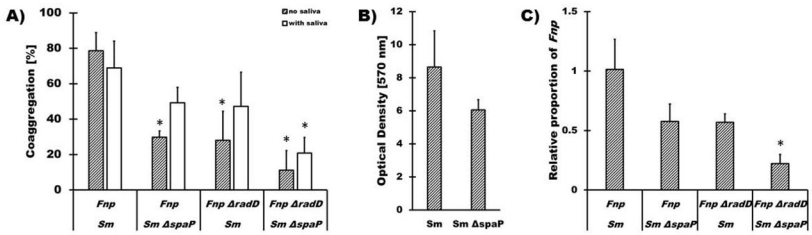

Fig. 6. Quantitative coaggregation and biofilm integration of *F. nucleatum* ssp. *polymorphum* and its*ΔradD* derivative with *S. mutans* and its Δ*spaP* derivative

A) Quantitative coaggregation of *Fnp* wildtype and the respective *ΔradD* mutant derivative with *S. mutans* strain UA140 (*Sm*) and its *ΔspaP* derivative (*SmΔspaP*) in the absence (striped bars) and presence (white bars) of 50% saliva; B) Quantitative crystal violet staining of biofilms formed by *S. mutans* strain UA140 (*Sm*) and its *ΔspaP* derivative (*SmΔspaP*) in the presence of 0.5% sucrose and 25% saliva; C) binding of *Fnp* wildtype and the respective *ΔradD* mutant derivative to *S. mutans* strain UA140 (*Sm*) and the *ΔspaP* derivative (*SmΔspaP*) biofilms in the presence (white bars) of 25% saliva. Significant differences to the respective combination of wildtype strains for each condition are indicated (* $p<0.05$).

Table 1

Bacterial strains and plasmids used in this study

Strain	Description	Antibiotic resistance[a]	Source
Fusobacteria			
ATCC33693	Wildtype *F. periodonticum*		Laboratory collection
ATCC23726	Wildtype *F. nucleatum* ssp *nucleatum*		Laboratory collection
ΔFn1526	ATCC23726 *ΔradD* mutant	Tap	Kaplan *et al.* 2009
ATCC51191	Wildtype *F. nucleatum*, ssp *animalis*		Laboratory collection
ATCC49256	Wildtype *F. nucleatum* ssp. *vincentii*		Laboratory collection
ATCC10953	Wildtype *F. nucleatum* ssp *polymorphum*		Laboratory collection
ATCC10953 *ΔradD*	ATCC10953 *ΔradD* mutant	Tap	This study
Streptococcus mutans			
UA159	Wildtype *S. mutans*		Laboratory collection
UA159 *srtA*	UA159 *srtA* mutant	Erm	Levesque *et al.* 2005
UA159 *fruA*	UA159 *fruA* mutant	Erm	Levesque *et al.* 2005
UA159 *wapA*	UA159 *wapA* mutant	Erm	Levesque *et al.* 2005
UA159 *wapE*	UA159 *wapE* mutant	Erm	Levesque *et al.* 2005
MZ159c	UA159 *gbpC* mutant	Spc	Zhu *et al.* 2009
UA140	Wildtype *S. mutans*		Laboratory collection
UA140 *srtA*	UA140 *srtA* mutant	Erm	This study
UA140 *fruA*	UA140 *fruA* mutant	Erm	This study
UA140 *wapA*	UA140 *wapA* mutant	Erm	This study
UA140 *wapE*	UA140 *wapE* mutant	Erm	This study
UA140 *gbpC*	UA140 *gbpC* mutant	Spc	This study
UA140 *spaP*	UA140 *spaP* mutant	Spc	This study
Lactococcus lactis			
MG1363	Wildtype *L. lactis*		Jakubovics *et al.* 2005
Plasmids			
pFW5	*S. mutans* gene inactivation vector	Spc	Podbielski *et al.* 1996
pFW5-*spaP*	pFW5 carrying *spaP66–1100*	Spc	This study
pJET1.2/blunt	PCR fragment cloning vector	Amp	
pHS31	*F. nucleatum* gene inactivation vector	Tap	Kaplan *et al.* 2009
pBS24	pHS31 carrying *radD4931–5962*	Tap	This study
pUB1000	*L. lactis* expression vector	Erm	Jakubovics *et al.* 2005
pUB1559	pUB1000 *pac-S. mutans* NG8	Erm	Jakubovics *et al.* 2005
pUB1660	pUB1000 *spaP-S. mutans* Guy's	Erm	Jakubovics *et al.* 2005
pUB1661	pUB1000 *spaP-S. mutans* Ingbritt 162	Erm	Jakubovics *et al.* 2005

[a]Amp – Ampicillin (50μg/ml); Erm – erythromycin (15μg/ml for *S. mutans*, 5μg/ml for *L. lactis*); Spc –spectinomycin (500μg/ml); Tap – Thiamphenicol (5μg/ml)