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Characterization of *aid1*, a novel gene involved in *Fusobacterium nucleatum* interspecies interactions

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Abstract

The oral opportunistic pathogen *Fusobacterium nucleatum* is known to interact with a large number of different bacterial species residing in the oral cavity. It adheres to a variety of Gram-positive bacteria, including oral streptococci via the arginine-inhibitable adhesin RadD. In this study, we describe a novel protein encoded by the predicted open reading frame FN1253 that appears to play a role in interspecies interactions of *F. nucleatum*, particularly with oral streptococci and related Gram-positive species. We designated FN1253 as *aid1* (Adherence Inducing Determinant 1). Expression analyses demonstrated that this gene was induced in *F. nucleatum* single species biofilms, while the presence of representative members of the oral microbiota known to adhere to *F. nucleatum* triggered its suppression. Inactivation as well as overexpression of *aid1* affected the ability of *F. nucleatum* to coaggregate with oral streptococci and the closely related *Enterococcus faecalis*, but not other Gram-positive oral species tested. Furthermore, overexpression of *aid1* led to a drastic change in the structure of dual species biofilms of *F. nucleatum* with oral streptococci. Aid1 function was abolished in the presence of arginine and found to be dependent on RadD. Interestingly, differential expression of *aid1* did not affect mRNA and protein levels of RadD. These findings indicate that RadD-mediated adhesion to oral streptococci involves more complex cellular processes than the simple interaction of adhesins on the surface of partner strains. Aid1 could potentially play an important role in facilitating RadD-mediated interaction with oral streptococci by increasing binding specificity of *F. nucleatum* to other microbial species.

Keywords

F. nucleatum; oral streptococci; interspecies interaction; RadD

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Introduction

The oral cavity is a great model system for studying polymicrobial interactions since it is home to over 600 different recognized species of bacteria [1] most of which are considered to be commensal [2]. The microorganisms in the oral biofilm have been categorized into early and late colonizers [3]. Early-colonizing species are primarily Gram-positive, able to adhere directly to the tooth surface and form the basal layers of the oral biofilm. Late colonizers are comprised of mostly Gram-negative bacteria, including certain periodontal pathogens such as *Treponema denticola*, *Tannerella forsythia* and *Porphyromonas gingivalis* as well as others. Bacteria within the oral biofilm, also known as the dental plaque, form a complex network of direct and indirect interactions. The spatial distribution of different bacterial species is important in oral biofilm formation and architecture. Many of the known oral bacterial species do not directly adhere to one another; instead they interact indirectly via their mutual attachment to *Fusobacterium nucleatum* [4]. *F. nucleatum* is a Gram-negative, anaerobic fusiform bacterium that has been associated with periodontal disease and a number of systemic diseases [5–11]. It is considered a “bridging organism” due to its ability to form a “colonization bridge” between species that do not directly interact, thus playing an integral role in the formation of a mature dental plaque. The physical attachment between interacting partner species is mediated by specific cellular adhesion proteins localized on their outer membranes. To characterize these important surface features in *F. nucleatum* on a molecular level, we employed a genetic system that was previously established in our laboratory and lead to discovery of the large outer membrane autotransporter protein RadD, which is required for effective binding to early colonizers [12]. In order to investigate the transcriptional responses of *F. nucleatum* upon interactions with other species, we conducted microarray analysis of *F. nucleatum* grown in the presence of representatives from both early and late colonizing species [13]. These microarray data revealed that a small hypothetical protein encoded by FN1253 according to annotation of *F. nucleatum* ATCC 25586 [14] is induced in *F. nucleatum* single species biofilms but ubiquitously repressed in the presence of both early and late colonizers. Downregulation of this gene in dual species biofilms was more pronounced upon interaction with early colonizing streptococci.

FN1253 homologues are highly conserved across all fusobacterial species sequenced to date and, with no homologues found in other species for which genome sequences are available, it appears to be unique to fusobacteria. In this study, we investigated the role of FN1253 in microbial interactions involving *F. nucleatum.* We demonstrated that FN1253, which we denoted as *aid1* (Adherence Inducing Determinant gene 1), plays a role in interaction of *F. nucleatum* with oral streptococci. Aid1 function requires the presence of the previously identified adhesin RadD [12]. To the best of our knowledge, this is the first hypothetical protein in the *F. nucleatum* genome that has been characterized thus far with a role in interspecies interactions.

Materials and Methods

Bacterial strains and culture conditions

F. nucleatum strains were grown on Columbia agar plates supplemented with 5% sheep blood or in Columbia broth (Difco, Detroit, MI) under anaerobic conditions (5% CO_2, 5% H_2, 90% N_2). Thiamphenicol (MP Biomedicals, Irvine, CA) at 5μg/ml was used for selection and maintenance of strains containing the *catP* determinant. Clindamycin (MP Biomedicals, Irvine, CA) at 0.4μg/ml was used for selection and maintenance of strains possessing the *ermB* determinant. *Streptococcus sanguinis* ATCC 10556 and *S. gordonii* ATCC 10558 were grown anaerobically in Todd-Hewitt (TH) broth (BD Difco, Detroit, MI) at 37°C. *Enterococcus faecalis* ATCC 19433 was grown aerobically at 37°C with shaking in Brain Heart Infusion (BHI) (BD Difco, Sparks, MD) broth. *Lactobacillus casei* ATCC 393 was grown aerobically in the presence of 5% CO_2 in Luria Berthani (LB) (BD Difco, Sparks, MD) broth supplemented with 1% yeast extract. *Staphylococcus epidermidis* ATCC 12228 was grown aerobically at 37°C with shaking at 250rpm in Tryptic Soy Broth (TSB) (BD Difco, Sparks, MD). *Veillonella atypica* PK1910 was grown anaerobically in TH broth (BD Difco, Detroit, MI) supplemented with 0.06% lactic acid at 37°C. *Porphyromonas gingivalis* W50 was grown in Columbia broth anaerobically at 37°C. *Treponema denticola* ATCC 35405 was grown in TYGVS anaerobically at 37°C as previously described [15]. *Tannerella forsythia* ATCC 43037 was grown in NAM medium (ATCC) anaerobically at 37°C.

Mutant construction

In this study we used *F. nucleatum* ATCC 23726, which in contrast to ATCC 25586 can be genetically modified [16]. FN1253 is annotated as HMPREF0397_0433 (Genbank ID: ADVK00000000.1, NCBI BioProject Accession: PRJNA31471 ID: 31471) in *F. nucleatum* ATCC 23726. The Δ*aid1* inactivation strain was constructed by double homologous recombination (Supplemental Figure 1A). A sequence including *aid1* and ~600–700bp flanking regions (that contain only truncated portions of the respective upstream and downstream genes) was amplified from wild type *F. nucleatum* ATCC23726 strain with *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) using forward (5′-TTTATTAAAACTTATGGGAGATAGATA-3′) and reverse (5′-TCCAGAAGGAAAACAACCATCA-3′) primers and subcloned into the pJET 2.1 cloning vector (Fermentas, Inc, Glen Burnie, MD, USA) to obtain construct pIP-*aid1* (Supplemental Figure 1A). The *catP* gene was amplified from plasmid pHS31 [16] using forward (5′-GTCACAGGATCCCAGTCGAAGTGGGCAAGT-3′) and reverse (5′-GTCACCGGATCCCCGTATTTCTACGATGTTTTTGC-3′) primers and subcloned into the pJET 2.1 vector. *catP* was excised with BamHI and ligated into a pIP-*aid1* digested with BamHI resulting in an insertion at nucleotide 38 within the *aid1* gene. The resulting plasmid pIP-*aid1::catP* was verified by restriction analysis and PCR. The plasmid was linearized with ScaI prior to transformation into *F. nucleatum.* For overexpression of *aid1* in *F. nucleatum* we constructed plasmid pEP-*aid1* as follows. The fragment carrying *aid1* including the upstream and downstream regions described above was excised from pIP-*aid1* using XbaI and XhoI and ligated into linearized pHS58 shuttle plasmid (Haake, personal communication) that carries an *ermB* erythromycin/clindamycin resistance cassette to

generate the pEP-*aid1* expression vector. Plasmid pEP-*aid1* was transformed into wildtype *F. nucleatum* strain ATCC 23726 as well as its derivative lacking *radD* according to previously described protocols [17] to yield *Fn/*pEP-*aid1* and Δ*radD/*pEP-*aid1*, respectively.

Transcriptional Analysis

Genomic DNA was extracted from stationary-phase cells following standard protocols and used for generating reference standard curves. Total RNA was extracted using High-Pure RNA Isolation Kit (Roche, Palo Alto, CA, USA) according to manufacturer's instructions. 2μg of total RNA was used for cDNA synthesis using Transcriptor Universal cDNA Master (Roche, Palo Alto, CA, USA) following the manufacturer's protocol. For qRT-PCR, SYBR green (Bio-Rad, Hercules, CA, USA) was used for fluorescence detection with the iCycler real-time PCR system (Bio-Rad), according to the manufacturer's instructions. *aid1* cDNA was amplified using 5′-TACAGGAGGTGCCGTAGCAG-3′ forward and 5′-TTTTTGTTAATTCTCCAGCTCCA-3′ reverse primers. Expression levels of 16S rRNA were determined using 5′-TTGGACAATGGACCGAGAGT-3′ forward and 5′-GCCGTCACTTCTTCTGTTGG-3′ reverse primers for normalization of the qRT-PCR data.

Coaggregation assay

Coaggregation assays were performed in coaggregation buffer (CAB) 150mM NaCl, 1mM Tris, 0.1mM $CaCl_2$, 0.1mM $MgCl_2$ [18]. Briefly, the cells were collected, washed and resuspended in phosphate-buffered saline (PBS). Equal numbers of bacterial cells were diluted in CAB or 25% pooled saliva in ddH_2O to a final concentration of 2×10^9 cell ml^{-1} in a 200μL clear PCR reaction tube. The tubes were vortexed for 5s and graded on a 0–4 scale after 10 min based on degree of coaggregation [19]. Scores were assigned as follows: 0 - no visible coaggregation; 1 - small aggregates that stay suspended; 2 - larger aggregates that settle slowly and leave the supernatant turbid; 3 - large aggregates that settle quickly but leave the supernatant turbid; 4 - complete sedimentation with a clear supernatant. No autoaggregation of individual strains was observed in our experimental controls. For coaggregation inhibition assays, 50mM final concentration of either D-galactose, L-arginine, D-glucose or N-acetyl galactosamine were added to the reaction tube containing the *F. nucleatum* strain and mixed by vortexing before addition of the coaggregation partner strain.

Spectrophotometric coaggregation assay

Spectrophotometric co-aggregation experiments were performed according to our published procedures [12]. Briefly, the cells were combined in CAB as described above and after 10 min of incubation, the co-aggregation reactions were centrifuged at low speed (100 *g*) for 1min to pellet co-aggregating cells while leaving the non-aggregated bacteria in suspension. The supernatant was then removed without disturbing the pellet, and the optical density of the recovered supernatant was measured at 600 nm. Relative co-aggregation of species A and B was determined by dividing the difference between the total turbidity of each partner strain and the coaggregation supernatant turbidity by the total turbidity of each partner strain using the $\{[OD_{600}(A)+OD_{600}(B)]-OD_{600}(A+B)\} / [OD_{600}(A)+OD_{600}(B)]$ formula.

Biofilm growth

Biofilms were grown in eight-well chambered coverglasses (Nunc, Rochester, NY) pre-coated with 100μl of 50% saliva diluted in ddH_2O that was centrifuged for 5 min at 10,000 x *g* to remove debris. The chambers were UV sterilized for 1hr before inoculation. Overnight cultures of *F. nucleatum* (~10^7 cells), *S. sanguinis* and *S. gordonii* (~10^5 cells) were inoculated into the growth chamber wells containing 400μl of filter-sterilized BHI saliva broth (BHIS: 25% BHI and 25% saliva). Samples were incubated overnight under anaerobic conditions (5% H_2, 5% CO_2, 90% N_2) at 37°C. After 22–24hrs samples were fluorescently labeled with the nucleic acid staining dye SYTO9 (Invitrogen, Carlsbad, CA) and visualized using a PASCAL 5 confocal laser scanning microscope (CLSM) (Zeiss, Jena, Germany). The scanning module of the system was mounted onto an inverted microscope (Axiovert 200M) and samples were viewed through a 40x oil-immersion objective (Achroplan/N.A. 1.3). Excitation of 488nm with an argon laser in combination with a 505–530nm bandpass emission filter was used for SYTO9 fluorescence imaging.

Statistical analysis

Student's *t*-test was performed using Excel 2010 (Microsoft, Seattle, WA)

Results

Confirmation of the *aid1* gene expression profile

Previous microarray analysis in *F. nucleatum* strain ATCC 23726 revealed a unique expression pattern for an ORF that corresponds to FN1253 in the published genome of *F. nucleatum* strain ATCC 25586 [13]. *F. nucleatum* ATCC 23726 was used for further characterization since it can be genetically manipulated [16]. Homology searches with the recently published draft genome of *F. nucleatum* ATCC 23726 confirmed the presence of the corresponding ORF (HMPREF0397_0433) in this strain, albeit the original annotation indicated a truncated version of the protein. Analysis of flanking sequences revealed that HMPREF0397_0433 exhibits 100% homology with FN1253 indicating a mis-annotation in the ATCC 25586 genome, where the protein is missing the N-terminal portion.

Expression of FN1253 was induced in single species biofilms, and repressed in the presence of representative species of both early and late colonizers [13]. Conditions tested included dual species biofilms of *F. nucleatum* ATCC 23726 with *S. sanguinis* ATCC 10556 and *S. gordonii* ATCC 10558 representing early colonizing streptococci, as well as *T. denticola* ATCC 35405 and *T. forsythia* ATCC 43037 as representatives of Gram-negative late colonizing species. The microarray data were validated via quantitative real-time PCR (qRT-PCR) with *aid1*-specific primers, which confirmed the differential regulation of *aid1* in single and dual species biofilms (Figure 1). Repression of *aid1* was most pronounced upon interaction with the streptococci.

Repression of *aid1* is time- and contact-dependent

Next, to determine whether physical interaction of *F. nucleatum* with streptococci plays a role in regulation of *aid1*, we analyzed the *aid1* mRNA levels in mixed culture. Cells were mixed together with and without arginine, a known inhibitor of interactions of *F. nucleatum*

with oral streptococci [12], and spun down into a pellet, since only few *F. nucleatum* cells start forming biofilms at early time points. qRT-PCR revealed a small decrease in *aid1* expression after 1hr incubation (Figure 2). At the 20hr time point the expression of *aid1* was significantly decreased. When 50mM arginine was added to the cell mixture there was no significant gene regulation present at both time points. Neither co-culture with *S. sanguinis* nor the addition of arginine affected the growth rate of *F. nucleatum.* Since fusobacterial interactions with streptococci are predominantly mediated by RadD, we performed similar analysis with a *F. nucleatum* mutant strain lacking this major adhesin. However, inactivation of *radD* did not have an effect on *aid1* levels, while addition of 50mM of arginine to the mixture blocked repression of *aid1* in a manner comparable to the wildtype parent.

Construction and basic characterization of *aid1* gene inactivation and overexpression derivatives

In order to characterize *aid1* on a molecular level, we constructed a gene inactivation mutant as well as a strain overexpressing the gene in wild type *F. nucleatum* ATCC 23726. Multiple attempts to obtain an inactivation mutant in which the gene was fully deleted were unsuccessful. As an alternative, we disrupted *aid1* by inserting a chloramphenicol/ thiamphenicol resistance cassette (*catP*) 38 nucleotides downstream of the gene start site. The mutation was introduced into the *F. nucleatum* wild type ATCC 23726 strain via double homologous recombination of a construct carrying the *catP* gene and the corresponding chromosomal regions (*aid1::catP*) (Supplemental Figure 1A). This results in a non-functional truncated and out-of-frame final gene product. The overexpression mutant was constructed by introducing an expression vector carrying *aid1* under its own endogenous promoter (pEP-*aid1*) into *F. nucleatum* ATCC 23726 (Supplemental Figure 1B). The respective mutant derivatives were selected with appropriate antibiotics, thiamphenicol for the inactivation mutant and clindamycin for the overexpression strain. Inactivation of *aid1* was confirmed by PCR (Figure 3A). Both mutants had growth rates comparable to the wild-type strain (data not shown). Expression levels of *aid1* were examined by qRT-PCR and the overexpression strain was found to produce approximately three times more *aid1* mRNA than the wild type parent strain (Figure 3B).

aid1 modulates the coaggregation ability of *F. nucleatum* with oral streptococci and related species in a RadD-dependent manner

The *aid1* encoding gene is noticeably repressed in the presence of oral streptococci and Gram-negative late colonizers. We therefore investigated the involvement of *aid1* in the ability of *F. nucleatum* to coaggregate with a wide array of oral bacterial species. Since *F. nucleatum* does not autoaggregate using standard protocols, we first tested if lack or overexpression of *aid1* would alter this behavior and found that this is not the case (Table 1). We then investigated the ability of the *aid1* mutants to interact with other species using a standard coaggregation assay [18]. Equal numbers of cells were added into a tube with coaggregation buffer (CAB) and coaggregation was scored on a 0–4 scale after 10 mins as described in the Experimental Procedures section. The *aid1* inactivation strain displayed a reduced ability to aggregate with oral streptococci as well as closely related *Enterococcus faecalis* while its ability to coaggregate with other Gram-positive and Gram-negative species

was not affected. The *F. nucleatum* strain overexpressing *aid1* exhibited an increased aggregation ability with oral streptococci, a feature that was not apparent during interaction with other Gram-positive and Gram-negative oral species (Table 1). Based on this data FN1253 was designated as *aid1* (Adherence Inducing Determinant gene 1). To further characterize the nature of this enhanced interaction with oral streptococci, we performed coaggregation assays with *S. sanguinis* as representative species in the presence of known inhibitors of fusobacterial interactions with other species as well as carbohydrates that generally do not interfere with coaggregation [20]. Enhanced coaggregation of the *aid1*-overexpressing mutant derivative with streptococci was only blocked by arginine (Table 2).

We reported previously that an arginine-inhibitable adhesion RadD plays an important role in mediating the coaggregation between *F. nucleatum* and a variety of Gram-positive oral bacteria, including oral streptococci [12]. To investigate whether Aid1 requires RadD for its function, we introduced the pEP-*aid1* plasmid into a *radD* mutant derivative of ATCC 23726, which is unable to coaggregate and form biofilms with oral streptococci [12]. Despite overexpression of *aid1*, lack of *radD* still abolished coaggregation of *F. nucleatum* with oral streptococci (Table 1). All coaggregation data were quantified by performing a spectrophotometric coaggregation assay, which verified the differences in coaggregation under the various conditions tested in the visual coaggregation assay (Figure 4).

aid1 overexpression enhances the ability of *F. nucleatum* to form biofilms with oral streptococci and alters biofilm morphology

Since *aid1* levels are significantly decreased after 20hrs of co-incubation with *S. sanguinis*, we investigated the ability of *aid1* mutant derivatives to form biofilms with *S. sanguinis*. Lack of *aid1* did not affect the ability of *F. nucleatum* to form dual species biofilms with *S. sanguinis.* The biofilms formed with the *aid1* inactivation mutant were indistinguishable from the ones formed by *S. sanguinis* and wild type *F. nucleatum*. In contrast, biofilms produced by the *aid1* overexpression mutant in the presence of *S. sanguinis* were consistently taller and morphologically different. Streptococcal cells were more evenly distributed throughout the height of the dual-species biofilm, as opposed to localizing closer to the bottom of the biofilm when grown with wildtype *F. nucleatum.* (Figure 5).

Discussion

In this study, we discovered that a small conserved hypothetical protein in *F. nucleatum* ATCC 23726, which we designated as Aid1 (Adhesion Inducing Determinant 1) plays a role in fusobacterial interspecies interactions and biofilm formation. According to the corresponding *F. nucleatum* ATCC 25586 annotation [14], Aid1 is encoded by a homolog of ORF FN1253. This ORF first caught our attention during microarray analysis, since it was significantly induced in *F. nucleatum* mono-species biofilms and repressed in the presence of other oral bacterial species [13]. We confirmed the expression pattern of *aid1* by qRT-PCR and demonstrated a much greater reduction of gene expression in the presence of the Gram-positive oral streptococci compared to Gram-negative species (Figure 1). Neither inactivation nor overexpression of *aid1* had any discernable effect on general cell physiology, appearance, overall membrane composition or single species biofilm growth

(data not shown), and interaction with Gram-negative species (Table 1). Aid1-dependent differences in coaggregation appear to be limited to oral streptococci and closely related species, since coaggregation of the *F. nucleatum aid1* mutant derivatives with more distantly related Gram-positive species such as *Lactobacillus casei* and *Staphylococcus epidermidis* was indistinguishable from the wildtype parent behavior. This apparent differential effect of Aid1 on binding to partner species was especially intriguing considering that *F. nucleatum* interacts with numerous oral bacterial species and serves as a colonization bridge between species that cannot attach to each other [3,21]. This unique characteristic of fusobacteria plays a major role in the formation and architecture of oral biofilms and fusobacterial interactions with other microorganisms have previously been characterized by specific inhibition with a variety of amino acids and carbohydrates [20,19]. Futhermore, we recently identified the large outer membrane protein RadD as the major adhesin for interactions with a number of Gram-positive oral bacterial species including oral streptococci, and demonstrated that lack of this large outer membrane protein disrupts dual-species biofilm architecture [12]. In this study we found that Aid1-mediated effects are dependent on the presence of RadD (Table 1).

Investigation of Aid1, the novel protein that appears to modulate in particular fusobacterial interactions with oral streptococci and the closely related *E. faecalis*, led to the hypothesis that interspecies interactions of *F. nucleatum* are more complex than the simple binding of a fusobacterial surface adhesin with the corresponding adhesin on its partner species. Specifically, we propose that this important bridging organism distinguishes its binding partners by employing large outer membrane proteins for general attachment to larger groups of bacteria and fine-tuning these interaction by providing binding specificity via differential expression of small membrane-associated proteins such as Aid1. The idea that *F. nucleatum* can distinguish its binding partners is also supported by previous work on the inhibition of coaggregation, which demonstrated that *F. nucleatum* interacts with oral streptococci in a multimodal manner since different combinations of inhibitors were necessary to block these interactions [22].

Even though the presence of RadD is required for Aid1 function both proteins overlap in their involvement in the same process on inter-cellular interactions, their gene expression is independent of each other. Increased abundance of *aid1* appears to induce the ability of *F. nucleatum* to bind to oral streptococci without significantly increasing *radD* mRNA or protein levels (data not shown). Furthermore, expression of *aid1* is regulated in a contact- and time-dependent manner that is RadD-independent (Figure 2). Addition of arginine completely blocks all cellular interactions between *F. nucleatum* and *S. sanguinis* (Table 2, Figure 4) even when cells are coincubated in pellet form and abolishes the typical dramatic decrease of *aid1* mRNA in the presence of this partner species (Figure 2), which suggests that cellular contact is required for activation of the signaling pathway resulting in regulation of the *aid1* gene. At the same time, inactivation of RadD, while still dramatic, does not completely eliminate coaggregation between *F. nucleatum* and *S. sanguinis*. This observation is more obvious in the more sensitive spectrophotometric coaggregation assay (Figure 4). Our data suggest that the reduced cell-cell contact in the absence of RadD is still sufficient to trigger the signaling cascade leading to repression of *aid1*, while addition of

arginine completely blocks all interactions and thus the downstream transcriptional effects. These findings further support that RadD and Aid1 act independently of each other but are involved in the same intracellular interaction process. The *aid1* gene seems to be required for initial interaction with oral streptococci, since the Δ*aid1* mutant displays decreased coaggregation with *S. sanguinis* ability (Figure 4, Table 1). Downregulation of *aid1* occurs over time and at the time of initial contact with *S. sanguinis* the protein is still present in its unchanged amounts in the cell, therefore in the coaggregation assay lack of Aid1 leads to reduction in binding ability. Based on our data Aid1 appears to allow *F. nucleatum* cells to distinguish between different Gram-positive species. RadD, while being the primary adhesin, does not possess the necessary specificity to distinguish between different Gram-positive species including different streptococci and the interactions rely on additional proteins that facilitate cell-cell recognition and further attachment.

The interactions between different bacterial species are important in the formation and maintenance of oral biofilms. The oral biofilm is a structured bacterial community of cells growing attached directly to tooth and tissue surfaces [23]. Within the oral biofilm architecture each species occupies a certain niche, which is crucial in the formation and maintenance of the mature plaque. The importance of the spatial distribution of the different species has been described both *in vitro* [24] and more recently *in vivo* [25]. Gram-positive species, primarily streptococci, usually occupy the basal layers of the biofilm and are able to directly attach to the tooth surface, while *F. nucleatum* is distributed throughout the biofilm, providing a scaffold by binding different species. To date only the fusobacterial adhesin RadD has been shown to play a role in the formation of a dual species biofilm between *F. nucleatum* and *S. sanguinis* [12]. Our data indicate that *aid1* also appears to play a role in dual-species biofilm architecture of *F. nucleatum* and *S. sanguinis.* The observed spatial distribution of *S. sangunis* in a dual species biofilm with *F. nucleatum* strain overexpressing *aid1* (Figure 5) suggests that the downregulation of *aid1* is necessary for the normal formation of the biofilm involving *F. nucleatum* and oral streptococci and overexpressing the gene disrupts this natural architecture of the oral biofilm and alters the proper distribution of the streptococcal species. This can have potential detrimental effects on the downstream maturation of the biofilm by not allowing other species, primarily late colonizers, to adhere and integrate into the growing plaque. Inactivation of *aid1* had no significant effect on biofilm formation and structure, which is consistent with its role in interactions with oral streptococci and the repression of the gene in the presence of species belonging to this genus.

The *aid1* gene is predicted to encode a small hypothetical protein comprised of 67 amino acids. BLAST searches against available databases revealed that it is highly conserved across oral fusobacterial species, while no significant homology to any gene within other species sequenced to date was apparent. Protein analysis tools such as InterProScan (version 4.8), PredictProtein and SABLE protein structure prediction server predict that Aid1 is membrane-associated based on the presence of a predicted short N-terminal signal sequence, short helices that are indicative of interaction with the cellular membrane as well as a lipoprotein motif at the N-terminal end of the protein followed by a glycine zipper motif. Glycine zipper sequences are common motifs in membrane proteins and are usually found in

transmembrane domains [26]. These domains are also thought to be involved in protein oligomerization [27,28], that may allow Aid1 to form a larger protein complex on the bacterial membrane. While Aid1 is the first small hypothetical protein being characterized in *F. nucleatum* interspecies interactions, another small fusobacterial membrane protein FadA has been previously identified and characterized for its ability to attach to eukaryotic cells [29]. FadA has been shown to oligomerize and thus form a large protein complex via leucine zipper motifs that allows it to act as an adhesin required in attachment and invasion of epithelial cells [30].

In summary, the nature of bacterial interactions involving *F. nucleatum* appears to be more complex than the contact between individual adhesins on the cell surfaces of the partner species. It appears that small proteins like Aid1 are able to define and fine-tune the specificity of large adhesins such as RadD. To best of our knowledge *aid1* is currently the first hypothetical fusobacterial protein identified to have a specific role in interspecies interactions of this important bridging organism with other species in the oral cavity. It appears to be required by *F. nucleatum* for recognition and discrimination for Gram-positive oral bacterial species. This study sheds light on the complexity of bacterial interactions in the oral cavity, suggesting that most of these interactions involve more than one adhesin. Bacteria in the oral cavity need mechanisms to identify and select their binding partners to create the large network of interactions that is present in the dental plaque. Other proteins may be involved in recognition of different streptococcal groups as well as specific species. Studies are currently underway to understand the deeper implications of *aid1* in the formation and maintenance of the oral biofilm as well as identification of other proteins involved in specific interactions of *F. nucleatum* with different oral bacterial species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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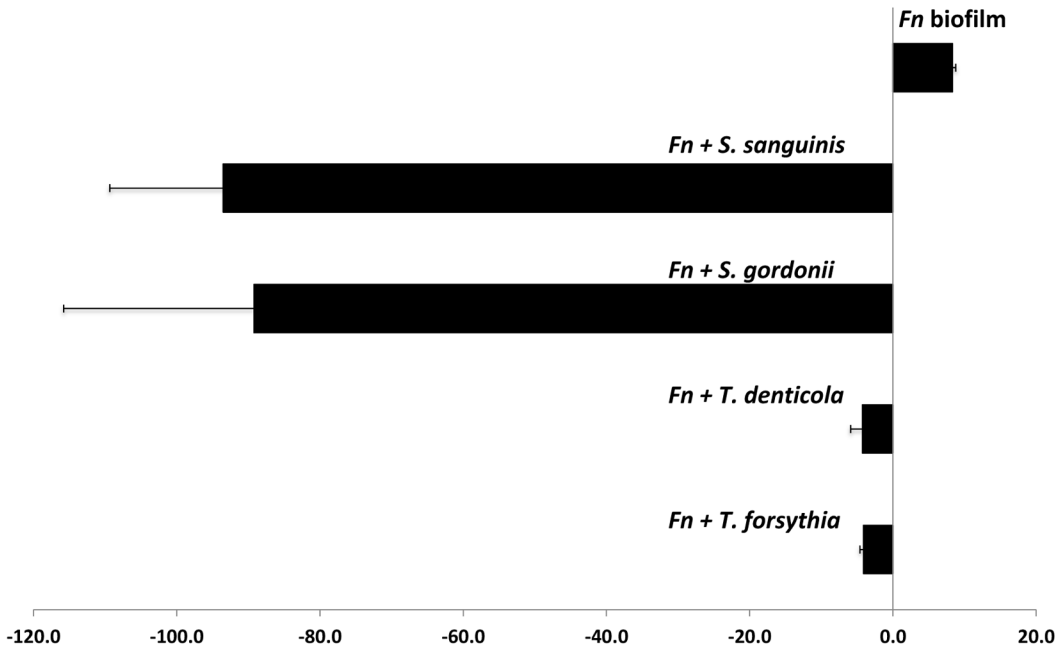

Figure 1. Differential expression of *aid1* in *F. nucleatum* single and dual species biofilms

F. nucleatum ATCC23726 cells were grown under biofilm conditions for 24hrs either alone or in the presence of known interacting partners. *aid1* gene expression was analyzed by quantitative real-time PCR (qRT-PCR). Three independent experiments were performed.

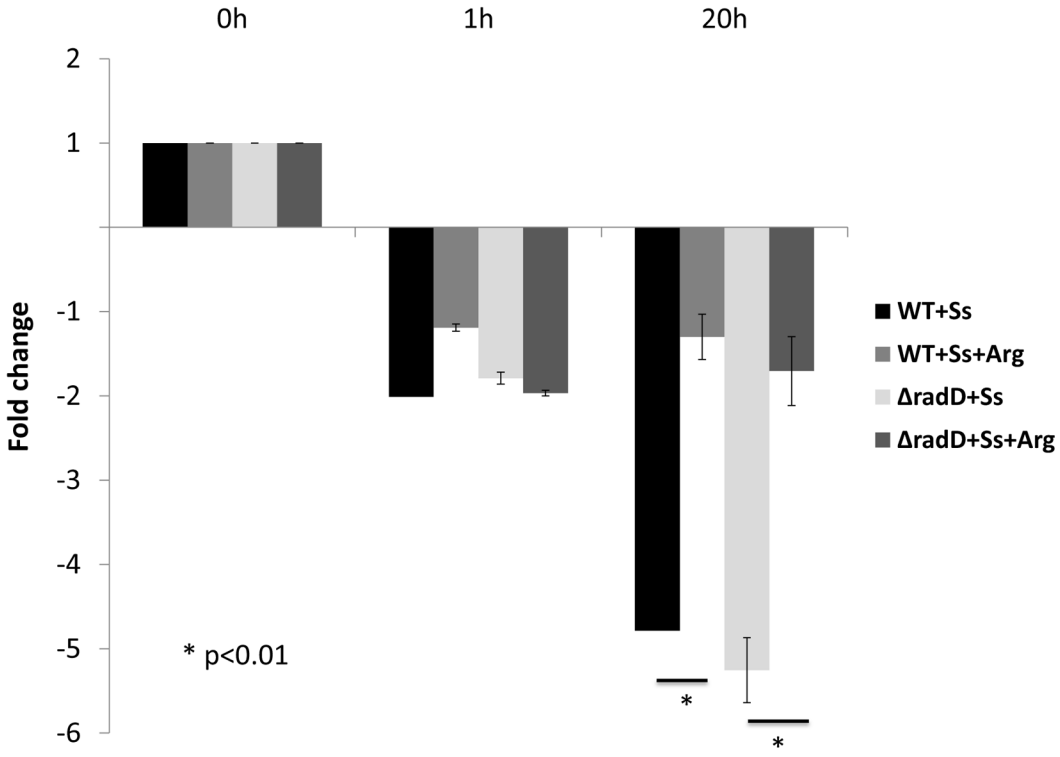

Figure 2. *aid1* gene expression is time- and touch-dependent

aid1 gene expression was monitored by qRT-PCR at early (1hr) and late (20hr) time points when *F. nucleatum* was coincubated with *S. sanguinis* after being pelleted by centrifugation. Arginine was added as a negative control to block physical interactions between the cells. The data represent the average of three independent experiments.

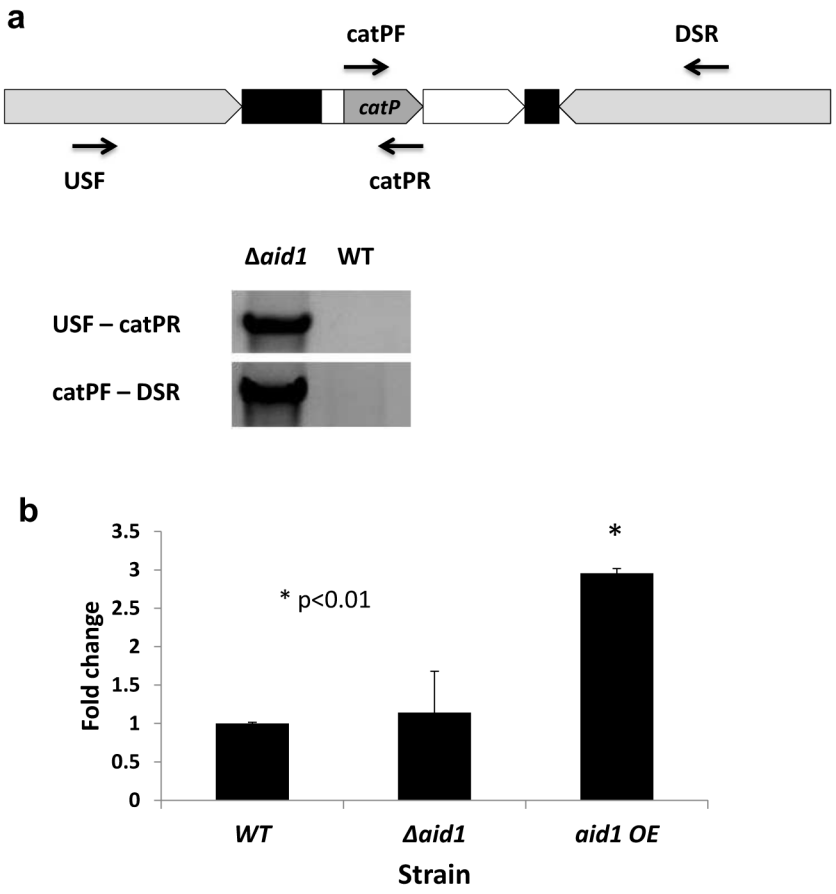

Figure 3. Analysis and verification of *aid1* mutants

A. Analysis of Δ*aid1* mutant strain. Diagram of gene inactivation linear vector introduced into WT *Fn* ATCC 23726 to obtain the Δ*aid1* inactivation mutant. Confirmation of insertion into *aid1* gene by PCR analysis. Arrows indicate the location of the primers used for PCR amplification. Fragments were amplified from the mutant strain but not from the wild-type control. **B. Gene expression of *aid1* in mutant strains and verification of *aid1* overexpression strain.** *aid1* gene expression was verified using quantitative real-time PCR (qRT-PCR) using *aid1*-specific primers.

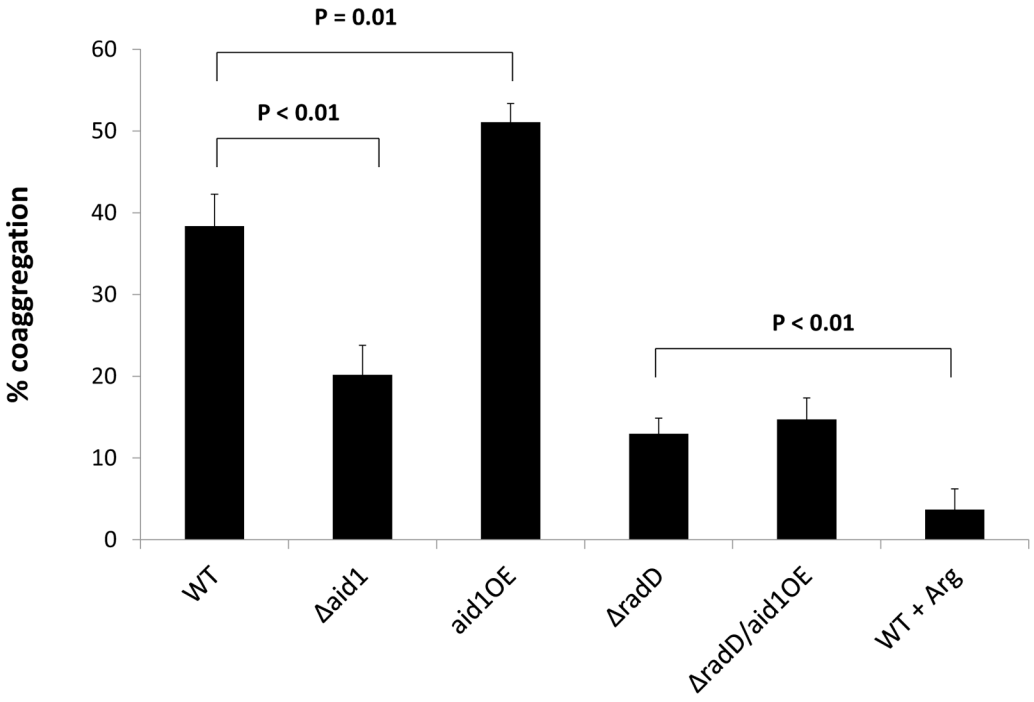

Figure 4. Quantitative coaggregation assay between *F. nucleatum* strains and *S. sanguinis*

Wild type *F. nucleatum* and *aid1* mutant derivatives were mixed with equal number of *S. sanguinis* cells in coaggregation buffer (CAB) and allowed to aggregate for 10 mins. OD_{600} absorption was measured before and after cells were allowed to aggregate. The data represent the average of three independent experiments.

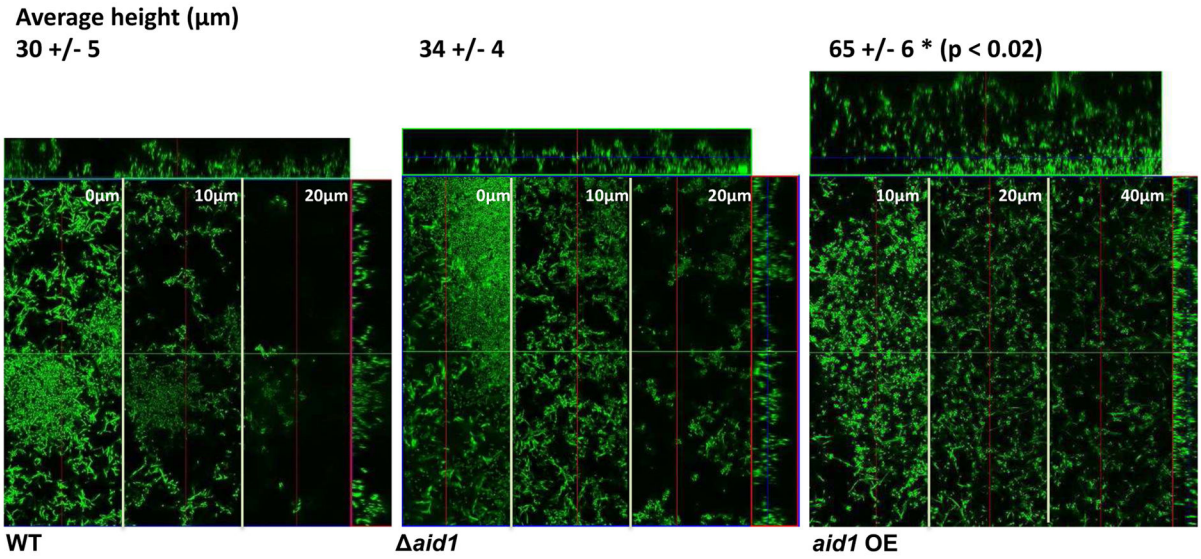

Figure 5. Biofilm growth and morphology of *aid1* mutants with oral streptococci

Dual species biofilms were grown with wild type *F. nucleatum*, mutants lacking and overexpressing *aid1* together with *S. sangunis.* Biofilms were grown for 24 hrs under anaerobic conditions and stained with SYTO9. Sections from 20µm above the growth surface demonstrate the variation in biofilm morphology as well as the differences in the height of the biofilm. Representative images chosen from three independent experiments are shown.

Table 1

Coaggregation of *aid1* mutant derivatives with oral bacteria

Aggregation ability of *aid1* mutants.

Wild type *F. nucleatum* and *aid1* mutant derivatives were mixed with other species in coaggregation buffer (CAB) and allowed to aggregate for 10 mins. The method of assigning coaggregation scores is described in the experimental procedures. Coaggregation experiments were performed at least five times.

	WT	**Δ*aid1***	***aid1* OE**	**Δ*radD***	**Δ*radD/aid1* OE**
Auto-aggregation	0	0	0	0	0
Gram-positive oral					
S. sanguinis	2	1	3	0	0
S. gordonii	2	1	3	0	0
E. faecalis	2	1	3	0	0
L. casei	2	2	2	0	0
S. epidermidis	4	4	4	2	2
Gram-negative oral					
P. gingivalis	3	3	3	3	3
T. denticola	0	0	0	0	0
T. forsythia	0	0	0	0	0
V. atypica	0	0	0	0	0

Table 2

Inhibition of coaggregation of *aid1* mutant derivatives with *S. sanguinis*

Inhibition of cellular coaggregation.

50mM final concentration of inhibitor was added to coaggregation mix in coaggregation buffer (CAB) and coaggregation was scored after 10 mins. Experiments were repeated independently at least five times.

	WT	**Δ*aid1***	***aid1* OE**
PBS	2	1	4
Arginine	0	0	0
Galactose	2	1	3
GlcNac	2	1	4
Glucose	2	1	4