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Determination of RadD-Dependent Gene Regulation in Fusobacterium nucleatum in Response to Streptococcus sanguinis

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Determination of RadD-Dependent Gene Regulation in *Fusobacterium nucleatum* in Response to *Streptococcus sanguinis*

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Oral Biology

By

Emil J. Simanian

2013

ABSTRACT OF THE THESIS

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in *Fusobacterium nucleatum* in Response to *Streptococcus sanguinis*

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Master of Science in Oral Biology

University of California, Los Angeles, 2013

Professor Wenyuan Shi, Chair

Periodontal disease is a highly common disorder believed to be triggered by colonization of bacteria on the surface of teeth. Although the relationship between many oral bacterial species has been shown to rely on physical adherence, the intracellular consequences that arise as a result of these intimate associations are currently unknown. *Fusobacterium nucleatum* (*F.n.*), a Gram-negative periodontal pathogen that has the unique capability to adhere to Gram-positive primary colonizing and Gram-negative secondary colonizing oral bacteria was chosen to assess differential gene regulation that may occur in response to a primary colonizer, *Streptococcus sanguinis* (*S.s.*). A mutant strain of *F.n.* that lacks RadD, an outer-membrane adhesin required for adherence and recognition of Gram-positive oral streptococci, was also used to determine if

RadD-dependent gene regulation in response to *S.s.* was present. RNA was extracted from wildtype *F.n.* and the ΔRadD mutant alone, as well as after co-incubation with *S.s.*, and the expression profile of twenty-four *F.n.* genes selected from a previous microarray study for validation was determined via Real-Time Polymerase Chain Analysis (RT-PCR). We found that of the twenty-four genes tested, seventeen showed differential regulation in wildtype *F.n.* in the presence of *S.s.*, twenty were regulated in the ΔRadD mutant in response to *S.s.*, while five exhibited transcriptional differences due to lack of RadD independent of S.s. when the expression profile of the ΔRadD mutant was compared to wildtype. Compared to wildtype *F.n.*, six genes showed opposite regulation in the ΔRadD mutant in response *S.s.*, indicating that there is contact-dependent gene regulation mediated by the RadD adhesin. Aside from providing an intracellular understanding of contact-dependent adherence between two prominent oral bacteria, to the best of our knowledge, this is the first discovery of expression of genes that rely on the presence of an interspecies recognition based outer membrane adhesin in a bacterial species.

The thesis of Emil J. Simanian is approved.

Shen Hu

Renate Lux

Wenyuan Shi, Committee Chair

University of California, Los Angeles

2013

DEDICATION

Dedicated to my father Mayer, to my mother Mitra,

to my brothers Matthew, Maurice, Allen,

to Ashley, to my friends, and to Luigi,

whose love and support during every step of

this journey has been critical to my success.

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INTRODUCTION

Periodontal disease, defined as a disorder of the tissues which surround and support teeth, is an extremely common condition affecting more than 50% of the elderly population [1]. The disease is an umbrella term for two conditions that are associated with inflammation: gingivitis and periodontitis [1]. Considered a milder form of periodontal disease, gingivitis can be easily reversed by proper oral hygiene, and only affects the portion of teeth adjacent to the gums [1]. On the other hand, periodontitis refers to inflammation that travels deep into tissues and impairs the connective tissue and alveolar bone which supports teeth [1]. This ultimately causes formation of deep crevices between the gums and the roots of teeth, impairment of the ability to chew, and pain that may eventually lead to loss of the tooth [1]. Although different factors have been identified to promote periodontal disease, the main cause has been attributed to a variety of microorganisms in the mouth that interact with tissues and cells of the host, eliciting an immune response [1, 2]. It is this response from the host immune system to oral pathogens that predominate in regions between the tooth and gingival margin that ultimately causes the release of chemokines and inflammatory cytokines [2]. The release of these chemicals ultimately leads to destruction of the periodontal ligament, alveolar bone, and tooth-supporting structures [2]. The trigger for the initiation of periodontal disease has been directly linked to oral bacteria, which specifically form complex structures known as biofilms [2]. While majority of the bacteria that constitute biofilms are commensals, a select few are considered pathogenic, and ultimately are responsible for periodontal disease.

Biofilms in the oral cavity, which constitute dental plaque, ultimately allow for a variety of different types of bacteria to be able to colonize tooth surfaces via distinct mechanisms. Studies have shown that although there are an estimated seven-hundred bacterial species that can inhabit and colonize the oral cavity, these species have the ability to structurally organize themselves in a highly specific manner within complex biofilms [2]. This organization allows for further characterization of the relationships among different species during the progression of periodontal disease, illustrating the importance of interspecies interactions in the development and sustenance of oral biofilms [3, 4]. Indeed, bacterial adherence is essential for efficient integration into the microbial community, and provides an important basis for the examination of ecological relationships that occur among oral bacteria and their host [4]. A clear example of this relationship is demonstrated by a phenomenon that was first seen to occur in bacteria isolated from human plaque samples, known as co-aggregation [5].

Co-aggregation is defined as a highly specific and social process where bacteria adhere to one another [5]. For two bacteria to have this ability, one must contain a specialized protein 'adhesin', while the other a complementary polysaccharide 'receptor' [5]. Currently, it is believed that oral biofilms develop on the surface of teeth through a succession of distinctive events that involve adhesion and integration of different bacterial species [5]. Co-aggregation is thought to be a critical aspect this process through two ways [5]. Firstly, it is believed to be the main reason why bacteria contained in suspension recognize and adhere to other distinct bacteria that are already attached to a developing biofilm [5]. Secondly, co-aggregation is believed to occur between bacteria in suspension, which then adhere together to the developing biofilm [5]. The bacterial cells that co-aggregate in both situations are able to adhere to cells within already

existing biofilm through a specific process defined as coadhesion, subsequently becoming part of the biofilm complex [5, 6, 7]. These co-aggregation events are believed to play an integral role by allowing bacteria to adhere and pair up with other species within the oral cavity (Figure 1) [5].

As the oral biofilm ecosystem has been extensively studied and characterized, the sequence of bacterial colonization to the tooth surface, which ultimately relies on co-aggregation, has been proposed to occur in a highly specific and coordinated manner (Figure 2) [5]. The first bacteria that colonize the surface of the tooth, which is coated with glycoprotein and other salivary proteins collectively known as the dental pellicle, are Gram-positive rods and streptococci, specifically *Actinomyces naeslundii* (*A.n.*), *Streptococcus sanguinis* (*S.s.*), *Streptococcus gordonii* (*S.g.*), *Streptococcus mitis* (*S.mi.*), and *Streptococcus oralis* (*S.o.*) [5, 8]. These first organisms are known as primary colonizers, and their attachment to the dental pellicle relies on specific and non-specific physio-chemical interactions [5]. Indeed, human saliva has been shown to be a necessary component that stimulates biofilm formation for many of these oral streptococci, and specifically, bacterial surface components such as pili have been characterized to bind to multiple salivary proteins [8]. One such protein, α-amylase, which catalyzes the hydrolysis of dietary starch, is abundant in human saliva and has been shown to bind to the pili of *S.s.* and facilitate its adherence to the dental pellicle [8]. As the environment of the early biofilm begins to change due to the presence of these primary colonizers, Gram-negative secondary colonizers then begin to adhere [5]. Thus, primary colonizers essentially serve as a substrate for the binding of secondary colonizers, allowing the biofilm to quickly develop into a vast oral microbial community [5]. These include *Treponema denticola* (*T.d.*),

Tannerella forsythia (*T.f.*), *Porphyromonas gingivalis* (*P.g.*), *Actinobacillus actinomecetemcomitans* (*A.n.*), and it is important to note that the interactions between primary and secondary colonizers are highly specific [5]. Although primary colonizers are able to co-aggregate with each other, only a known few secondary colonizers, such as *P.g.*, are able to directly bind to primary colonizers [5]. After only twenty-four hours, the oral biofilm is transformed into a conglomerate of bacteria with different morphologies, and is a stable reservoir that can ultimately counterbalance the shear forces contained in the oral cavity, aid in nutrient availability, and confer microbial resistance among different species [5]. Thus, it is clear that the ability of oral species to coaggregate with one another plays a very important role in the temporal sequence of biofilm formation and plaque development [5].

One aspect in oral biofilm formation that cannot be overlooked is the importance of organisms that can co-aggregate with both primary and secondary colonizers. The Gram-negative anaerobic microorganism *Fusobacterium nucleatum* (*F.n.*) has been proposed to serve as a bridging organism in oral biofilms due to this unique ability. As early biofilms contain predominately Gram-positive facultative species, *F.n.* is one of the first Gram-negative anaerobes that colonize the oral cavity [9]. Indeed, it has been demonstrated that many of the secondary colonizers cannot adhere to oral biofilms in the absence of *F.n.,* and that these bacteria must co-aggregate with *F.n.* in order to survive in a planktonic state [5]. For these reasons, *F.n.* has been identified an essential organism for the initial development and maturity of oral biofilms, by facilitating interspecies interaction [5]. Epidemiologically, increasing numbers of *F.n.* in the oral cavity has been associated with periodontal disease, and recent studies have shown that *F.n.* contains outer-membrane proteins that induce apoptosis in human lymphocytes,

an ability that has been suggested to increase its pathogenesis [13]. *F.n.* has been isolated from both subgingival and supragingival plaque, and the bacterium may further serve as a connecting link between the two types of plaque due to its ability to enhance the survival of strict anaerobic pathogens, such as *P.g.* and *Prevotella nigrescens*, demonstrating its importance in the progression of periodontal disease [9]. The specific cell surface components of *F.n.* that mediate their interaction with neighboring bacteria has been attributed to adhesin molecules, which specifically allow co-aggregation between different oral species as well as attachment to host proteins [5].

In *F.n.*, two distinctive forms of adherence, based on type of inhibition and partner strain, have been identified [10]. The adherence to Gram-negative secondary colonizing bacteria has been found to be inhibited by the monosaccharide sugar D-galactose, while adherence to Gram-positive primary colonizers has been found to be inhibited by the amino acid L-arginine [11]. Through previous biochemical techniques, both adhesins were characterized to be quite large, with the D-galactose and L-arginine inhibitable proteins having a molecular mass of 300-330 kDa, and 370 kDa, respectively [10]. Furthermore, a spontaneous mutant strain of *F.n.* (ATCC 10953) identified to have deficient interactions with Gram-positive early colonizers was found to lack a 360kDa outer membrane protein, further confirming the function of the L-arginine-inhibitable adhesin [10]. Through these findings, our lab had previously generated several outer-membrane protein gene inactivation mutants in the genetically transformable strain *F.n.* ATCC 23726, in order to identify and characterize adhesin mutants defective in co-aggregation with Gram-positive primary colonizers and *in vitro* biofilm formation [10]. Through this method, only one outer-membrane protein *F.n.* mutant showed visual and quantitative co-aggregation

deficiencies with Gram-positive primary colonizers (*S.s., S.o., S.g., A. n.*), which paralleled what is seen with L-arginine inhibition [10]. When this mutant, which lacks the 350kDa gene product of FN1526, was grown in a dual-species biofilm with *S. s.* ATCC 10556, the morphology differed compared to the same biofilm formed with wildtype *F.n.*. [10]. Briefly mentioned earlier, *S.s.* is a Gram-positive early colonizer that is prevalent in supragingival plaque and has been found to co-aggregate with a variety of oral bacteria [12]. This suggested that FN1526 also is necessary for the formation of elaborate dual-species biofilms with *S.s. in vitro* [10]. It was determined from these experiments that FN1526, also known as *radD* (arginine(R)-inhibitable adhesin D), is an arginine-inhibitable outer-membrane autotrasporter present in *F.n.* ATCC 23726 that is required for inter-species adherence, as well as biofilm formation [10].

With the understanding that it harbors unique social characteristics and plays an important role in periodontal disease, *F.n.* was used a model organism in order to further study community interactions within the oral cavity. Subsequently, to determine if transcriptional alteration is present in *F.n.* in response to bacteria associated with periodontal disease, (*T.d.*, *S.s.*, *S.g*, and *T.f.*) a comprehensive microarray study was performed in our lab (unpublished data). The study additionally set out to determine if differential regulation is present between wildtype *F.n.* compared to its mutant lacking the L-arginine-inhibitable adhesin (ΔRadD), in response to its adhering partner strain, *S.s.*. Part of the aims of the microarray was to determine which molecular components were behind *F.n.*'s adherence to neighboring species in the oral cavity, and if differential gene regulation in *F.n.* depended on the outer-membrane RadD adhesin. Indeed through the analysis, gene regulation was seen in a variety of different tested conditions,

indicating that there may be differential regulation in response to *S.s.* in both wildtype *F.n.* and the ΔRadD mutant.

The ultimate goal of this study is to validate the unpublished results of the microarray analysis using Real-Time Polymerase Chain Reaction (RT-PCR), an established method used to accurately and reliably quantitate RNA levels using a fluorogenic reporter molecule [14, 15]. Twenty-four genes that showed differential regulation in two different conditions tested in the microarray were ultimately selected for RT-PCR analysis. These included genes regulated when wildtype *F.n.* was co-incubated with *S.s.*, as well as those regulated when the ΔRadD mutant was co-incubated with *S.s.*. Among the studies that have been done on *F.n.* and its interacting partner *S.s.*, there remain two key questions, which will be addressed in this study. The first question is what gene regulation occurs in *F.n.* in response to *S.s.*? Since we are using *F.n.* as a model organism in this study, confirmation of genes that were differentially regulated in the microarray study will present a new perspective as to what occurs at the molecular level when these two species interact with each other. The next main question surrounds the outer membrane L-arginine-inhibitable adhesin of *F.n.*, RadD, and how an inactivation mutant of the gene that encodes this protein could impact *F.n.*'s response to *S.s.* at the molecular level. Is RadD simply an adhesin that allows *F.n.* and *S.s.* to bind to each other in oral biofilms? Or does RadD activate an intermediate step that triggers differential gene regulation within *F.n.*? These particular questions will be addressed through the testing of wildtype *F.n.* and its ΔRadD mutant, with and without the presence of *S.s.*, which allows for determination of RadD dependent gene regulation in response to *S.s.* via RT-PCR. By addressing these two questions, this study will demonstrate that there does indeed appear to be a gene regulation event that goes beyond bacterial co-

aggregation, and that the RadD adhesin may indeed be involved in altering gene regulation in *F.n.* through a hypothesized signaling transduction mechanism.

MATERIALS AND METHODS

Bacterial strains and co-incubation conditions

Strains in this study are listed in Table 1. *Fusobacterium nucleatum* (*F.n.*) ATCC 23726 and its mutant derivative lacking a large outer membrane protein Fn1526 (ΔRadD mutant) were cultivated in Tryptone-Yeast Extract-Gelatin-Volatile Fatty Acids-Serum (TYGVS) medium and *Streptococcus sanguinis* (*S.s.*) ATCC 10556 was cultivated in artificial saliva solution (ASS) [16, 17]. 5 ml of each strain was grown in 15 ml centrifuge tubes in an anaerobic chamber (5% CO_2, 5% H_2 and 90% N_2) at 37°C overnight. Thiamphenicol (MP Biomedicals, Irvine, CA, USA) at 5 μg/ml was used for the selection and maintenance of the *F. nucleatum* mutant strain possessing the *catP* determinant. Co-incubation experiments were performed as follows: an overnight culture of *F.n.* ($1x10^9$ cells) was pelleted at 4,600xg for 10 minutes at 4°C, and then resuspended in equal volumes of pre-reduced ASS with 25% TYGVS. Approximately $1x10^7$ *F. nucleatum* cells was then inoculated in each well of a six-well plate. The same procedure was repeated for the *F.n.* ΔRadD mutant grown overnight ($1x10^9$ cells) in a separate plate. Final volumes were adjusted to 2 ml per well using pre-reduced ASS containing 25% TYGVS. The cells were then allowed to grow anaerobically at 37°C for approximately 5 hours, at which point the plates were taken out of chamber. $1x10^5$ cells of *S. sanguinis* was then inoculated in three wells for each plate. Cells were then allowed to co-incubate anaerobically 37°C over the course of approximately 18 to 20 hours.

RNA extraction and purification

Following co-incubation, bacteria from each well were transferred to 2 ml eppendorf tubes and pelleted at 4,600×g for 15 minutes at room temperature. Supernatant was removed and cells were resuspended in 200 µl 10mM Tris, pH 8.0. Cells were lysed by flash freezing for 30 seconds in an ethanol bath chilled at -80°C, and then thawed in an ice bath for approximately 5 minutes. This procedure was repeated three times. For additional lysis, 4µl of lysozyme (50mg/ml) was added and cells were incubated at 37°C for 10 minutes. RNA was subsequently extracted using High Pure Isolation Kit (Roche) according to the manufacturer's instructions. RNA quality was analyzed by electrophoresis in 1% agarose gels with 1X Tris-acetate-EDTA (TAE) buffer and visualized by staining with ethidium bromide. Extracted RNA was then treated with Turbo DNAse I (2U/µl, Ambion) for 1 hour at 37°C for removal of any residual genomic DNA. Absence of DNA contamination in the RNA samples was verified with polymerase chain reaction (PCR) utilizing *F.n.* 16S ribosomal RNA specific primers and GoTaq DNA Polymerase (M300), using the following cycle conditions: 1 cycle at 95°C for 2 minutes followed by 30 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 5 min. Each PCR reaction included 0.75 units of GoTaq DNA Polymerase (Promega), 1X Green GoTaq Reaction Buffer including 1.5 mM MgCl2, 0.75 pmol of each primer, 200 µM each of dATP, dGTP, dCTP, and dTTP, nuclease-free water (<0.5 µg/50 µl) and the template for a final volume of 20 µl. PCR amplicons were analyzed by electrophoresis in 2% agarose gels. After ruling out any DNA contamination, further purification of RNA samples was performed using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. Quality of purified RNA was analyzed via electrophoresis in a1% agarose gel and visualized by staining with ethidium bromide.

Genomic DNA isolation

For DNA isolation, *F. nucleatum* ($1x10^9$ cells) was harvested by centrifugation at 4,600 x g for 20 minutes at 4°C. Pellets were resuspended in 250 µl PBS and lysed by incubation with 3 ml Tissue and Cell Lysis Solution containing 500 µg proteinase K at 65°C for 20 min, with vortexing every 5 minutes. DNA isolation was performed using MasterPure DNA purification kit (Epicentre). The samples were cooled to 37°C, and RNA was degraded by incubation at 37°C for 40 min with 50 µg RNase A. After incubation on ice for 10 min, 1.75 ml MCP Protein Precipitation Reagent was added, and the mixture was vortexed for 10 sec. Cellular debris was pelleted by centrifugation at 4,600 x g for 20 min. To the supernatant, 5 ml isopropanol was added and mixed by inverting the tube 30-40 times. The DNA was pelleted by centrifugation at 4,600 x g at 4°C for 20 min. The pellet was rinsed twice with 75% ethanol, resuspended in 50 µl water. 1 µl of DNA was used for quantification using the Nanodrop 2000 microvolume spectrophotometer (Thermo Scientific).

Synthesis of complementary DNA (cDNA)

1 µl of purified RNA from the bacteria samples was quantified using Nanodrop 2000 (Thermo Scientific). Single stranded cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Briefly, reverse transcription of 1µg of RNA was performed using random hexamer primers (600 pmol/µl) at a final concentration of 60µM. The template-primer mixture was denatured at 65°C for 10 minutes in a thermal block cycler, after which 4 µl Transcriptor Reverse Transcriptase Reaction Buffer (1x of 5x concentration), 0.5 µl Protector RNase Inhibitor (20U of 40U/µl), 2 µl Deoxynucleotide Mix (1mM of 10mM) and 0.5 µl Transcriptor Reverse Transcriptase (10U of 20U/µl) was added for a final volume of 20 µl, mixed carefully. Each µl in the 20 µl reaction contained 50 ng of RNA. The reverse transcriptase reaction was then incubated for 10 minutes at 25°C, followed by 60 minutes at 50°C. The Transcriptor Reverse Transcriptase was inactivated by being heated to 85°C for 5 minutes, after which the reaction was placed stopped and placed in ice. As a negative control, reactions were also run without Reverse Transcriptase. The reaction product was diluted 1:20, and 1 µl of this diluted product was used for PCR verification of cDNA synthesis using 16S *F.n.* primers and the following cycle conditions: 1 cycle at 95°C for 2 minutes followed by 30 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 5 min. Each PCR reaction included 0.75 units of GoTaq DNA Polymerase (Promega), 1X Green GoTaq Reaction Buffer including 1.5 mM MgCl2, 0.75 pmol of each primer, 200 µM each of dATP, dGTP, dCTP, and dTTP, nuclease-free water (<0.5 µg/50 µl) and cDNA template (2.5 ng) for a final volume of 20 µl. Synthesized cDNA was analyzed by electrophoresis in a 2% agarose gels with 1X Tris-acetate-EDTA (TAE) buffer and visualized by staining with ethidium bromide.

Real-Time Polymerase Chain Reaction (RT-PCR) Analysis

From an unpublished microarray study, twenty-four *F.n.* genes were selected that represented various levels of predicted induction or repression. PCR primers (Table 2) that specifically amplified between 90-120 base pairs for each gene were designed using PrimerQuest (www.idtdna.org), and primers were used at a final concentration of 100 pmol. iQ SYBR Green Supermix (2x concentration, Biorad) was mixed thoroughly with forward and reverse primers, and RNase-free H_2O for dye-based Real-Time PCR, with a total reaction master mix volume of 19 µl per well. 2.5 ng of cDNA template (1 µl of a 1:20 dilution) was first added into the wells of a 96-well RT-PCR plate, followed by addition of an equal aliquot master mix. cDNA templates were added as independent triplicates in three separate wells for each gene tested. The 20 µl mixtures in each well were resuspended thoroughly via pipetting. Plates were sealed with optically transparent film, and RT-PCR was performed with a MyiQ Real Time PCR Detection System (Biorad) and the accompanying program Biorad iQ5. The thermal cycling protocol was as follows: polymerase activation and DNA denaturation at 95°C for 3 min, followed by amplification: denaturation at 95°C for 15 seconds and annealing/extension at 55°C for 60 seconds for 34 cycles. When warranted, a melt curve analysis was done from 55-95°C with a 0.5°C increment every 5 seconds. Standard curves for each primer set were generated by the iQ5 software using four 10-fold dilutions of genomic *F.n.* genomic DNA, starting with 10^{-3}, to allow quantification of transcript levels for each condition tested. The respective fold change for each tested was calculated by dividing the transcript abundance of each test sample by the transcript abundance of the corresponding control sample. In addition, a standard curve was generated using different dilutions of *F.n.* genomic DNA, and RT-PCR starting quantity values generated from the standard curves were normalized to the 16S Ribosomal RNA gene. Data analysis was

performed using these normalized values, and specific calculations allowed for determination of genes differentially regulated for each condition. All values are represented in fold-changes, and cut-off values for induction and repression for each gene were set to 2 and -2, respectively.

RESULTS

A comprehensive Real-Time PCR analysis was carried out in order to identify genes regulated in *F.n.* that respond to *S.s.*. Previous studies have already shown that the outer membrane adhesin RadD is required for cell to cell interaction of *F.n.* with *S.s.* [10]. Our study covers *F.n.*'s response to *S.s.* that may or may not be mediated through RadD. A previous microarray analysis (unpublished data) revealed differential gene regulation in *F.n.* and the ΔRadD mutant responding to *S.s*. amongst other periodontal pathogens. Based on this microarray analysis, twenty-four genes were selected for further analysis through RT-PCR which had shown differential regulation in *F. nucleatum* and possibly a specific response to *S.s.* via outer membrane adhesin RadD. These genes were then separated into different classes depending on their function: thirteen genes are involved in cellular processes, two genes are related to transcription, five genes are involved with transport, two genes are membrane-associated, and two genes have a currently unknown hypothetical function. Additionally two housekeeping genes, encoding the 16S Ribosomal RNA and DNA-directed RNA polymerase beta chain (*rpoB*) of *F.n.*, were used as reference points to ensure all the conditions tested did not show regulation pertaining to growth conditions or other environmental factors (Table 3).

Genes regulated in *F. nucleatum* in response to *S. sanguinis*

The first condition tested pertains to the genes regulated in *F.n.* in response to *S.s.* when both organisms were co-incubated. (Figure 3). To determine this using RT-PCR, a calculation was used where *F.n.* ATCC 23726 grown with *S.s.* was taken as the experimental condition, and *F.n.* ATCC 23726 was taken as the control condition (Wt + *S.s.* / Wt). This calculation allows for determination of a specific response to *S.s.*. The differential regulation determined through RT-PCR was compared to its microarray fold change, as indicated in Table 4. Of the twenty-four genes tested for *F.n.*'s response to *S. s.*, seventeen showed differential regulation. Four genes involved in cellular process, including chaperone protein dnaK (FN0116), enoyl-CoA hydratase (FN0271), neutrophil activating protein-A (FN1079) and V-type sodium ATP synthase subunit D (FN1733), and one gene involved in transport, glycerol uptake facilitator protein (FN1838), were all induced. Twelve genes, including six genes involved in cellular processes, two genes involved in transport, two membrane-associated and two hypothetical genes tested were repressed. Additionally, seven out of twenty-four genes, which had shown differential regulation in the microarray study, did not show regulation through RT-PCR in this condition.

Determining the role of RadD in gene regulation in the presence of *Streptococcus sanguinis*

Seventeen *F.n.* genes showed regulation in the previous condition. It can be suggested that since RadD is required for cell-to-cell interaction between *F.n.* and *S.s.*, some of these genes may be regulated through the RadD adhesin (Figure 4). In order to investigate if the regulation of genes is attributed through an interaction via RadD, a calculation was used where the ΔRadD mutant co-incubated with *S.*s was taken as the experimental condition, and the regulation was tested with wildtype *F.n.* co-incubated with *S.s.* (ΔRadD + *S.s.* / Wt + *S.s*). Under these conditions tested, twenty out of twenty-four genes showed differential regulation (Table 5). Four genes were induced in the ΔRadD mutant compared to wildtype *F.n.* in response to *S.s.*, including two genes involved in cellular processes (FN0116, FN0392), and two genes involved in transport (FN0205, FN1860). In contrast, sixteen genes showed repression, including eight genes involved in cellular processes, two genes involved in transcription, two genes involved in transport, two membrane associated genes, and two hypothetical genes. It is important to note this is a mutant condition when the RadD adhesin is absent, and thus repression in this condition indicates that in wildtype *F.n.* these genes are no longer repressed in response to *S.s.*

Determination of gene regulation dependent on the outer membrane adhesin RadD

In order to determine which genes depend on the RadD adhesin for induction or repression in response to *S.s.*, a comparison was made, in which the genes that showed regulation from the first tested condition (Wt + *S.s.* / Wt) were compared to those which showed regulation in the second tested condition (ΔRadD + *S.s.* / Wt + *S.s.*). Once the data were combined, only the genes that overlapped and showed regulation in both conditions were taken into consideration for RadD-dependency. As shown in Table 5b, out of seventeen genes that showed differential regulation in the first tested condition and twenty-one in the second, fifteen genes showed an overlap. These include eight genes involved in cellular processes, three genes involved in transport, two membrane-associated genes, and two hypothetical genes. These genes were subsequently separated into three categories.

The first category pertains to genes that show RadD dependent gene regulation in response to *S.s.*. It is important to note that in order to confirm that the responses to *S.s.* were truly RadD dependent and not just due to the deletion of the RadD adhesin, the overlapping genes were compared to a third condition, where the ΔRadD mutant was taken as the experimental condition, and the regulation was tested with wildtype *F.n.* (ΔRadD / Wt), shown in Table 6, and illustrated in Figure 5. Only five tested genes showed differential regulation in this third condition, two involved in cellular process (FN0652, FN1079), two involved in transport (FN0242, FN1838), and one membrane associated (FN1899). Therefore, upon closer examination, the genes that were RadD dependent in response to *S.s.* were determined to have opposite regulation between the (Wt + *S.s.* / Wt) condition and the (ΔRadD + *S.s.* / Wt + *S.s.*) condition, and no regulation in the (ΔRadD / Wt) condition. Specifically, this category includes

three cellular process genes, (FN0271, FN0392, FN1733), and one gene involved in transport (FN1860).

The second category shows gene regulation in response to *S.s*. that does not depend on the RadD adhesin. These genes were determined to have the same response between the (Wt + *S.s.* / Wt) and (ΔRadD + *S.s.* / Wt + *S.s.*) conditions, and either no regulation or an induction in the (ΔRadD / Wt) condition. The genes that fall in this category include four involved in cellular processes (FN0116, FN0559, FN0652, FN1380), one involved in transport (FN1989), one that is membrane-associated (FN0471), and two that are hypothetical (FN1230, FN1253).

The last category involves genes that may show regulation independent of the RadD adhesin and the presence of *S.s.*. These genes show two trends: an induction in the (Wt + *S.s.* / Wt) and (ΔRadD / Wt) conditions and a repression in the (ΔRadD + *S.s.* / Wt + *S.s.*) condition, or a repression in all three conditions. The genes that fall in this category include one involved in cellular processes (FN1079), one involved in transport (FN1838), and one that is membrane-associated (FN1899).

DISCUSSION

Over the past decade, microarray technology has quickly become a widespread tool to measure gene expression, allowing numerous samples to be tested on a single chip [18]. Although advances in arrays have allowed for superior probe density, structural layout, and quality of generated data, various limitations inherent to the technology remain [18, 19, 20]. Notably, depending on the technical and biological procedures being used, as well as the different platforms available, the gene expression data generated can be of variable quality [21]. Additionally, non-specific background signals and cross-hybridization of target cDNAs to other DNA probes within the array must also be taken into account, as each phenomenon can provide false positives that may ultimately generate misleading data [22]. Therefore, before results from a microarray can be considered accurate, it is important to rule out any erroneous data generated by subsequently using methods that do not rely on hybridization [22].

To account for this, a tool most often used to validate the results obtained from microarray analyses is real-time PCR [21]. Versus other common methods, such as northern blot or *in situ* hybridization, the main advantages of using real-time PCR for validation of microarray data relies on the fact that it is relatively inexpensive, requires a minimal starting template, and provides rapid results that are fairly reproducible [22, 23, 24, 25]. By using the term validation in this context, the main task at hand involves determining whether results of the biological system in question were accurate [22]. In doing so, the first goal of this study was to experimentally perform independent gene-expression verification through the selection of genes which had shown greater than a 2-fold differential regulation from the microarray study.

Although there were quantitative differences between the values generated real-time PCR compared to the microarray data, the overall regulation trend for most, though not all, of the genes tested matched. This not only gave a strong implication that the generated data from the microarray, with all of its potential limitations, was experimentally valid and accurate, but confirms that the real-time PCR data generated is accurate as well.

Aside from validation, the second main goal of this study was to perform a comprehensive real-time PCR analysis to further investigate the interaction between two bacterial species prominent in the oral microbial community: *F. nucleatum* (*F.n.*) and *S. sanguinis* (*S.s.*). The twenty-four *F.n.* genes (Table 2) that were ultimately selected for analysis by RT-PCR had derived from the microarray analysis used to investigate *F.n.*'s interactions with streptococcal species and periodontal pathogens (unpublished data). These genes were separated into five classes based on their function (Figure 6), and subsequently tested in three different conditions, as outlined in the results. Through analysis of the generated data, the first aim of this study was to determine which genes in *F.n.* are regulated in the presence of *S.s*. that may provide potential evidence towards a downstream effect (Figure 3).

Detailed analysis of the data set demonstrated that majority of the differentially regulated genes in *F.n.* responding to *S.s.* showed an overall trend of repression (Table 4). Out of twenty four genes tested, only five genes were found to be induced. Three of these genes include an enoyl-coA hydratase (FN0271), a V-type sodium ATP synthase subunit D (FN1733), and a glycerol uptake facilitator protein (FN1838), all of which are associated with metabolic function. These genes may be indicative of the downstream processes that *S.s.* may ultimately trigger in

F.n., which is suggestive of significant events that may occur when the two bacteria come in contact with one another. Another induced *F.n.* gene, chaperone protein DnaK encoded by FN0116, is a predicted heat shock protein and chaperone system, and has been proposed to be linked to the 30S ribosomal subunit in *Escherichia coli*, specifically binding to pre-30S molecules and ultimately facilitating in assembly of components of the subunit [26]. Furthermore, it has been suggested that inhibiting the effect of the 30S subunit assembly through an aminoglycoside antibiotic such as paromomycin and neomycin inhibited viable cell numbers, protein synthesis, and growth rates of *E. coli* cells, having a similar effect of inhibiting translation within these cells [27]. Through induction of this gene, an increase in the assembly of the 30S ribosomal subunit may be indicative of cell growth of *F.n.* in a dual-species context, which may offer an alternate perspective from the stress response connotation of chaperone protein DnaK.

Moreover, it has been demonstrated that *F.n.* is sensitive to the presence of oxygen and hydrogen peroxide secreted within oral microbial communities, particularly those present in supragingival plaque [28, 29]. Although *F.n.* is a Gram-negative bacterium typically identified with subgingival plaque, studies have shown that it is often isolated from Gram-positive dominated supragingival plaque as well [30, 31, 32]. Characterized as an adaptation that helps oral streptococci survive and compete in oral microbial communities, *S.s.* has been shown to synthesize and secrete hydrogen peroxide, particularly in response to certain membrane-associated proteins of *F.n.* yet to be identified [28, 29]. This adaptation allows early colonizing Gram-positive bacteria to form a barrier, essentially creating a "colonization resistance" that must be overcome by Gram-negative bacteria that wish to integrate themselves into these

communities [28]. Thus, *F.n.*'s role as a bridging organism in multi-species oral communities between early and late colonizers implies that the Gram-negative bacterium must harbor certain adaptations which allow it survive forms of oxidative stress. Indeed, oxidative stress that is induced by oxygen or hydrogen peroxide has been shown to modify amino acid residues and drastically misfold proteins, rendering them functionally inactive, and eventually leading to their degradation [29, 33]. Subsequently, one way in which *F.n.* may alleviate the potentially lethal effect of protein misfolding is by upregulating chaperone proteins such as DnaK, allowing proteins to be refolded and functional once again, subsequently mediating binding between the two species [29]. This survival mechanism may allow *F.n.* to survive a hostile environment as it eventually binds with early colonizing Gram-positive bacteria to ultimately integrate within the oral microbial community.

To further expand on this idea of colonization resistance, repression of the remaining twelve genes during co-incubation with *S.s.* may be indicate that these genes may be involved in the initial interaction between the two species. It can be hypothesized that once contact is established between *F.n.* and *S.s.*, these genes are repressed, suggesting that they may no longer be required for any further interaction. Indeed, it has been demonstrated that another way *F.n.* evades the community resistance barriers generated by oral streptococci, and ultimately survives and integrates into an already established Gram-positive dominated microbial community, is by adhering to *S.s.* [28]. This adherence has been shown to reduce production of hydrogen peroxide from *S.s.*, by most likely inhibiting, or "camouflaging" the surface components of *F.n.*, while simultaneously increasing *F.n.*'s resistance to hydrogen peroxide [28]. Moreover, *S.s.* has been shown to aggregate with *F.n.* by forming specific morphological structures labeled as "corncobs"

[34]. This "corncob" formation between the two species has been hypothesized to act as a connecting link between Gram-positive dominated supragingival plaque and Gram-negative dominated subgingival plaque [34]. Thus, adhering and decorating itself with *S.s.* may allow *F.n.* to avoid detection by other Gram-positive species in the oral cavity that may produce reactive oxygen species [28]. Additionally, this adherence strategy may elicit a response in *F.n.* that promotes increased resistance to environmental stresses, as well as generate a differential gene expression pattern which may enhance the bacteria's survival in a mixed-species community [28]. This theory may explain the repression seen in this condition, and implies that *F.n.*'s response to the presence of *S.s.* is not stress-related. Rather, *F.n.* may be camouflaged by the adherence of *S.s.*, a phenomenon which may cause downregulation of metabolic genes. Thus, thus an upregulation of genes related to metabolic activity may not be required, which may be why an opposite trend is seen in majority of the genes in Table 4. It is important to note that this "evasion through adherence" strategy is not specific to *F.n.* and *S.s.*. Repression of over four-hundred proteins was seen in a proteomics study when the Gram-negative periodontal pathogen *Porphyromonas gingivalis* (*P.g.*) was co-aggregated with its interacting partners, *S. gordonii* (*S.g.*) and *F. nucleatum*, compared to *P.g.* cultivated alone [36]. Additionally, many pathogens are able to evade host immune responses via a "camouflage" method, in which they bind to proteins of the host and are ultimately recognized as "self" instead of "foreign" antigens. This implies that there are complex intracellular responses that are related to aggregate formation and cell-to-cell contact that are common in other oral microbial species as well.

If this "evasion through adherence" hypothesis is true, by removing an outer membrane protein of *F.n.* (RadD) that has been shown to be required for inter-species adherence between

F.n and *S.s.*, an opposite trend should be seen (Figure 4). This would be due to the fact that *F.n.* would not be able to bind to *S.s.*, and may no longer be decorated by streptococci that are still present in the surrounding environment. As a result, a majority of the genes that were repressed in wildtype *F.n.* co-incubated with *S.s.* may be induced when compared to the regulation seen in ΔRadD mutant. To further validate this point, when the ΔRadD mutant strain of *F.n.* was co-incubated with *S.s.*, an opposite trend was indeed seen. Nine out of fifteen genes, which showed an overlapping regulation compared to the same condition in wildtype *F.n.*, (FN0116, FN0559, FN0652, FN1380, FN1989, FN0471, FN1230, FN1253, FN1899), followed this trend, as shown in Table 5b. These genes encode proteins ultimately involved in cellular processes, transport, and membrane-association, with an additional two having a hypothetical function. Among them, the only one induced was chaperone protein DnaK (FN0116). As discussed earlier, just one possibility may be due to the presence of hydrogen peroxide produced by *S.s.* in response to *F.n.*. While it has been demonstrated that the ΔRadD mutant is defective in co-aggregation with Gram-positive primary colonizers, and that hydrogen peroxide production by *S.s.* is contact dependent, the exact *F.n.* protein that triggers this phenomenon in *S.s.* remains unknown [10, 28]. Thus, as previous studies have shown, hydrogen peroxide is still produced in *S.s.* even when co-incubated with the ΔRadD mutant, and this may be a stress response that relates to why DnaK is upregulated [28]. The other eight aforementioned genes show a repression in the ΔRadD mutant in response to *S.s.*. This would indicate that in comparison, these genes are upregulated in wildtype *F.n.*, demonstrating that when a large outer membrane protein responsible for binding to *S.s.* is missing, opposite regulation occurs.

Interestingly, the generated data may indicate that in the presence of *S.s.*, the outer membrane adhesin RadD of *F.n.* may play a key role in gene regulation, although it is not known to what extent. A hypothesis may be that the adhesin may act as a sensor that mediates the regulation of other genes through signal transduction pathways, thus playing an important part in gene expression in *F.n.*. Due to the fact that RadD is an outer membrane adhesin, there may also be a two-component system involved, which along with RadD allows *F.n.* to sense the presence of *S.s.*, and subsequently mediate a signaling response through the differential expression of target genes [35]. Ultimately, how a signaling pathway is able to utilize the RadD adhesin and mediate gene expression after *F.n.* adheres to *S.s.* warrants further studies. Furthermore, studies have indicated that RadD is the last gene of a putative four gene operon, with the first three genes characterized as RadA (FN1529) , RadB (FN1528), and RadC (FN1527) [10]. It has been demonstrated (unpublished data) that a RadABC knockout strain expresses RadD at a considerably higher level, and has increased co-aggregation with many common Gram-positive and Gram-negative oral species compared to wildtype *F.n.*. This increase in RadD expression may in fact be a compensatory mechanism to overcome the loss of proteins encoded by RadABC, all of which may play an additional role in this signaling process.

Perhaps the most important aspect of this study surrounds the fact that the conditions tested looked at wildtype *F.n.* and its ΔRadD mutant in the presence of *S.s.*. Through the data generated, it is possible that the missing RadD adhesin could be responsible for the some of the differential regulation that is seen (Table 5a). To rule out this possibility, regulation seen from the ΔRadD mutant was compared to that of wildtype *F.n.*., and ultimately only five genes showed differential regulation (Table 6). Majority of the genes in the ΔRadD background that

showed a dramatic difference when co-incubated with *S.s.* did not show any regulation in a ΔRadD background. This implies that these genes are strictly related to the presence of *S.s.*, and that the RadD adhesin alone has a minimal impact to the regulation of genes when *S.s.* is no longer present. Furthermore, this data set allowed for the determination of two types of gene expression: those that are RadD-dependent and those that are RadD independent in response to *S.s.* (Table 5b). Exactly how RadD is responsible for the induction or repression for a variety of genes in response to *S.s.*, and how RadABC may have an additional intermediate role in this process, warrants further studies.

CONCLUSION

Individual gene inactivation methods in *Fusobacterium nucleatum* (*F.n.*), in conjunction with co-aggregation assays and *in-vitro* biofilms, have been important tools to elucidate the mechanisms behind cell-to-cell contact and adherence. However, not many studies have been done in *F.n.* to investigate the intracellular events that occur as a consequence of these interactions. Subsequently, our goal was to use transcriptional analysis to determine the type of differential gene regulation occurring in *F.n.* in the presence of a primary colonizer. In this study, we have validated a microarray analysis previously done to study transcriptional changes in *F.n.* along with its outer-membrane L-arginine-inhibitable adhesin mutant, ΔRadD, in response to *Streptococcus sanguinis* (*S.s.*). By doing so, we have successfully developed an effective Real-Time PCR assay to quantify gene expression in *F.n.*.

Through the investigation of twenty-four genes selected from the microarray analysis, majority of the genes demonstrated the same trend of regulation between microarray and Real-Time PCR. A minority of genes showed discrepancy in regulation and fold change, which may be due to experimental shortcomings of microarray analyses. Seventeen genes had shown differential regulation in wildtype *F.n.* responding to *S.s*. (Table 4). As many of these genes were repressed, it was hypothesized that these genes may no longer be required for further interaction once contact is established between the two species. Furthermore, compared to wildtype *F.n.*, twenty genes showed regulation in the ΔRadD mutant in response to *S.s.*, and five genes showed regulation in the ΔRadD mutant grown in the absence of *S.s.* (Tables 5 & 6). As a result of these analyses, there is evidence that transcriptional changes do indeed occur when *S.s.* associates with

F.n., and these changes may result from adherence during co-incubation. As the *F.n.* RadD adhesin has been shown to be responsible for co-aggregation with *S.s.*, removing this adhesin showed opposite regulation for six genes in the presence of to *S.s.* compared to wildtype *F.n.*, indicating that there is regulation dependent on the RadD adhesin. The ΔRadD mutation itself was shown to have a minimal impact on *F.n.* cells when *S.s.* was not present, confirming that the dramatic differences in gene regulation occur only in response to *S.s.*. Most excitingly, to the best of our knowledge, this is the first discovery of gene expression that relies on the presence of an interspecies recognition based outer membrane protein.

Although our lab has successfully characterized the importance of the RadD adhesin in *F.n.* using both genetic and global approaches, precisely how gene expression may be mediated through this adhesin is currently unknown. It has been hypothesized in this study that RadD may ultimately act as a mediator in a signal transduction pathway, and that the differentially regulated genes may be involved in species-specific recognition within the context of an oral biofilm. As such, there are a variety of follow-up studies that can be done to further investigate *F.n.*'s RadD adhesin and interspecies interaction in the oral cavity. The first future study should be centered on the ΔRadABC mutant generated in our lab, which has shown increased RadD expression and co-aggregation with Gram-positive primary colonizers (unpublished data). It would interesting to look at the twenty-four *F.n.* genes tested in this study in an *F.n.* ΔRadABC mutant background, with and without the presence of *S.s.* and compare the data. This may allow us to further understand what role, if any, the RadABCD operon may play in this hypothesized signaling process, and how higher RadD expression may affect gene regulation in the presence of *S.s.*. Another future study should focus on genes that are RadD dependent. It would be interesting to

test another Gram-positive primary colonizer such as *Streptococcus gordonii.* or *Streptococcus oralis* and perform a similar study to determine if the gene regulation seen in *F.n.* and the ΔRadD mutation followed a similar pattern to this study. Furthermore, it has been demonstrated previously that the receptor on *S.s.* that adheres to RadD is most likely a surface protein, since no co-aggregation was observed with the RadD mutant [10]. Additional study on this *S.s.* protein would allow us to delve into both the *F.n.* and *S.s.* aspect of co-aggregation, and give us a better picture on how these two species co-exist in biofilms. Each of these future studies would ultimately provide greater insight into the social behavior of bacteria within the oral microbial community, and provide us with a deeper understanding of the differential gene regulation that consequently occurs among bacterial colonizers.

FIGURES

Figure 1. Role of co-aggregation in biofilm development

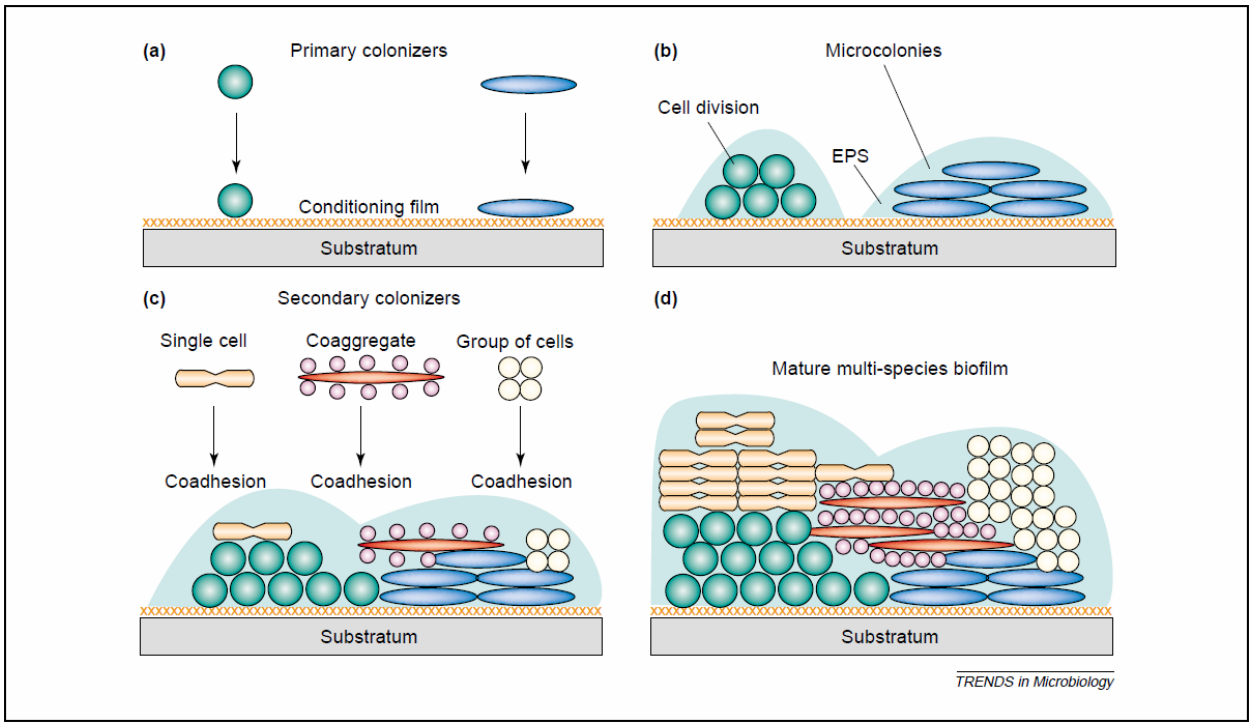

Figure 1. Diagram adopted from Rickard, et. al. [5], which illustrates the proposed role of co-aggregation in the development of oral biofilms. A) Represents primary colonization of a substratum, analogous to the tooth surface, that is covered in a conditioning film, analogous to the dental pellicle, consisting of proteins and polysaccharides. B) Represents cell growth and division of these primary colonizers. C) Represents co-aggregated cells which coadhere to the already established biofilm. D) Represents a mature biofilm in which co-aggregated cells have integrated and proliferated into the niche.

Figure 2: Temporal nature of human oral bacterial colonization on tooth surfaces

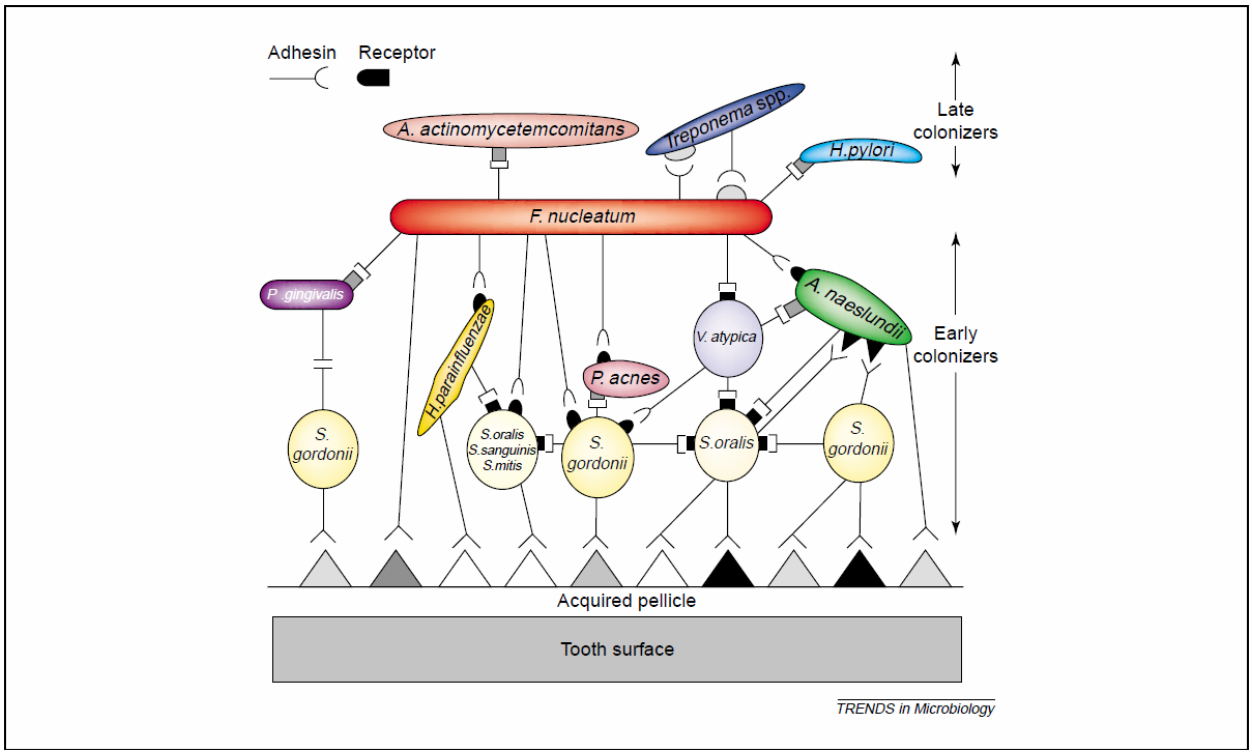

Figure 2. Diagram adopted from Rickard, et. al. [5], which illustrates the succession of oral bacteria on the tooth surface which ultimately initiates biofilm formation. The species that are represented here have been characterized according to when they colonize the tooth surface. Primary colonizers include *Streptococcus, gordonii, Streptococcus mitis, Streptococcus oralis, Streptococcus sanguinis, Actinomyces naeslundii.* Secondary colonizers include *Helicobacter pylori, Treponema denticola,* and *Actinobacillus actinomycetemcomitans*, the last two which are associated with periodontal disease. Fusobacterium *nucleatum* is a well characterized intermediate colonizer that can bind to both primary and secondary colonizers. In this diagram, known interactions between bacterial species such co-aggregation and adherence is represented by sets of adhesin-receptor symbols.

Figure 3. Gene expression of *Fusobacterium nucleatum* in response to *Streptococcus sanguinis*

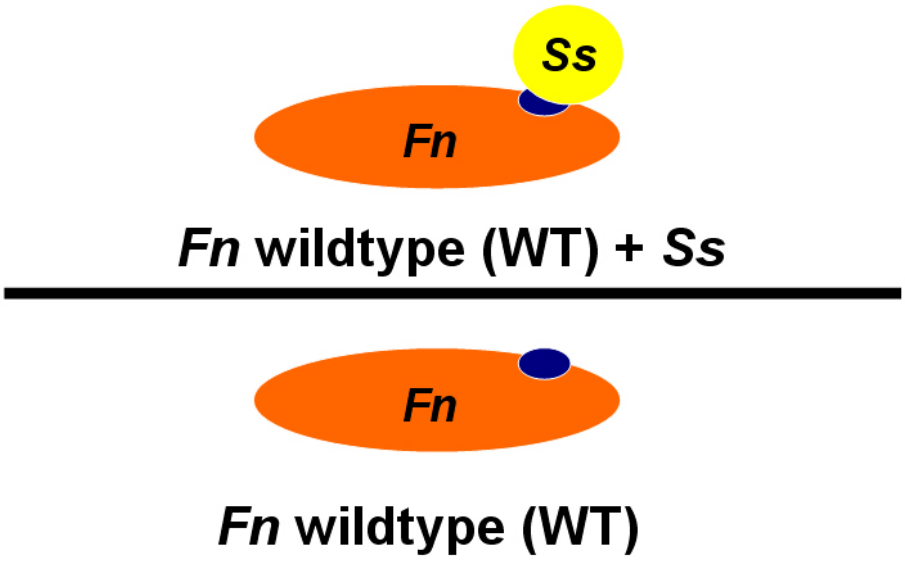

Figure 3. This figure illustrates the calculation used to determine gene regulation in *Fusobacterium nucleatum* (*F.n.*) in response to *Streptococcus sanguinis* (*S.s.*). The blue circle represents the RadD outer membrane adhesin on the surface of *Fusobacterium nucleatum*. To determine this using Real-Time PCR, a calculation was used where *F.n.* ATCC 23726 grown with *S.s.* was taken as the experimental condition, and *F.n.* ATCC 23726 was taken as the control condition. The starting quantity values were then normalized, and calculated using the following equation: (Wt + *S.s.* / Wt). Positive values indicate upregulation in response to *S.s.*, while negative values indicate downregulation in response to *S.s.*.

Figure 4. Determining the role of RadD in gene regulation in the presence of *Streptococcus sanguinis*

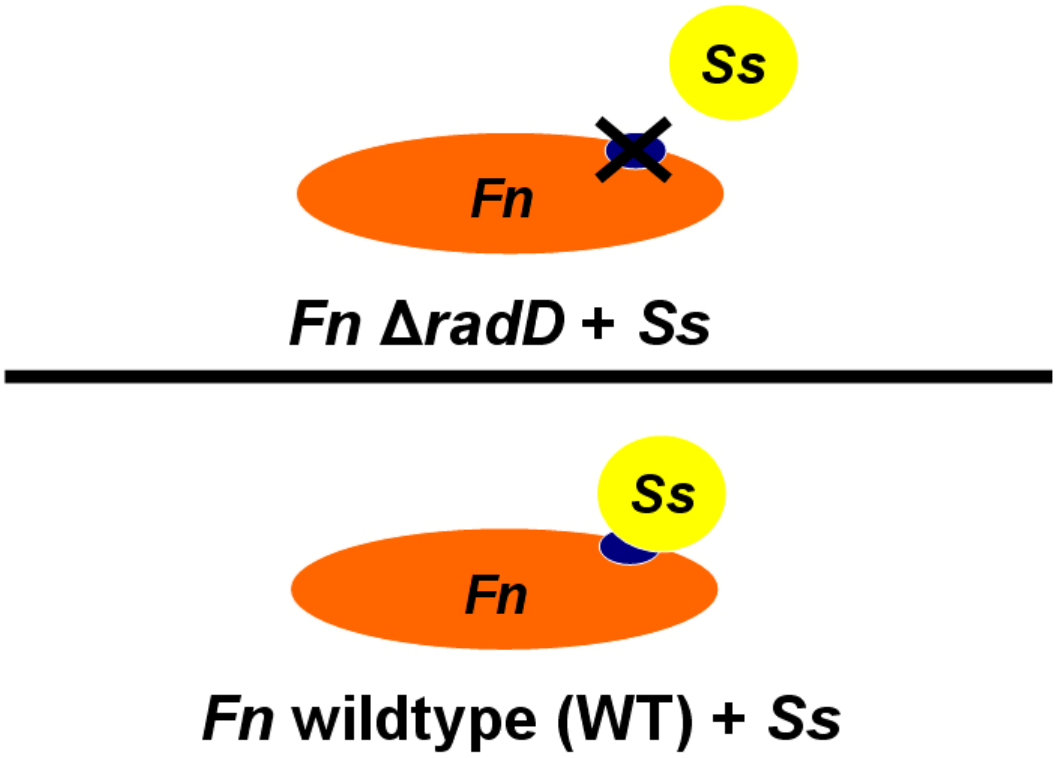

Figure 4. This figure illustrates the calculation used to determine gene regulation that may or may not be dependent on the RadD adhesin in response to *Streptoccocus* sanguinis. In order to investigate if the regulation of genes is attributed through an interaction via RadD using Real-Time PCR, a calculation was used where the ΔRadD mutant co-incubated with *S.*s was taken as the experimental condition, and the regulation was compared to wildtype *F.n.* co-incubated with *S.s.* The starting quantity values were then normalized, and calculated using the following equation: (ΔRadD + *S.s.* / Wt + *S.s*).

Figure 5. Determination of RadD-dependent Gene Regulation

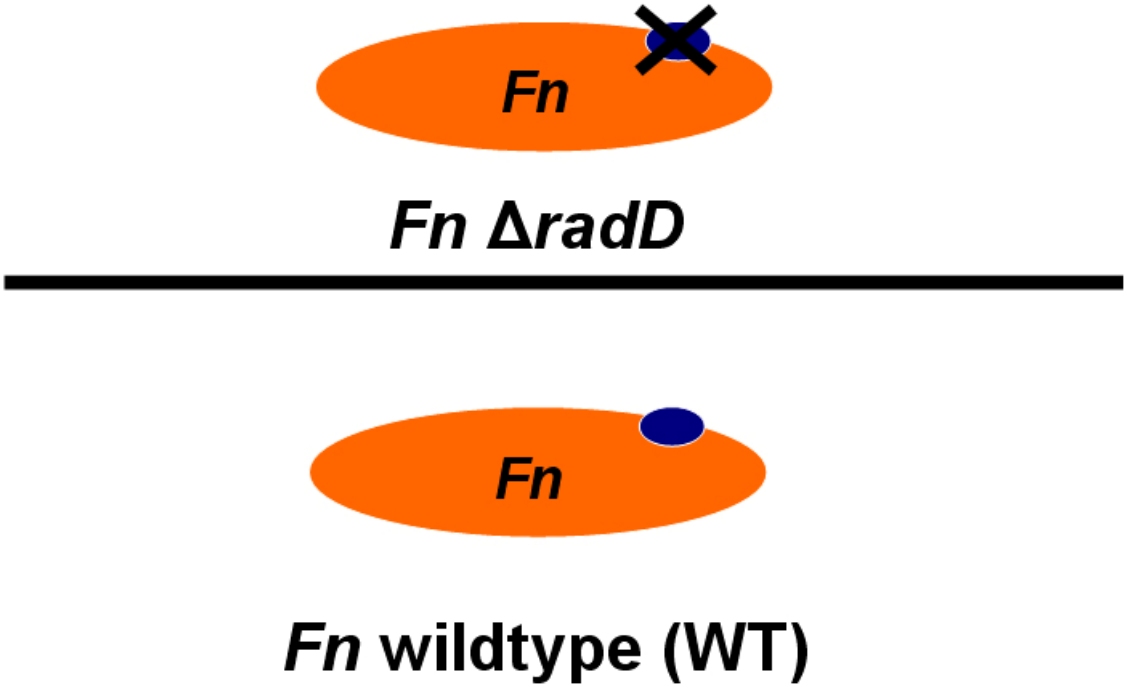

Figure 5. This figure illustrates the calculation used to determine gene regulation specific to the RadD adhesin in comparison to wildtype *Fusobacterium nucleatum*. To determine this using Real-Time PCR, a calculation was used where the ΔRadD mutant grown alone was taken as the experimental condition, and *F.n.* ATCC 23726 was taken as the control condition. The starting quantity values were then normalized, and calculated using the following equation: (ΔRadD / Wt).

Figure 6. Classes of genes tested in *Fusobacterium nucleatum*

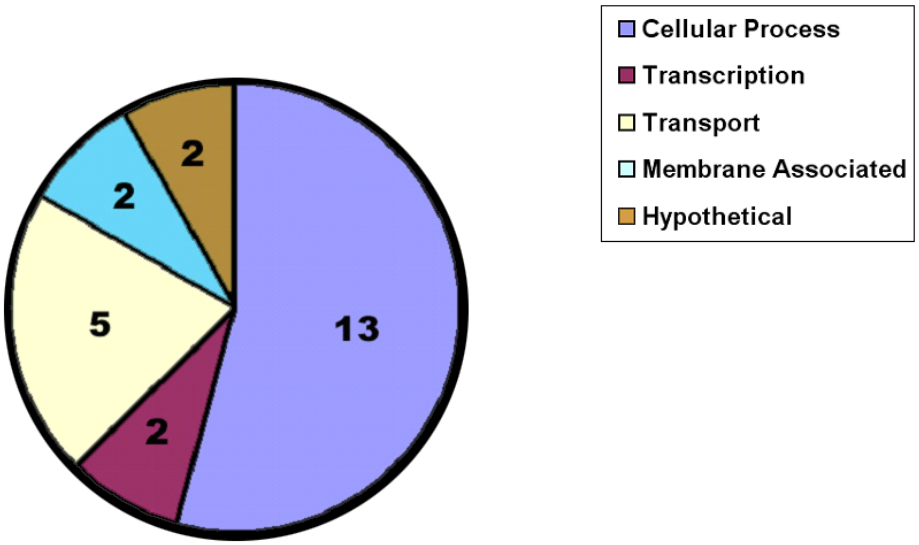

Figure 6. This is a pie chart illustrating the different classes of genes tested in Fn. Majority of the genes tested were involved in cellular process, representing 13 out of the 25 genes tested. Genes involve in transport were the next highest, representing five genes tested. Membrane associated genes, transcriptional genes, and genes encoding hypothetical protein represented 2 of the 25 genes tested, respectively.

TABLES

Table 1: Bacterial strains used in this study

Strain	Source	Characteristics
Fusobacterium nucleatum spp. nucleatum	**ATCC 23726**	**Intermediate Colonizer**
***Fusobacterium nucleatum spp. nucleatum* ΔFN1526**	**Shi Lab** [10]	**Outer membrane adhesin (RadD) Mutant**
Streptococcus sanguinis	**ATCC 10556**	**Primary Colonizer**

Table 2: Primers used in this study

Gene ID	Forward primer 5'-3'	Reverse primer 5'-3'
FN16SrRNA	TTGGACAATGGACCGAGAGT	GCCGTCACTTCTTCTGTTGG
FN0116	AGCAACACAAGGACCTACAA	CCATTCTTGAACTGCTGGTATTC
FN0205	AGGTTGTATGCTTGGAGGAC	CCTGATTCACCTTCTGCATCTA
FN0242	TTGCTCAAGCAGAAGGAATAA	TCAGCAGCAGCACCTAAA
FN0271	GTCTTGCACTTGGAGGAGATT	CCACCAGTATCAGGAACAAGTC
FN0392	GGGTTTCTATCTATGGAACAGCTA	TCCACGCCTAAATAGACAAGAC
FN0471	GGTTCTTTAAGTGCAGCTCTTGCTGG	GTCCTAATGCAGCCATAACTTGCG
FN0559	ATGCAAACCCAGAGGATACAA	CAAGAACTGCCAAACCAACTC
FN0652	GCACCAGCTACTGGAGATTT	CCATTGGAGCAAGACAGTTAGT
FN1079	TGGCTCAAGCAGAAGGAATAA	TCAGCAGCAGCACCTAAA
FN1089	GTTGCTGCCTCTTCTTGAATTT	GTCCACCACTGCAACCTATT
FN1230	TTCAAGGTGGAACAGGTGAC	CTCCAGGCTTTGTACTTTCCT
FN1253	TACAGGAGGTGCCGTAGCAG	TTTTTGTTAATTCTCCAGCTCCA
FN1317	GGAGTAGAAGATTGCTGTGGAA	AAGTTCATGTGCCTTAATCAATCC
FN1318	AGGGAACATAGGCCTTATGAAAG	GCCTGTCTTATCCACCAAGTA
FN1380	GTGCTTGTGGTTCTATGGGATA	GCTGGTAAGTTAGGGAAAGGAA
FN1733	TGGCTTTGTTCAAACTTCAGC	AGAGCATTAACTCTTCTTCTTGTCT
FN1838	CCAGAACCTGCAGTGAAATTAG	TCCTACTTCGCCATAACCAATA
FN1839	ATAGCAGGAGTAGCAGGAGAT	GCAAGAAACAACCTGTTCCATAA
FN1840	TGGCTCAAGCAGAAGGAATAA	TCAGCAGCAGCACCTAAA
FN1860	AGACTGTTGCAGTTGTAGGAGCCA	TCCTCCTGCTATAGCTGCTGTTGT
FN1899	TGAAGGAATCCCAGGAACTTATC	CTTGTACAGGATCTTCGTGAGTAT
FN1989	TGTTGGATCTGCTGTTGGAATGGC	CCAAGTGTTCCTGTTTCAGCTCTTC
FN2036	CAAAAACTCATTGAAAGACTTGATTTT	GAATGCTAATTCAAATCCTTTTTCTTC
FN2103	GGCTGATAGCCAATTCCAAAC	GGTTAGAAGCACCTGTACCATTA

Table 3. Housekeeping genes regulated in *Fusobacterium nucleatum* showing regulation in tested conditions

Class	Locus	Common Name	Wt + *Ss* / Wt	ΔRadD + *Ss* / Wt + *Ss*	ΔRadD / Wt
Housekeeping Gene	FN2036	DNA-directed RNA polymerase beta chain (*rpoB*)	1.2	0.97	0.70
Housekeeping Gene	FN16SrRNA	Fusobacterium nucleatum 16S ribosomal RNA	0.75	1.2	1.4

Table 4. Genes regulated in *Fusobacterium nucleatum* in response to *Streptococcus sanguinis* - (Wt + *S.s.* / Wt) -

Class	Locus	Common Name	RT-PCR		Microarray	
			Induced	Repressed	Induced	Repressed
Cellular process	FN0116	chaperone protein dnaK	2.33		18.1	
Cellular process	FN0271	Enoyl-CoA hydratase	6.72		8.3	
Cellular process	FN0392	Oxygen-independent coproporphyrinogen III oxidase		-23.3		-5.3
Cellular process	FN0559	Phosphoglucomutase		-30.4		-5.0
Cellular process	FN0652	Glyceraldehyde 3-phosphate dehydrogenase		-105.7		-6.4
Cellular process	FN1079	Neutrophil-activating protein A	5.50		18.4	
Cellular process	FN1089	ATP-binding protein		-9.7		-3.0
Cellular process	FN1380	Citrate lyase beta chain		-5.7		-3.1
Cellular process	FN1733	V-type sodium ATP synthase subunit D	2.70		5.4	
Cellular process	FN2103	RecA protein		-55.2		-5.0
Transport	FN1838	Glycerol uptake facilitator protein	2.49		3.1	
Transport	FN1860	NA+/H+ antiporter NHAC		-4.9	2.4	
Transport	FN1989	Sodium-dependent tyrosine transporter		-7.0	2.2	
Membrane-Associated	FN0471	Outer Membrane Protein		-3.9	2.2	
Membrane-Associated	FN1899	Hypothetical lipoprotein		-98.4		-5.7
Hypothetical	FN1230	Hypothetical cytosolic protein		-11.6		-3.3
Hypothetical	FN1253	Function Unknown		-14.9		-6.9

Note: Majority of genes tested demonstrated the same trend of regulation between microarray and Real-Time PCR. Discrepancy in regulation and fold change was seen for a small number of genes in this study.

Table 5a. Determining the role of RadD in gene regulation in the presence of *Streptococcus sanguinis* - (ΔRadD + *S.s.* / Wt + *S.s*) -

Class	Locus	Common Name	RT- PCR		Microarray	
			Induced	Repressed	Induced	Repressed
Cellular process	FN0116	chaperone protein dnaK	8.3		81.6	
Cellular process	FN0271	Enoyl-CoA hydratase		-19.4		-33.0
Cellular process	FN0392	Oxygen-independent coproporphyrinogen III oxidase	2.2			-2.9
Cellular process	FN0559	Phosphoglucomutase		-6.8		-12.2
Cellular process	FN0652	Glyceraldehyde 3-phosphate dehydrogenase		-10.1		-5122.9
Cellular process	FN1079	Neutrophil-activating protein A		-3.2		-10.5
Cellular process	FN1380	Citrate lyase beta chain		-6.8		-5.2
Cellular process	FN1733	V-type sodium ATP synthase subunit D		-70.7		-8.6
Cellular process	FN1839	Glycerol kinase		-3.8		-3.6
Cellular process	FN1840	Dihydroxyacetone kinase		-4.9		-3.0
Transcription	FN1317	RNA polymerase sigma factor		-2.2		-14.0
Transcription	FN1318	RNA polymerase sigma factor rpoD		-2.5		-6.4
Transport	FN0205	Sodium/glutamate symport carrier protein	2.6		6.2	
Transport	FN1838	Glycerol uptake facilitator protein		-28.9		-3.0
Transport	FN1860	NA+/H+ antiporter NHAC	2.7		1.7	
Transport	FN1989	Sodium-dependent tyrosine transporter		-3.1		-48.0
Membrane-Associated	FN0471	Outer Membrane Protein		-3.4		-495.7
Membrane-Associated	FN1899	Hypothetical lipoprotein		-3.6		-7.1
Hypothetical	FN1230	Hypothetical cytosolic protein		-10.82		-32.8
Hypothetical	FN1253	Function Unknown		-3.70		-26444.0

Table 5b. Genes showing overlapping differential regulation between different tested conditions

Class	Locus	Common Name	Wt + *Ss* / Wt	ΔRadD + *Ss* / Wt + *Ss*	ΔRadD / Wt	Type of Regulation
Cellular process	FN0271	Enoyl-CoA hydratase	↑	↓	NR	RadD-Dependent Gene Regulation in Response to *Ss*
Cellular process	FN0392	Oxygen-independent coproporphyrinogen III oxidase	↓	↑	NR	
Cellular process	FN1733	V-type sodium ATP synthase subunit D	↑	↓	NR	
Transport	FN1860	NA+/H+ antiporter NHAC	↓	↑	NR	
Cellular process	FN0116	chaperone protein dnaK	↑	↑	NR	RadD-Independent Gene Regulation in Response to *Ss*
Cellular process	FN0559	Phosphoglucomutase	↓	↓	NR	
Cellular process	FN0652	Glyceraldehyde 3-phosphate dehydrogenase	↓	↓	↑	
Cellular process	FN1380	Citrate lyase beta chain	↓	↓	NR	
Transport	FN1989	Sodium-dependent tyrosine transporter	↓	↓	NR	
Membrane-Associated	FN0471	Outer Membrane Protein	↓	↓	NR	
Hypothetical	FN1230	Hypothetical cytosolic protein	↓	↓	NR	
Hypothetical	FN1253	Function Unknown	↓	↓	NR	
Cellular process	FN1079	Neutrophil-activating protein A	↑	↓	↑	Genes Regulated Independent of RadD & *Ss*?
Transport	FN1838	Glycerol uptake facilitator protein	↑	↓	↑	
Membrane-Associated	FN1899	Hypothetical lipoprotein	↓	↓	↓	

Note: Arrows represent induction (↑) or repression (↓), adapted from respective RT-PCR values. **NR** = No Regulation

Table 6. Determination of RadD-dependent Gene Regulation - (ΔRadD / Wt) -

Class	Locus	Common Name	RT- PCR	
			Induced	Repressed
Cellular process	FN0652	Glyceraldehyde 3-phosphate dehydrogenase	2.61	
Cellular process	FN1079	Neutrophil-activating protein A	8.80	
Transport	FN0242	Trk system potassium uptake protein trkA	8.07	
Transport	Fn1838	Glycerol uptake facilitator protein	2.18	
Membrane-Associated	FN1899	Hypothetical lipoprotein		-2.5

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