Published in final edited form as:

Mol Oral Microbiol. 2017 October ; 32(5): 432–442. doi:10.1111/omi.12184.

Survival of an *Aggregatibacter actinomycetemcomitans* quorum sensing *luxS* mutant in the mouths of Rhesus monkeys: Insights into Ecological Adaptation

Senthil Kumar Velusamy^{1,+}, Vandana Sampathkumar^{1,+}, Dipti Godbole¹, and Daniel H. Fine¹

¹Department of Oral Biology, Rutgers School of Dental Medicine, Rutgers University, Newark, New Jersey, USA

SUMMARY

Experiments were designed to explore a prominent autoinducer-2 (AI-2) producing gene (*luxS*) related to colonization and survival of *Aggregatibacter actinomycetemcomitans*, a low abundance member of the indigenous flora, that forms a key component of the dysbiotic flora in localized aggressive periodontitis. The *luxS* gene was disrupted in a primate strain of *A. actinomycetemcomitans* prior to implantation into the oral cavity of Rhesus monkeys (Rh). The colonization efficiency of the *luxS* mutant (RhAa-VS4) was compared to the parental wild-type strain (RhAa3) (positive control) and a *ltxA* mutant (RhAa-VS2) (negative control). The *in vivo* results showed that the *luxS* mutation had minimal impact on *A. actinomycetemcomitans* colonization as compared to the wild-type RhAa3 strain. *In vitro* studies revealed that there was a significant up-regulation of attachment related *aae*, *apiA* and *flp* in the RhAa-VS4 strain as compared to RhAa3. Biofilm forming ability was also significantly increased in RhAa-VS4 strain compared to RhAa3, while the AI-2 signal was ablated. The addition of the AI-2 precursor dihydroxy pentanedione (DPD) allowed the RhAa-VS4 strain to achieve RhAa3 biofilm levels. This is the first primate study to test the relevance of *LuxS* *in vivo*. *In vitro* assessment suggests that survival of RhAa-VS4 strain was due to the production of signaling autoinducer molecules derived from other members of the flora as well as the up-regulation of genes related to attachment and biofilm formation.

Keywords

Rhesus monkey; Autoinducer-2; Quorum sensing; Biofilm; Oral colonization

INTRODUCTION

Aggregatibacter actinomycetemcomitans is a Gram-negative oral bacterium implicated in localized aggressive periodontitis (LAP), an aggressive form of periodontal disease that

Correspondence: Daniel H. Fine, 185 South Orange Avenue, Medical Science Building Rm C-636, Newark New Jersey, 07103, Phone: 973-972-3728, FAX: 973-972-0045, finedh@sdm.rutgers.edu.

⁺These authors contributed equally

DR DANIEL FINE (Orcid ID : 0000-0001-6242-6451)

causes loss of front teeth and molars in African American adolescents¹. *A. actinomycetemcomitans* is often thought as an exogenous pathogen. However, it has been shown that *A. actinomycetemcomitans* is a member of the indigenous oral microflora^{1, 2}. In spite of these diverse opinions, an in-depth evaluation of colonization and survival of *A. actinomycetemcomitans* is likely to reveal microbial strategies used by *A. actinomycetemcomitans* to cope with environmental stressors³.

Much has been learned about *A. actinomycetemcomitans* key virulence attributes by mutating its virulence genes and studying the altered phenotype in an idealized *in vitro* environment⁴⁻⁸. However, these virulence attributes have not been studied in an ecologically competitive mammalian environment resembling that seen in humans^{4, 9-11}. The survey of *A. actinomycetemcomitans* in Old World monkeys shown that 80% of monkeys harbored *A. actinomycetemcomitans* strains in their oral cavity¹². The biological principle supporting this fact is derived from the evidence that adhesins on the surface of *A. actinomycetemcomitans* show specificity for cells derived from humans and Old World primates¹¹.

In our lab, a Rhesus monkey (Rh) model was designed to determine how *A. actinomycetemcomitans* can attach, colonize, and be retained in the primate oral cavity in a real-world environment where *A. actinomycetemcomitans* occurs naturally¹¹. The current experiment was designed to further explore the ability of specific virulence traits that could influence *A. actinomycetemcomitans* survival in a competitive *in vivo* environment. To accomplish this task we mutated, *luxS*, an autoinducer-2 (AI-2) producing gene¹³.

The purpose of these experiments was to 1) compare the oral colonization efficiencies of a *luxS* mutant in comparison to wild-type strains of *A. actinomycetemcomitans* in an animal model, and 2) to examine the effects of the *luxS* mutation *in vitro* to understand the *in vivo* results observed. The present study showed that the *luxS* mutation had minimal impact on *A. actinomycetemcomitans* colonization. The *in vitro* study confirmed expectations that the *luxS* disruption, can act as a global regulator, upregulating genes responsible for supragingival attachment and biofilm formation. The results show that *in vivo* experiments supplemented by *in vitro* data confirms the global regulatory effects of LuxS as seen in a dynamic microbial ecosystem.

MATERIALS AND METHODS

Bacterial strains, growth conditions and plasmids

The strains and plasmids used in this study are listed in Table 1. Rh monkey derived fimbriated, serotype 'a' *A. actinomycetemcomitans*, wild type RhAa3 was the parental strain used in this study¹⁴. A spectinomycin resistant wild-type strain was developed by sub-culturing the strain in the media with increasing concentration of spectinomycin (Sigma-Aldrich, St. Louis, MO) until it reaches 50 µg resistance. *A. actinomycetemcomitans* strains were grown on Brain Heart Infusion (BHI) agar plates containing 0.6% yeast extract (Beckton Dickinson, Franklin Lakes, NJ) supplemented with 0.8% dextrose and 0.4% sodium bicarbonate. For liquid biofilm cultures, BHI broth with supplements was used. For the selection of mutant strain, 50 µg/ml of spectinomycin was supplemented in the growth

media. The strains were grown in a 10% CO₂ incubator for 16/48 h at 37°C. *E. coli* strains were grown on LB agar or LB broth supplemented with appropriate antibiotics in an aerobic 37°C incubator.

DNA procedures

All DNA manipulations were carried out as previously described¹⁵. The PCR amplifications were performed using Phusion DNA polymerase as per manufacturer's instructions (ThermoFisher, Waltham, MA). All primers used in this study are listed in Table 2 and were synthesized from Integrated DNA technologies (Coralville, IA). Genomic, plasmid DNA isolation and gel extraction kits (Qiagen, Foster City CA), and restriction enzymes (New England Biolabs, Ipswich, MA) were used in this study as per the manufacturer's directions. Plasmids were transformed into One Shot Mach-1 T1 *E. coli* (Life technologies, Grand Island, NY) or Stellar Competent Cells (Clontech Laboratories, Mountain View, CA) as described by the manufacturer and selected on LB agar plates supplemented with the appropriate antibiotics. Recombinant plasmids were transformed into *A. actinomycetemcomitans* by electroporation. All plasmid constructs and *A. actinomycetemcomitans* disrupted strains were verified by DNA sequencing (Macrogen Inc, New York NY).

Construction of an *A. actinomycetemcomitans luxS* mutant

A *luxS* disrupted strain was constructed by insertional inactivation of a spectinomycin cassette (*aadA*) gene as described for creating the RhAa-VS2 strain¹⁴ and the resultant strain was designated as RhAa-VS4. The primers used in this study are listed in Table 2. Briefly, a 3-kbp Phusion DNA polymerase amplified PCR fragment comprising the *luxS* gene and its flanking regions using primers *KpnI luxSF* and *SacI luxSR* was digested with *KpnI* and *SacI* and ligated into restriction digested plasmid pBluescript II KS (+) (Agilent Technologies, Santa Clara, CA). This ligation reaction mixture was transformed into One Shot Mach-1 *E. coli* competent cells to obtain the recombinant plasmid pVS20. To introduce *XbaI* and *PstI* sites within *luxS* ORF, a PCR reaction was carried out with primers *XbaI luxSF* and *PstI luxSR* using pVS20 as the template. The amplified product was restriction digested with *XbaI* and *PstI*. Then the *aadA* gene was amplified from the pJAK12 blue plasmid using primers *PstI aadAF* and *XbaI aadAR* and the PCR product was restriction digested, ligated and transformed into One Shot Mach-1 *E. coli* competent cells. The resultant recombinant plasmid was designated as pVS23. The insertion of the *aadA* cassette within the *luxS* ORF in pVS23 was confirmed by sequencing. The final *luxS* disruption plasmid was electroporated into RhAa3 as described previously¹⁴. The electroporated cells were selected against spectinomycin (50 µg/ml). The cells that had undergone double crossover event were screened using primers *luxSscreenF* and *luxSscreenR* and the PCR products were confirmed by sequencing (Macrogen USA, New York, NY). The resultant *luxS* knockout strain was designated as RhAa-VS4.

Congo red and crystal violet staining of biofilm

A. actinomycetemcomitans strains were grown in a 96 well plate for 16 h. The supernatant was discarded and the biofilm was stained with 2mg/ml of congo red (Fisher Scientific, Fair Lawn, NJ) and Gram crystal violet (BD& Company, Sparks, MD) independently for 20 min.

Excess stain was removed by rinsing the wells with distilled water and after drying the plates the intensity of the congo red and crystal violet were read at OD_{419 nm} and OD_{495 nm} respectively. Experiments were conducted in triplicate and the significant difference in EPS production between the strains was calculated by one-way ANOVA with Tukey's post-hoc multiple comparison test. A $P < 0.05$ was considered as significant.

Confocal microscopic analysis of the biofilms

Confocal microscopy was performed as described previously⁹. Confocal imaging of biofilm structures were carried out using a Nikon A1R-A1 confocal microscope with Plan Apo VC 20× or 60× WI DIC N2 objective lenses in 10% CO₂ atmospheric condition throughout the image capture. The biofilm 3D architecture was evaluated in the 16 h biofilms. The 48 h biofilms were captured and analyzed in 9–11 different zones. To calculate the ratio of live/dead cells, NIS Elements software (Version 4.2, Nikon, Melville, NY) was used as described previously¹⁴.

RNA isolation and qRT-PCR

The total RNA isolation, purification, and genomic DNA removal were carried out as previously described⁹. The total RNA was converted to cDNA using the High Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Light Cycler 480 system (Roche Life Science, Indianapolis, IN) was used for quantitative detection of cDNA by appropriate dilutions. Typically, 1:100 diluted cDNA was used for 5S rRNA, the reference gene and a 1:25 dilution was used for targeted genes. The PCR cycling conditions were used as follows: initial denaturation at 95°C for 10 min followed by 40 cycles of amplification (94°C, 10 sec; 55°C, 10 sec, and 72°C, 20 sec). Following the amplification cycle, melting curve analysis was carried out to assess the PCR product specificity. Each amplicon specific for the target and reference genes were sequenced before the data analysis. All the primers used for the qRT-PCR detection experiment were obtained from our previous study¹⁴.

Chemical complementation of *luxS* mutant

The complementation experiments were carried out with chemically synthesized dihydroxy pentanedione (DPD), a precursor of AI-2 molecule (CRO laboratories Inc, Dallas, TX). Briefly 100 nM of DPD was added to the freshly inoculated cells and the biofilm was allowed to grow for 16/48 h in a 10% CO₂ incubator at 37°C. This biofilm was analyzed by confocal microscopy imaging, congo red staining, crystal violet staining and qRT-PCR analysis as described previously.

AI-2 bioassays using the indicator strain *Vibrio harveyi* BB170

A. actinomycetemcomitans strains were grown in BHI broth at 37°C in a 10% CO₂ incubator. Samples of the biofilm culture supernatants were withdrawn from the plate every hour over a period of 8 hours. Cell-free culture supernatant samples were 0.22µ filter sterilized. The samples were stored at -20°C until used for the AI-2 assay. The detection of AI-2 from different *A. actinomycetemcomitans* culture supernatants using *V. harveyi* BB170 (*luxN*) was performed as previously reported^{16–18}. Briefly, an overnight culture of BB170

was diluted 1: 2,000 in autoinducer bioassay (AB) medium and 100 µl of cell-free *A. actinomycetemcomitans* culture fluid was added to 900 µl of diluted *V. harveyi* cells. Cell-free culture fluid of DPD (100 nM) supplemented *A. actinomycetemcomitans* was included as a positive control, and the sterile AB medium was included as a negative control. The reaction was carried out at 30°C, and light production was monitored in a Tecan Infinite M200 Pro plate reader (Mannedorf, Switzerland) and the luminescence induction was expressed as a percentage relative to luminescence units.

***In vivo* oral colonization in a non-human primate**

The *in vivo* colonization study using the non-human primate model was designed to compare the colonization abilities of RhAa3, RhAa-VS2, and RhAa-VS4. The detailed experimental plan was designed as described in Fig. 1. The experimental groups were comprised of 2 to 5-year old male Rhesus monkeys (*Macaca mulatta*) born and bred in the New England Primate Research Center, Southborough, MA. All experimental procedures were conducted in accordance with the Institutional Animal Care and Use Committees (IACUC) at Rutgers School of Dental Medicine, Newark, NJ, and the New England Primate Research Center (NEPRC). Rh monkeys were divided into four groups, each group comprised of 5 monkeys ($n=5$). *A. actinomycetemcomitans* strains were inoculated after thorough cleaning and oral prophylaxis as described previously¹¹. The monkeys were caged separately after inoculation to avoid cross contamination of strains between the monkey groups. All monkeys were served with soft food to facilitate colonization of the newly inoculated labeled strains of *A. actinomycetemcomitans*. Briefly, 5 monkeys were used in each group. Initial baseline sampling was carried out to test the presence of any spectinomycin resistant *A. actinomycetemcomitans* before inoculation of spectinomycin labeled *A. actinomycetemcomitans* strains. Then a thorough cleaning and prophylaxis was followed by application of Listerine®. After decontamination with Listerine®, the oral cavity was rinsed with water to remove any residual Listerine® which could potentially affect the newly inoculated *A. actinomycetemcomitans*. The monkeys in groups 1–3 were inoculated with RhAa3, RhAa-VS2, and RhAa-VS4 respectively on day1. Monkeys were then fed their respective *A. actinomycetemcomitans* strains smeared pancakes on days 2–6. The control monkeys in group 4 (5 monkeys) were neither inoculated nor fed *A. actinomycetemcomitans*. After inoculation, oral plaque sampling was carried out on days 7, 21, 28 and 35. Samples were stabbed onto BHI soft agar vials and transported from NEPRC, Southborough, Boston to Rutgers School of Dental Medicine, Newark campus. The plaque sample was recovered from the vial and suspended in 1 ml of PBS. A 100 µl sample was plated on BHI agar plates supplemented with the appropriate antibiotics. At the end of the study, a thorough oral prophylaxis and swabbing of the hard and soft oral tissues with Listerine® were carried out to eliminate the inoculated strains¹¹. Sampling on day 60 was also performed to determine whether the inoculated foreign *A. actinomycetemcomitans* was removed from the Rh monkey's oral flora.

Supragingival plaque sampling and enumeration of *A. actinomycetemcomitans*

The dental plaque samples from monkey's teeth were collected using 13/14 Columbia dental scaler (Hu-Friedy Mfg. Co., LLC, Chicago, IL). The plaque samples were dispersed in 2 ml Phosphate Buffered Saline (PBS) and vigorously vortexed for 1 min. Then a 100 µl sample

was plated on BHI agar supplemented with bacitracin (75 µg/ml) (Sigma-Alrich, St. Louis, MO) and vancomycin (5 µg/ml) (Sigma-Alrich, St. Louis, MO) to select total *A. actinomycetemcomitans* colonies. To recover the inoculated *A. actinomycetemcomitans* strains, 100 µl of plaque suspension was plated onto BHI agar plate supplemented with bacitracin (75 µg/ml) vancomycin (5 µg/ml) and spectinomycin (50 µg/ml). The total bacterial population was enumerated by plating 100 µl of the plaque sample suspension on blood agar plates. All plates were incubated for 48 h in a 10% CO₂ chamber. The recovered *A. actinomycetemcomitans* strains were identified by PCR amplification of the leukotoxin and spectinomycin cassette as described previously¹².

Statistical analysis

Data derived from the full experimental *in vivo* protocol designed to assess the presence or absence of inoculated and labeled *A. actinomycetemcomitans* (groups 1–3) in the primate samples were compared and analyzed by ANOVA using the Kruskal-Wallis analysis of variance by ranks to compare the three groups. Tukey's method was used for multiple comparisons to determine intergroup differences between RhAa-VS4, RhAa-VS2, and RhAa3 groups. The RhAa3 group was used as the control group in the analysis with the significance value set at $P<0.05$. The level of recolonized native unlabeled *A. actinomycetemcomitans* and the labeled *A. actinomycetemcomitans* between the groups were assessed by using Tukey-Kramer setting $P = 0.05$ as the level of significance. The level of statistical significance was depicted as * $P<0.1$, ** $P<0.001$, *** $P<0.0001$.

RESULTS

Oral colonization of *A. actinomycetemcomitans* strains in the mouths of Rh monkeys

The purpose of this study was two-fold: 1) to understand the role of *luxS* in a Rh monkey's oral ecosystem resembling humans, and 2) to assess microbial/microbial interactions in the *luxS* mutated strain in greater detail by means of *in vitro* studies. In the present Rh monkey oral colonization study, we related the initial colonization efficiencies of a *ltxA* mutant (RhAa-VS-2, the negative control) and the *luxS* mutant strain (RhAa-VS-4) in comparison to the RhAa3 (the wild-type strain). We observed that the RhAa-VS4 strain colonized comparable to RhAa3 wild-type strain in 4 weeks post inoculation time. The RhAa-VS2 strain was not able to colonize and survive (Fig. 2A). The results showed that at 2-weeks post inoculation, 4 out of 5 monkeys inoculated with RhAa3 showed supra-gingival colonization; 2 out of 5 monkeys inoculated with RhAa-VS4 showed colonization, but only 1 out of the 5 monkeys inoculated with RhAa-VS2 showed colonization and only at 2-weeks post inoculation. At the 4-weeks post inoculation time, 4 out of 5 monkeys inoculated with RhAa3 and 3 out of 5 monkeys inoculated with RhAa-VS4 showed colonization; while no RhAa-VS2 was recovered from monkey's oral cavity at 4 weeks post inoculation.

One-way ANOVA showed that RhAa3 and RhAa-VS4 strains were not significantly different but that both colonized significantly better than the negative control (RhAa-VS2 ($P=0.008$)). Comparative analysis of *A. actinomycetemcomitans* recovery showed that the recovery of inoculated *A. actinomycetemcomitans* was about $8.9\pm0.1\%$ in RhAa3 group and $8.1\pm0.1\%$ in RhAa-VS4 group (Table. 3). Further, the native *A. actinomycetemcomitans*

strains were shown to recolonize the oral cavity within 2 weeks (Fig. 2B). Assessment of the total bacterial counts in all the monkey groups showed that there was an increase at 4 weeks post inoculation compared to the 2 weeks post inoculation. However, differences between groups were not statistically significant (ANOVA; $P=0.27$) (Fig. 3A). Visual determination of plaque levels using a Plaque Index indicated that plaque did not differ when groups were compared (Fig. 3B).

Characterization of *luxS* mutant strain

To demonstrate that there was no *luxS* mRNA expression in RhAa-VS4 strain, RT-PCR was performed at the exponential phase of growth. The result has shown that there was no amplicon in the RhAa-VS4 strain corresponding to the RhAa3 PCR product. The 16S rRNA served as the internal control (Fig. 4A). Further, to confirm ablation of AI-2 in RhAa-VS4 strain, a standard AI-2 assay was performed using *V. harveyi* as the quorum sensing reporter strain. The results showed that the *luxS* mutant RhAa-VS4 strain did not produce the AI-2 molecule, while the wild type RhAa3 as expected did show AI-2 production. In the DPD supplementation experiment, RhAa-VS4 showed a significant increase in bioluminescence as compared to the RhAa-VS4 strains. AB medium was used as the negative control (Fig. 4B).

Expression of adhesin genes in RhAa-VS4

To determine regulatory effects of *luxS* disruption, several genes were examined related to *A. actinomycetemcomitans* attachment. qRT-PCR was performed in RhAa-VS4 and compared with RhAa3 to assess expression of *A. actinomycetemcomitans* attachment genes. The results of qRT-PCR analysis of autotransporter adhesins *aae*, *apiA* and *flp* showed that there was a significant increase in the expression levels of *aae* ($P=0.006$) *apiA* ($P=0.0002$) and *flp* ($P=0.0003$) in RhAa-VS4 compared to RhAa3 (Fig. 5). The functional complementation of RhAa-VS4 strain with DPD resulted in significantly decreased gene expression levels of *aae* ($P=0.02$), *apiA* ($P=0.001$), and *pgaC* ($P=0.001$) levels. However, the *flp* gene expression was not altered due to the addback of DPD in RhAa-VS4 strain (Fig. 5).

Biofilm formation and EPS production in RhAa-VS4

Biofilm formation is an important determinant of bacterial colonization and survival in a plaque environment. To evaluate biofilm forming abilities, RhAa3, RhAa-VS4, and RhAa-VS4+DPD biofilms were grown for 16 h in a CO₂ incubator and the biofilm formation was analyzed by confocal microscopy and crystal violet staining. The RhAa-VS4 strain showed a different morphological biofilm appearance as compared with RhAa3, and DPD supplemented RhAa-VS4 (6A). The results of biofilm formation revealed a significantly increased ($P=0.0007$) biofilm forming ability by the RhAa-VS4 strain as compared to RhAa3. When the mutant RhAa-VS4 strain was supplemented with the precursor of AI-2 (DPD in the growth medium) biofilm formation was reduced but was still comparable to the wild-type (Fig. 6B). This was further confirmed by confocal microscopic analysis of biofilm depth. The cross-sectional view of the biofilm (Fig. 6C) results showed that there was a significant increase in the biofilm depth compared to RhAa3 strain ($P=0.0002$). However, an addition of DPD to the growth medium during inoculation decreased biofilm depth as compared with mutant RhAa-VS4 without supplementation of DPD (Fig 6C). Biofilm

volume measurement analysis showed that there was no significant difference between RhAa-VS4 and RhAa3 strains at 16 h.

Another major component of biofilms, exopolysaccharide (EPS), was also analyzed by congo red staining. It was observed that RhAa-VS4 produced significantly increased ($P<0.0001$) level of EPS compared to RhAa3 (Fig. 7A). Furthermore, there was a significant increase in the *pgaC* (the gene responsible for exopolysaccharide synthesis) expression in RhAa-VS4 as compared to RhAa3 using qRT-PCR ($P=0.0004$). Exogenous supplementation of DPD significantly decreased the *pgaC* gene expression in RhAa-VS4 strain as compared to untreated RhAa-VS4 strain (Fig 7B).

Biofilm cell viability of RhAa-VS4 strain

The viability of 16 h RhAa3, RhAa-VS4 and RhAa-VS4+DPD biofilm cells were assessed by live/dead staining and confocal imaging. It was observed that there was no change in biofilm cell viability at 16 h of growth. However, when the biofilm was grown for 48 h, the confocal image analysis showed that there was a significant decrease in biofilm cell viability in RhAa-VS4 ($15.7\pm4.5\%$ live; $84.3\pm7.8\%$ dead cells) ($P=0.0002$) (Fig. 8). Further, confocal microscopic analysis at 48 h showed typical biofilm colonies in RhAa-VS4. Further at 48 h, the cell death was uniform all around the biofilm colonies and fewer live cells were seen on the surface of the biofilm colony in the RhAa-VS4 cells suggesting that the 48 h growth time was not appropriate for biological assessment. This conclusion was confirmed when the addition of DPD to the growth media for 48 h did not alter the viability or the biofilm architecture of the RhAa-VS4 strain.

DISCUSSION

The current study was designed to assess the effect of an *A. actinomycetemcomitans* quorum sensing related autoinducer-2 producing gene, *luxS*, on colonization and survival in an *in vivo* model. This *in vivo* model has allowed us to determine the colonization efficiency of *luxS* as compared to its wild-type strain. It is well known that *A. actinomycetemcomitans* uses chemical signals between and within species in the oral cavity^{18, 19}. This communication between the microbes through chemical signaling is known as quorum sensing (QS)²⁰. Our *in vitro* experiments were designed to provide a more in-depth understanding of why mutation of *luxS* failed to reduce the *in vivo* colonization of our RhAa-VS4 strain and why our RhAa-VS2 strain (the *ltx* mutant) failed to colonize and survive¹⁴. We did not expect that the RhAa-VS4 strain would colonize at almost the same frequency as the wild type strain of *A. actinomycetemcomitans* since this strain did not produce autoinducer AI-2 molecule. However, these results were substantially clarified by *in vitro* analysis of changes seen in the mutant strains. In *Mannheimia* sp, it has been shown that the *luxS* knock out resulted in increased attachment properties²¹. As such the present *in vitro* studies also demonstrated global up-regulation of biofilm formation, polysaccharide production, and overall adherence.

Quorum sensing (QS) is a mechanism used for both inter and intra-species communication in the oral cavity by means of signaling molecules which include: acylated homoserine lactones²², small peptides²³, quinolones²⁴, or furan derivatives²⁵. QS has been shown to

have a positive or negative effect on the expression of bacterial biofilm phenotypes²⁶. In *A. actinomycetemcomitans*, AI-2 molecules are recognized by QseB, LsrB and RbsB systems^{18, 27, 28}. It has been shown that a *luxS* mutant of *E. coli* had pleiotropic effects on the production of autoinducer-3 (AI-3), which serves as the QS signal for virulence genes and the AI-3 molecule were chemically distinct from AI-2. It was also shown that AI-3 cannot activate bioluminescence in *V. harveyi*, but it is able to activate transcription of *LEE1*, which encodes the Ler regulator that activates the other LEE genes. In contrast, AI-2 activates bioluminescence in *V. harveyi* but does not affect transcription of *LEE1*²⁹. The *lsrR* and *rbsB* mutants of *A. actinomycetemcomitans* strains produced fewer biofilms that were thinner than the wild-type strain, however, these studies did not show whether AI-2 is metabolized¹⁸. AI-2, which has been proposed to be a general interspecies signal, is concentration dependent and is synthesized by LuxS as a precursor, 4,5,-dihydroxy-2,3-pentanedione (DPD). This synthesis is typically followed by secretion into the medium where DPD spontaneously undergoes cyclization into AI-2. Interestingly, the precursor of AI-1, DPD can be converted into several structures that can be recognized by different bacterial species.³⁰. Moreover, AI-2 molecule derived from other oral microbes such as *Porphyromonas gingivalis*³¹ can be substituted for the autoinducer produced by *A. actinomycetemcomitans*²⁰. However, complementation with chemically synthesized DPD has produced varied results that appear to depend on the experimental conditions used³². This could be attributed to the inherent instability of the DPD molecule and thus *luxS* mutations were not always responsive to chemically synthesized DPD under the conditions tested³³. In the present study, we showed that the functional complementation of *luxS* mutant with chemically synthesized DPD was effective in recovering the RhAa-VS4 strain up to 16 h. However, at 48 h *A. actinomycetemcomitans* was not viable and no effect was observed due to the supplementation of DPD. Thus, we proposed that genes responsible for recognizing DPD may fail due to massive cell death observed at the 48 h time accounting for the lack of recognition of DPD.

Thus, to reiterate results seen in our *in vivo* studies could be attributed to; 1) the presence of alternate signaling molecules from the other oral microbiota sufficient to compensate for deficiencies in the *A. actinomycetemcomitans luxS* mutant strain, and 2) increased adherence and biofilm capacity demonstrated *in vitro* in the mutated strain. More pointedly, the *luxS* deficiency could have been overcome by the native *A. actinomycetemcomitans* strains, or by other microbes that could have produced similar autoinducer molecules. This could provide the mutant strain with sufficient protection in the *in vivo* oral environment³¹.

In vitro experiments also showed that the *luxS* mutant expressed an increased ability to form biofilm, EPS and attachment qualities such as up-regulation of *flp*. Since laboratory strains such as the JP2 strain of *A. actinomycetemcomitans* have been shown to lack expression of *flp*, it is possible that reduced biofilm growth in *luxS* mutants in the JP2 strain could partially explain differences seen in our results as compared to those seen in the experiments reported¹⁸. Taken together and as suggested by others, the loss of quorum sensing could be balanced by providing alternative signaling coupled with a more protective biofilm environment thus compensating for defects in the *luxS* mutated strain. These results confirmed the global regulatory features related to quorum sensing signals and support the

unique advantage of our *in vivo* model for studying the intricacies of quorum sensing and quorum quenching in a complex ecology.

We used the *ltxA* mutant strain as a negative control in these experiments since previous experiments indicated that mutation of the *ltxA* gene influenced the widespread colonization island related to attachment and colonization. Thus, the failure to colonize *in vivo* reflected a broader effect of the *ltxA* mutation on other genes in the *A. actinomycetemcomitans* genome. We have previously shown that the RhAa-VS2 strain (the *ltxA* mutant) had a decreased binding to hydroxyapatite beads and this was mediated by genes in the *tad* operon. These *in vitro* findings were supported by the *in vivo* experiments clarifying the failure of RhAa-VS2 strain to colonize the Rh monkeys oral cavity¹⁴.

The present *in vivo* experiments illustrate that the mutation can cause suppression of seemingly unrelated genes (*ltx* and the *tad* genes) in RhAa-VS2 strain and cause up-regulation of genes that can compensate for the deficiencies caused by the mutation of *luxS* (and upregulation of biofilm EPS and attachment genes). Overall these experiments indicated that coupling *in vitro* data with *in vivo* data could provide useful supplemental and supportive information. Taken together these studies support previous work suggesting that AI-2 molecule can act as a global regulator^{34, 35}.

Results from this study can be summarized in two ways. First, *in vitro* studies cannot reflect the true effect of any virulence factor since it fails to consider the ecological interactions that occur in an *in vivo* environment. Secondly, our *in vitro* data supports the belief that one gene can have pleiotropic effects. In the context of the biological aspects of the small genome of a simple bacterium such as *A. actinomycetemcomitans*, it would appear to make sense that a single gene can interact with a complex network of genes. As such if anyone gene is altered at any given time then others will be affected. This can best be examined by RNA seq, which is currently underway. A limitation of this study results from the fact that the impact of *luxS* on colonization could have been supplemented by studies of *LsrB* and *RbsB* gene knock-outs in *A. actinomycetemcomitans* and their effects on AI-2 signaling. This approach can be used in future studies.

In conclusion, colonization and adaptation are critical characteristics required for the survival of microbes in a complex oral ecosystem. We have disrupted two genes related to *A. actinomycetemcomitans* colonization and survival. The RhAa-VS4 strain colonized and survived successfully. In contrast, the RhAa-VS2 strain failed to colonize. Analysis from *in vitro* studies, derived in part from previously published work, showed that the RhAa-VS2 failed to express genes in the *Tad* locus which negatively affected colonization¹⁴; while the *luxS* mutant, as presented in this work was compensated for by upregulation of other genes related to biofilm formation. In the context of *A. actinomycetemcomitans*, a bacterium with a small genome, gene disruptions can either positively or negatively affect autologous and exogenous genes that can affect colonization and survival. These observations provide a powerful rationale for using a relevant *in vivo* model in combination with *in vitro* assessments when examining gene alternations associated with ecological harmony.

Acknowledgments

We thank the animal care staff at the New England Primate Research Center that includes; Ernest Neal, Leah Makaron, and Keith Mansfield. The authors would like to acknowledge support derived from grant R21 DE 021172 to DHF from the National Institutes of Health, National Institute of Dental and Craniofacial Research (NIDCR) for support for the work performed in this study. In addition, DHF would like to thank Dean Cecile Feldman Rutgers SDM for added support for these studies. Authors also like to thank Dr. David H Figurski for providing plasmids pJAK12 plasmid; Mr. Luke Fritzky, Manager of the Confocal Imaging Facility at NJMS, Rutgers for assistance with the confocal imaging.

References

1. Fine DH, Markowitz K, Fairlie K, et al. A consortium of *Aggregatibacter actinomycetemcomitans*, *Streptococcus parasanguinis*, and *Filifactor alocis* is present in sites prior to bone loss in a longitudinal study of localized aggressive periodontitis. *J Clin Microbiol.* 2013; 51(9):2850–2861. [PubMed: 23784124]
2. Bartold PM, Van Dyke TE. Periodontitis: a host-mediated disruption of microbial homeostasis. Unlearning learned concepts. *Periodontol 2000.* 2013; 62(1):203–217. [PubMed: 23574467]
3. Chahboun H, Arnau MM, Herrera D, Sanz M, Ennibi OK. Bacterial profile of aggressive periodontitis in Morocco: a cross-sectional study. *BMC Oral Health.* 2015; 1525
4. Schreiner HC, Sinatra K, Kaplan JB, et al. Tight-adherence genes of *Actinobacillus actinomycetemcomitans* are required for virulence in a rat model. *Proc Natl Acad Sci U S A.* 2003; 100(12):7295–7300. [PubMed: 12756291]
5. Juarez-Rodriguez MD, Torres-Escobar A, Demuth DR. ygiW and qseBC are co-expressed in *Aggregatibacter actinomycetemcomitans* and regulate biofilm growth. *Microbiology.* 2013; 159(Pt 6):989–1001. [PubMed: 23519160]
6. Shanmugam M, El Abbar F, Ramasubbu N. Transcriptome Profiling of Wild-Type and pga-Knockout Mutant Strains Reveal the Role of Exopolysaccharide in *Aggregatibacter actinomycetemcomitans*. *PLoS One.* 2015; 10(7):e0134285. [PubMed: 26221956]
7. Yue G, Kaplan JB, Furgang D, Mansfield KG, Fine DH. A second *Aggregatibacter actinomycetemcomitans* autotransporter adhesin exhibits specificity for buccal epithelial cells in humans and Old World primates. *Infect Immun.* 2007; 75(9):4440–4448. [PubMed: 17620359]
8. Tang G, Ruiz T, Mintz KP. O-polysaccharide glycosylation is required for stability and function of the collagen adhesin EmaA of *Aggregatibacter actinomycetemcomitans*. *Infect Immun.* 2012; 80(8):2868–2877. [PubMed: 22689812]
9. Shanmugam M, Gopal P, El Abbar F, et al. Role of exopolysaccharide in *Aggregatibacter actinomycetemcomitans*-induced bone resorption in a rat model for periodontal disease. *PLoS One.* 2015; 10(2):e0117487. [PubMed: 25706999]
10. Schreiner H, Markowitz K, Miryalkar M, Moore D, Diehl S, Fine DH. *Aggregatibacter actinomycetemcomitans*-induced bone loss and antibody response in three rat strains. *J Periodontol.* 2011; 82(1):142–150. [PubMed: 20681810]
11. Fine DH, Karched M, Furgang D, Sampathkumar V, Velusamy S, Godbole D. Colonization and Persistence of Labeled and “Foreign” Strains of *Aggregatibacter actinomycetemcomitans* Inoculated into the Mouths of Rhesus Monkeys. *J Oral Biol (Northborough).* 2015; 2(1)
12. Karched M, Furgang D, Sawalha N, Fine DH. Rapid identification of oral isolates of *Aggregatibacter actinomycetemcomitans* obtained from humans and primates by an ultrafast super convection based polymerase chain reaction. *J Microbiol Methods.* 2012; 89(1):71–75. [PubMed: 22326236]
13. Donlan RM. Biofilms: microbial life on surfaces. *Emerg Infect Dis.* 2002; 8(9):881–890. [PubMed: 12194761]
14. Velusamy SK, Sampathkumar V, Godbole D, Fine DH. Profound Effects of *Aggregatibacter actinomycetemcomitans* Leukotoxin Mutation on Adherence Properties Are Clarified in in vitro Experiments. *PLoS One.* 2016; 11(3):e0151361. [PubMed: 26977924]
15. Sambrook, JRD. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press; 2001.

16. Bachtiar EW, Bachtiar BM, Jarosz LM, et al. AI-2 of *Aggregatibacter actinomycetemcomitans* inhibits *Candida albicans* biofilm formation. *Front Cell Infect Microbiol*. 2014; 494
17. Chung WO, Park Y, Lamont RJ, McNab R, Barbieri B, Demuth DR. Signaling system in *Porphyromonas gingivalis* based on a LuxS protein. *J Bacteriol*. 2001; 183(13):3903–3909. [PubMed: 11395453]
18. Shao H, James D, Lamont RJ, Demuth DR. Differential interaction of *Aggregatibacter (Actinobacillus) actinomycetemcomitans* LsrB and RbsB proteins with autoinducer 2. *J Bacteriol*. 2007; 189(15):5559–5565. [PubMed: 17526716]
19. Periasamy S, Kolenbrander PE. *Aggregatibacter actinomycetemcomitans* builds mutualistic biofilm communities with *Fusobacterium nucleatum* and Veillonella species in saliva. *Infect Immun*. 2009; 77(9):3542–3551. [PubMed: 19564387]
20. Novak EA, Shao H, Daep CA, Demuth DR. Autoinducer-2 and QseC control biofilm formation and in vivo virulence of *Aggregatibacter actinomycetemcomitans*. *Infect Immun*. 2010; 78(7):2919–2926. [PubMed: 20404080]
21. van der Vinne AN, Lo RY, Shewen PE. Construction and analysis of a *Mannheimia haemolytica* A1 luxS mutant. *Vet Microbiol*. 2005; 110(1–2):53–66. [PubMed: 16061331]
22. Parsek MR, Greenberg EP. Acyl-homoserine lactone quorum sensing in gram-negative bacteria: a signaling mechanism involved in associations with higher organisms. *Proc Natl Acad Sci U S A*. 2000; 97(16):8789–8793. [PubMed: 10922036]
23. Dunne GM, Leonard BA. Cell-cell communication in gram-positive bacteria. *Annu Rev Microbiol*. 1997:51527–564.
24. Pesci EC, Milbank JB, Pearson JP, et al. Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A*. 1999; 96(20):11229–11234. [PubMed: 10500159]
25. Xavier KB, Bassler BL. LuxS quorum sensing: more than just a numbers game. *Curr Opin Microbiol*. 2003; 6(2):191–197. [PubMed: 12732311]
26. Biradar B, Devi P. Quorum sensing in plaque biofilms: challenges and future prospects. *J Contemp Dent Pract*. 2011; 12(6):479–485. [PubMed: 22269240]
27. Juarez-Rodriguez MD, Torres-Escobar A, Demuth DR. Transcriptional regulation of the *Aggregatibacter actinomycetemcomitans* ygiW-qseBC operon by QseB and integration host factor proteins. *Microbiology*. 2014; 160(Pt 12):2583–2594. [PubMed: 25223341]
28. Gonzalez Barrios AF, Zuo R, Hashimoto Y, Yang L, Bentley WE, Wood TK. Autoinducer 2 controls biofilm formation in *Escherichia coli* through a novel motility quorum-sensing regulator (MqsR, B3022). *J Bacteriol*. 2006; 188(1):305–316. [PubMed: 16352847]
29. Walters M, Sircili MP, Sperandio V. AI-3 synthesis is not dependent on luxS in *Escherichia coli*. *J Bacteriol*. 2006; 188(16):5668–5681. [PubMed: 16885435]
30. Miller ST, Xavier KB, Campagna SR, et al. *Salmonella typhimurium* recognizes a chemically distinct form of the bacterial quorum-sensing signal AI-2. *Mol Cell*. 2004; 15(5):677–687. [PubMed: 15350213]
31. James CE, Hasegawa Y, Park Y, et al. LuxS involvement in the regulation of genes coding for hemin and iron acquisition systems in *Porphyromonas gingivalis*. *Infect Immun*. 2006; 74(7):3834–3844. [PubMed: 16790755]
32. Hardie KR, Heurlier K. Establishing bacterial communities by ‘word of mouth’: LuxS and autoinducer 2 in biofilm development. *Nat Rev Microbiol*. 2008; 6(8):635–643. [PubMed: 18536728]
33. Federle MJ. Autoinducer-2-based chemical communication in bacteria: complexities of interspecies signaling. *Contrib Microbiol*. 2009:1618–32.
34. LaSarre B, Federle MJ. Exploiting quorum sensing to confuse bacterial pathogens. *Microbiol Mol Biol Rev*. 2013; 77(1):73–111. [PubMed: 23471618]
35. Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewski BH. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *J Bacteriol*. 2003; 185(7):2080–2095. [PubMed: 12644477]

Fig 1. Schematic illustration of labelled *A. actinomycetemcomitans* colonization study

The study was conducted for 60 days from the day of inoculation. Baseline plaque samples were collected from the monkeys prior to cleaning. A thorough debridement and decontamination of the oral cavity was carried out and Listerine® was applied to the oral cavity and then removed by washing three times with water. A total of 20 monkeys were divided into 4 groups ($n=5$). The monkeys were inoculated with RhAa3, RhAa-VS2 and RhAa-VS4. The un-inoculated control group monkeys were not swabbed with bacteria. The respective strains of *A. actinomycetemcomitans* smeared pancakes were fed from days 2–5

to the treatment groups. The control group was fed pancakes with no *A. actinomycetemcomitans*. Samples were collected on days 7, 14, 21 and 28. Following final sampling, on day 33, decontamination was performed and analyzed for residual labeled *A. actinomycetemcomitans* on day 60.

Fig 2. Recovery of *A. actinomycetemcomitans* strains from plaque sample

Plaque samples collected were plated on BHI agar plates supplemented with bacitracin and vancomycin to assess the presence of total *A. actinomycetemcomitans* in the oral cavity. All 5 Rh monkeys in the RhAa3 group showed *A. actinomycetemcomitans* on their supragingival plaque sample. At 4 weeks post inoculation, 4 out of 5 monkeys were colonized with RhAa3. RhAa-VS4 was recovered from 3 out of 5 monkeys at 4 weeks post inoculation. No RhAa-VS2 was recovered at 4 weeks post inoculation time. (A). Native *A. actinomycetemcomitans* was seen at 2 weeks post decontamination of the oral cavity in all animals in all groups. Native *A. actinomycetemcomitans* recolonized at much higher levels than the inoculated and labeled *A. actinomycetemcomitans* (B).

Fig 3. Plaque formation in Rh monkeys after *A. actinomycetemcomitans* inoculation

The total bacteria were enumerated on blood agar plates and the CFU/ml from each group were calculated by Kruskal-Wallis test. Total bacteria were elevated in all groups (A).

Monkeys inoculated with RhAa3 showed change in the visible plaque formation at 2 and 4 weeks post inoculation. The representative picture showing the visible and elevated plaque formation after 4-weeks post-inoculation (B).

Fig 4. Confirmation of *luxS* disruption

RT-PCR was performed using cDNA from RhAa3 and RhAa-VS4 using *luxS* gene specific primers. The genomic DNA template was used as the positive control and the total RNA with no RT was used as the negative control. Note that the *luxS* transcript corresponding to RhAa-VS4 did not amplify suggesting the disruption of *luxS*. The experiment was repeated at least three times with independent cultures (A). To confirm the ablation of the AI-2 molecule by *luxS* mutant, the light production by *V. harveyi* BB170 in response to RhAa3 and RhAa-VS4 were examined. RhAa-VS4+DPD was used as a positive control. The uncultured *V. harveyi* AB medium was used as a negative control. The percentage of relative luminescence units were calculated using the *luxS* mutant strain as 0. Data presented were from independent biological triplicate experiments and the significant difference was calculated using Kruskal-Wallis test (B).

Fig. 5. Comparative expression profile of selected adhesin genes

The relative expression levels of the genes involved in attachment such as *aae*, *apiA*, and *flp* were quantified by qRT-PCR as described in the methods section. The results are presented as means $\pm$ SD for independent triplicate cultures normalized to 5S rRNA. The significant relative expression folds ($P < 0.05$) between the strains were calculated by ANOVA using Kruskal-Wallis test. Note that *aae*, *apiA* and *flp* were up-regulated in RhAa-VS4. The *aae* and *apiA* adhesins genes were responsible for BEC binding while *flp* was responsible for hydroxyapatite binding. The functional complementation of DPD (100 nM) significantly decreased the expression of *aae* and *apiA*.

Fig 6. Biofilm formation

Biofilms were grown for 16 h and stained with bacterial live/dead stain. Representative confocal micrographs from the 16 h wild type, RhAa-VS4 and RhAa-VS4+DPD biofilm are shown (A). The biofilm was also stained with crystal violet dye. The amount of bound crystal violet (absorbance at 595 nm) was proportional to biofilm formed which was significantly increased in RhAa-VS4 as compared to RhAa3(* $P < 0.05$) (B). The biofilm depth was measured in 11 different spots on the glass bottom plate for each strain and significant differences were analyzed by ANOVA (* $P < 0.05$) (C). All experiments were conducted in independent triplicates.

Fig. 7. Production of exopolysaccharide and *pgaC* expression

Biofilms were grown for 16 h and stained with congo red solution. The bound congo red was suspended in DMSO and the intensity of stain was read at OD_{419nm} representing the exopolysaccharide production. The values are the mean±SD for triplicate wells (**P*<0.05) (A). The qRT-PCR result showing that the *pgaC* expression was significantly up-regulated in the RhAa-VS4 strain as compared to the wild-type strain (Significant differences were calculated between the strain by Tukey's method **P*<0.05) (B). All experiments were conducted in independent triplicates.

Fig 8. Viability of RhAa3 and RhAa-VS4 strain biofilm at 48 h biofilms

Live/Dead staining of biofilm cells were performed at 48 h. Cell death (propidium iodide stain, red channel) was substantially higher at 48 h. At 48 h, however, there was significantly more death in RhAa-VS4 cells and the cell death is apparent throughout the biofilm colonies. In contrast, in RhAa3 biofilm cells, showed more live cells. The percentage live/dead cells were calculated at 9 different spots in the biofilm by calculating the intensity of SYTO and PI staining. Addition of DPD to RhAa-VS4 had no effect. The significant difference between the strains were calculated by ANOVA test ($P < 0.05$).

Table 1

Strains and plasmids used in this study

S. No	Plasmids/strains	Relevant characteristics/genotype	Source/reference
1	RhAa3	Wild type strain	¹⁴
2	RhAa-VS2	<i>ltxA</i> insertionally inactivated in RhAa3 with <i>aadA</i> cassette	¹⁴
3	RhAa-VS4	<i>luxS</i> insertionally inactivated in RhAa3 with <i>aadA</i> cassette	This study
4	<i>E. coli</i> Mach1-T1®	F- Φ80 <i>lacZ</i> M15 <i>lacX74 hsdR</i> (rK-, mK+) <i>recA1398 endA1 tonA</i>	Invitrogen
5	<i>Vibrio harveyi</i> BB170	<i>luxN::tn5Kan</i> , AI-1 ⁻ AI-2 ⁺	ATCC@BAA-1117
6	pJAK12 blue	Shuttle vector, Sp ^R	A generous gift from Dr. David Figursky, Columbia University, NY
7	pBluescript KS II (+)	Cloning vector, Amp ^R	Agilent technologies
8	pVS20	<i>luxS</i> gene and its flanking regions cloned in to pBluescript KS II (+), Amp ^R	This study
9	pVS23	<i>luxS</i> insertionally inactivated with <i>aadA</i> in pVS20	This study

Table 2

Oligonucleotides used in this study

Primer	Sequence 5'→3'	T _m °C	Source
<i>PstI</i> adAF	ACCTTCCTGCAGTTATTTGCCGACTACCTTGG	64.3	This study
<i>XbaI</i> adAR	AGAATTCTAGAAAGTGCGGTCATTCAAATATGTATCCGCTCATG	63.5	This study
<i>KpnI</i> luxSF	CAGTAGGTACCGCTGCGTGGTCTCGCCCATCAGCCTTAC	70.5	This study
<i>SacI</i> luxSR	TCATGAGCTCGAGAGAACATCACGACTTTTGCGCCTAG	66.2	This study
<i>XbaI</i> luxSF	AAGTCTCTAGAGTGATCTCGCATAAAACCG	59.3	This study
<i>PstI</i> luxSR	TGAATCTGCAGGGTTTTTATATGTCCTTAATCGG	59.8	This study
luxScreenF	ATTTGAAACGCAAATACGGCAAACAGC	59.3	This study
luxScreenR	AATAACCTCGCTTATGACGTATAAAGTGC	57.0	This study

Sequences underlined are restriction enzyme sites

Table 3

Percentage recovery of *A. actinomycetemcomitans* in 2 and 4 weeks post inoculation from Rh monkey's plaque samples

Strains	<u>Recovery of <i>A. actinomycetemcomitans</i> post inoculation time</u>	
	2 weeks	4 weeks
RhAa3	3.7±0.019%	8.9±0.1%
RhAa-VS2	0.1±0.0029%	0.0±0%
RhAa-VS4	1.7±0.0003%	8.1±0.1%