PNAS
www.pnas.org

Supplementary Information for

Title: *Aggregatibacter actinomycetemcomitans* colonization and persistence in a primate model.

Classification: Biological Sciences: Microbiology

Short Title: *Aggregatibacter actinomycetemcomitans* colonizes Rhesus monkeys

Senthil Kumar Velusamy[1], Vandana Sampathkumar[1], Narayanan Ramasubbu[1], Bruce J. Paster[2], Daniel H. Fine[1]*

1. Department of Oral Biology, Rutgers School of Dental Medicine, 110 Bergen Street, Newark NJ 07103
2. The Forsyth Institute, 245 First Street, Cambridge MA 02142

Corresponding author:
Daniel H. Fine
Oral Biology Department
Rutgers, The State University of New Jersey
110 Bergen Street, Room C- 850
Newark, NJ 07103
Phone: 973-972-3728

www.pnas.org/cgi/doi/10.1073/pnas.1905238116

Email: finedh@sdm.rutgers.edu

Key words: *Aggregatibacter actinomycetemcomitans*, leukotoxin, Rhesus monkey, L-lactate, Microbiome

Leukotoxin expression levels of *A. actinomycetemcomitans* strains:
The expression levels were assessed by ltxA transcripts via qRT-PCR as described below. As for the Western Blot (Fig S6), we used this method in our initial effort to demonstrate that the hyper-producer (RhAa-ltxP530) secretes elevated levels of Leukotoxin as compared to wild-type and the ltx mutant strain (see below).

***In vivo* Rh monkey colonization experimental design**
Prior to anesthetizing Rhesus (Rh) monkeys, vital signs and weights were obtained. Rh monkeys were anesthetized with 10-20n mg/kg ketamine and medetomidine intramuscularly. Before any sampling procedure, monkeys were screened for the presence of *A. actinomycetemcomitans*, for possession of any spectinomycin resistant ($Spec^R$) strains, and/or for *A. actinomycetemcomitans* with a deletion in the 530 bp *ltx*A promoter region by means of PCR (1).

Human Oral Microbiome Next Generation Sequencing (HOMINGS) of Rh monkeys plaque sample:
For each sample, an average of >60,000 sequences of about 441 bp per sequence were obtained. Low abundance reads were quality filtered (Q25) to ensure the highest quality reads.
Sequence analyses: Species-specific, 16S rRNA-based oligonucleotide probes were used in a BLAST program, called ProbeSeq for HOMINGS, written for MatLab to identify the frequency of oral bacterial targets. At present, about 600 oligonucleotide probes of 17 to 40 bases target individual oral bacterial species or, in some cases, a few closely-related species are available. For species not identified 129 genus-level probes that target multiple species within that genus were used in a second pass. This technique has been used previously in studies of Rh primate dental plaque (2) (3). Typically, 1×10^5 cells/ml or greater was necessary to isolate sufficient amplifiable DNA for 16S rRNA gene sequencing.

Sample Acquisition and Oral Inoculation:

Samples were transported from the New England Primate Research Center (NEPRC) to the laboratories at Rutgers. At Rutgers, the portion of the sample designated for cultural analysis was plated on to BHI BV and BHI BV and spectinomycin plate to enumerate the indigenous *A. actinomycetemcomitans* strain and inoculated labeled *A. actinomycetemcomitans* strains respectively. The second half of the sample was stored at -80^{o}C for subsequent DNA analysis. For microbiome analysis, DNA was extracted from stored samples using the DNeasy blood and tissue kit (Qiagen) and then sent to the Forsyth Institute where microbiome analysis was performed (2, 3).

Recovery of labeled *A. actinomycetemcomitans* strains in plaque (Fig 2): Since the hyper-ltx producing strain shows an elevated expression of genes related to attachment (flp, tad, rcpB; Fig 4; and rcpB & apiA; Fig S3) this enables the hyper-ltx producer to outcompete the wild-type strain for a place on the tooth surface early on and as such allows the hyper-ltx producer strain initially to attach more rapidly and at greater numbers. This interpretation is supported by our in vitro hydroxyapatite binding data (Fig 4 panel A) and is also shown in attachment of the hyper-Ltx producer at weeks 1 and 2 in our in vivo data (week 1 is now added to the supplementary data Fig S1). However, at 4 weeks the already attached *Aa* reaches a plateau on the tooth surface and thus the levels of attached *Aa* tend to equalize among the groups where *Aa* has already attached. However, the wild-type *Aa* strain differentiates itself from the hyper-ltx producer over time because only 3 of the 5 monkeys colonize as opposed to the 5 of 5 in the hyper-ltx producing strain. It therefore appears as if the expression of attachment genes in the hyper-producer is critical in the initial phase of attachment and this is shown by the increased binding in vitro and in vivo in the earlier phases of colonization; however, once colonized, the growth of the microbe already attached proceeds independent of the initial attachment .

Estimation of patterns of association of *A. actinomycetemcomitans* and other members of the Rh monkeys oral microbiota by means of WGCNA and CoNET:

Both the WCGNA and CoNet methods can be used to unravel complex relationships among microorganisms based on their abundance across samples in an unbiased manner. First these assessments are based on total microorganisms by means of species-specific, 16S rRNA-based oligonucleotide probes used in a BLAST program, called ProbeSeq for HOMINGS, written for MatLab to identify the frequency of oral bacterial targets. The microbial tracking is taken from all Rh monkey samples at any given time period (1, 2 and 4 weeks). From each of these time points we select the top 50 microbes and then determine the co-occurrence of these microbes with each other. The input used in these analyses is the abundance matrix, which consists of a calculated and weighted correlation analysis of various microorganisms and the time point of their collection. Please note that the amount of *A. actinomycetemcomitans* can and does vary during the various sampling time points. In the analyses we calculate the presence of total *A. actinomycetemcomitans* and do not differentiate between the indigenous and inoculated strains of *A. actinomycetemcomitans*. In this analysis we do not differentiate between groups and combine all samples at each time point.

As for methodology, the interaction network as generated by WCGNA (and CoNet) is imported into Cytoscape, a program used to analyze the network topology by means of a Density of Maximum Neighborhood Component calculation (Chin CH, et al., BMC Syst Biol 2104;8 (Suppl 4): S11.doi:10.1186/1752-0509-8-S4-S11, Epub 2014 Dec 8. PubMed PMID: 25521941; PubMed Central PMCID: PMC4290687). The network topology sets up groupings of microbes that interact. Since we are interested in *Aa* we select the grouping (or subnetwork) that contains *Aa*. A node color scheme from highly essential (red) to less essential (green),, is generated by the software relative to each of these nodes to provide a first glance at the node essentiality (or in our case strength) evaluated by the scoring method. This method was originally applied to protein-protein interaction analysis in cancer biology and defines an essentiality of a protein based on its interactions. In our case, the color scheme can be considered a rough estimation of the number of interactions among the

top 50 microbes in the dental biofilm. As stated in the figure legend, red means stronger association whereas yellow means weaker association. Our analyses using these methods show that *Aa* interacts with other microorganisms and is colored red if it has a higher weight and relatively more interactions than others (the interaction in Fig 3a and 3c as shown in table generated by Cytoscape for Fig. 3 provided as an Appendix, Table. S2). In Fig. 3b, it has a low number of interaction and hence colored yellow.

For the sake of simplicity this is an unbiased weighted correlation analysis that we feel is very useful because it shows how microorganisms can interact with each other in a complex climax community. Additionally, it allows us to focus on the microbial interactions of particularly important microbes, such as *Aa*. It suggests in an unbiased manner that these organisms co-occur and we can perhaps understand the biological plausibility of that co-occurrence (in this case lactate production by one group and lactate utilization by *Aa*). We are putting this explanation in the Appendix with the sense that this adds clarity to these analyses.

As stated the 50 most abundant species at the genus level were used. An ensemble approach utilizing two measures of correlation (Pearson and Spearman) combined with two dissimilarity measures (Bray-Curtis and Kullback-Leibler) were used using a threshold for the correlations and dissimilarity set at 0.6. To accommodate for the relatively small dataset, the number of methods used to support a link connecting the nodes were set at 2 as a minimum. The data matrix was randomized using 100 row-wise permutations to obtain the edge Score. The P values <0.05 were retained after adjusting for Benjamini-Hochberg false-discovery rate corrections. The resulting networks were visualized using Cytoscape v 3.6 (4, 5).

In vitro analysis of attachment to salivary coated hydroxyapatite (sHA) discs:

The saliva sample collected from healthy human volunteer subject was clarified by centrifugation at 10,000 × g for 10 min. The HA discs (5 mm dia × 1.8 mm

thick, HiMed inc., Old Bethpage, NY) were washed 3 times with PBS and coated with 500 µl of clarified saliva by incubation for 30 min with rotation (Roto Torque from Cole Palmer Instruments, Chicago IL). The salivary coated HA (sHA) discs were then washed twice with 500 µl of PBS, air-dried, and reacted with a 1 ml bacterial suspensions in PBS (RhAa3, RhAa-VS2 and RhAa-ltxP530). The sHA/bacteria reacted discs were placed on the rotating device for 30 min at 37^{o}C. After incubation the discs were washed 3 times in PBS and then the bound bacterial cells were removed by brief sonication. The bacterial suspension removed from the discs were serially diluted and plated on to BHI agar plates supplemented with bacitracin and vancomycin. Plates were incubated in 10% CO_2 incubator for 48 h and the colonies enumerated were counted and expressed in CFU/discs. The results of the experiments were derived quadruplicates (Fig. 4A in the manuscript) and the significant difference in binding between the strains were calculated using Tukey HSD post-hoc analysis. P>0.05 is considered as statistical significant.

Gene expression of *A. actinomycetemcomitans* attachment related genes by qRT-PCR

A 60 µl of *A. actinomycetemcomitans* cell suspension was added to 20 ml of media with dextrose and L-lactate independently and grown under anaerobic conditions for 48 h. Biofilm cells were collected, total RNA extracted and the total RNA was treated with DNAse I for eliminate DNA contamination as described (6). The cDNA synthesis from total RNA (2.0 µg) was performed using the High capacity reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. A 25 µl qPCR reaction using the cDNA template (2.0 µl) was performed using Roche SYBR green 2X master mix in LightCycler 480 system as described in the user manual. 5s rRNA was used as the normalization control. Melting curve analysis was performed to analyze the specificity of the amplified product. Data analysis was carried out using LightCycler 480 software (Version

1.2.9.11). A reaction without reverse transcriptase was always performed as a negative control. Results were shown as the standard errors of the means (±SEM) calculated from independent triplicate experiments. Data was analyzed by Students t test. A $P<0.05$ was considered as significance.

***In vitro* analysis of biofilm growth and viability**:

Briefly, *A. actinomycetemcomitans* strains were inoculated into 35mm glass bottom micro well culture dishes (Cat. # P35G-0-10-C. s, MatTek Co. Ashland, MA) and grown for 16 and 48 h in BHI broth and, or, in CDM with either dextrose or L-lactate. The biofilm thus obtained was washed with fresh media followed by staining with Film tracer biofilm LIVE/DEAD stain (Life Technologies, NY). Confocal imaging was performed using a Nikon A1R-A1 confocal microscope with a Plan Apo VC 60X objective lens. For 16 h biofilms, a 20X image was used to analyze biofilm depth at 9 different zones. The 48-h biofilm image (60X) was used for live/dead cell assessment. In addition, the 16 h and 48 h biofilms were serially diluted, plated, and CFU's were enumerated to validate the live/dead confocal image (6).

Comparison of biofilm and exo-polysaccharide production of *A. actinomycetemcomitans* strains in L- lactate and/or dextrose

After staining, plates were washed 3 times in distilled water and dried. The de-stained crystal violet (OD_{595nm}) and congo red (OD_{415nm}) dyes were measured as the measure of biofilm and exo-polysaccharide respectively in a micro plate reader (Tecan, Austria GmbH, Austria). Data was presented from at least 8 different wells.

Western blot analysis of leukotoxin production by *A. actinomycetemcomitans* strains:

This method was used to demonstrate that the Hyper-ltx producer generates Ltx and that the Ltx-knock-out does not. Since we have repeatedly shown that the THP1 killing assay is more sensitive to Ltx, we use this assay for quantitative

comparative assessment of Ltx levels in the three Rh strains. This data is supported by the Ltx expression data (Fig 1 A and B).

The extracellular cell free supernatant from each of the three *A. actinomycetemcomitans* strains were processed for Western blot analysis as previously described (1). Here we equalized each sample by adding a standard quantity of 45 μg of protein. Briefly, protein samples run in SDS-PAGE analysis were transferred onto a nitrocellulose membrane, probed with monoclonal anti-LtxA antibody (1: 20,000, ProMab Biotechnologies Inc, Richmond, CA) and peroxidase labeled goat-anti-rabbit secondary antibody (1: 10,000 Sigma, St. Louis, MO). Reactive leukotoxin specific bands were visualized by treating the membrane with SuperSignal™ West Femto substrate (Pierce, Rockford, IL) and exposing the membrane to a FluorChem E imaging system (ProteinSimple, San Jose, CA).

Statistical analysis

Comparison of two variables were calculated using a standard Student's *t*-test to assess differences. A statistical value of $P<0.05$ was used to measure significance. Where more than two variables were tested such as in the case of gene expression, sHA binding assay, biofilm depth and L-lactate growth, statistical difference was calculated using ANOVA and Tukey HSD post-hoc analysis between the biological triplicates setting P=0.05 as the level for statistical significance. In data derived from the *in vivo* protocol recovery of *A. actinomycetemcomitans* in primate samples were analyzed by ANOVA using the Kruskal Wallis analysis of variance by ranks to compare groups. The un-inoculated group served as the control in the analysis setting the significance value at $P<0.05$. Specifics of data analysis for *in vivo* estimates of lactate availability are described in their appropriate sections.

Acknowledgements

The authors would like to acknowledge the assistance in all the work with Primates to The New England Primate Center and especially to Ernest Neal, Leah Makaron, and Keith Mansfield. We thank Mr. Luke Fritzky, Manager of the Confocal Imaging Facility at NJMS, Rutgers for assistance with the confocal imaging.

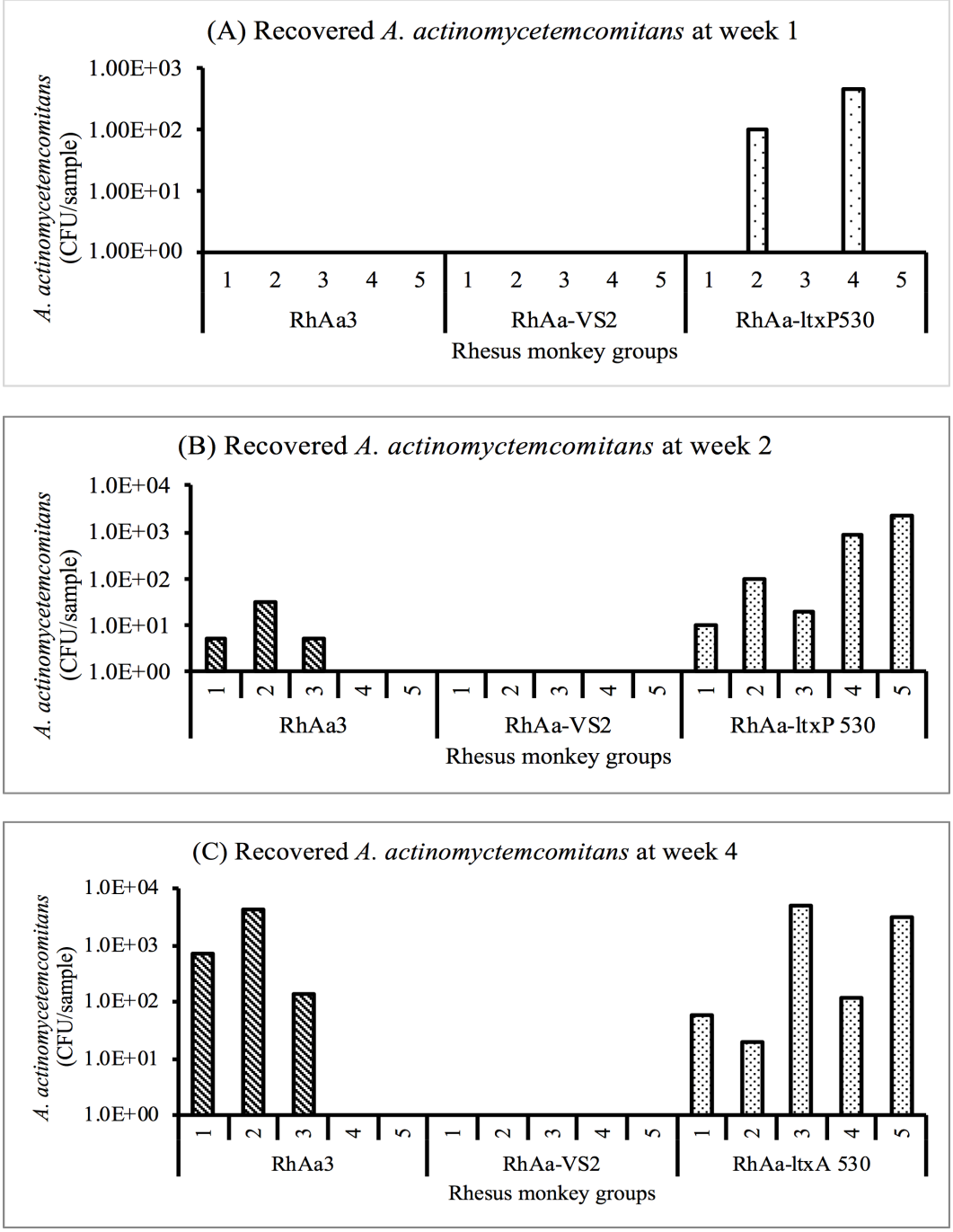

Fig. S1. Recovery of *A. actinomycetemcomitans* strains from plaque Sample

Plaque samples collected were plated on BHI agar plates supplemented with bacitracin, vancomycin and spectinomycin to assess the presence of spec^R labelled *A. actinomycetemcomitans* strains in the oral cavity. Plaque samples collected were plated on BHI agar plates supplemented with bacitracin,

vancomycin and spectinomycin to assess the presence of spec^R labeled *A. actinomycetemcomitans* strains in the oral cavity. At one week post inoculation time (A) RhAa-ltxP530 strain colonized in 2 out of 5 monkeys. Where as in 2 weeks post inoculation (B) RhAa-ltxP530 strain colonized in 5 out of 5 monkeys and 3 out 5 monkeys were colonized with RhAa3 strain. Similar trend was observed at 4 weeks of post inoculation time (C). No RhAa-VS2 (leukotoxin knock-out strain) was recovered at any post inoculation time. Numbers on the x-axis indicates the individual monkeys used in each group.

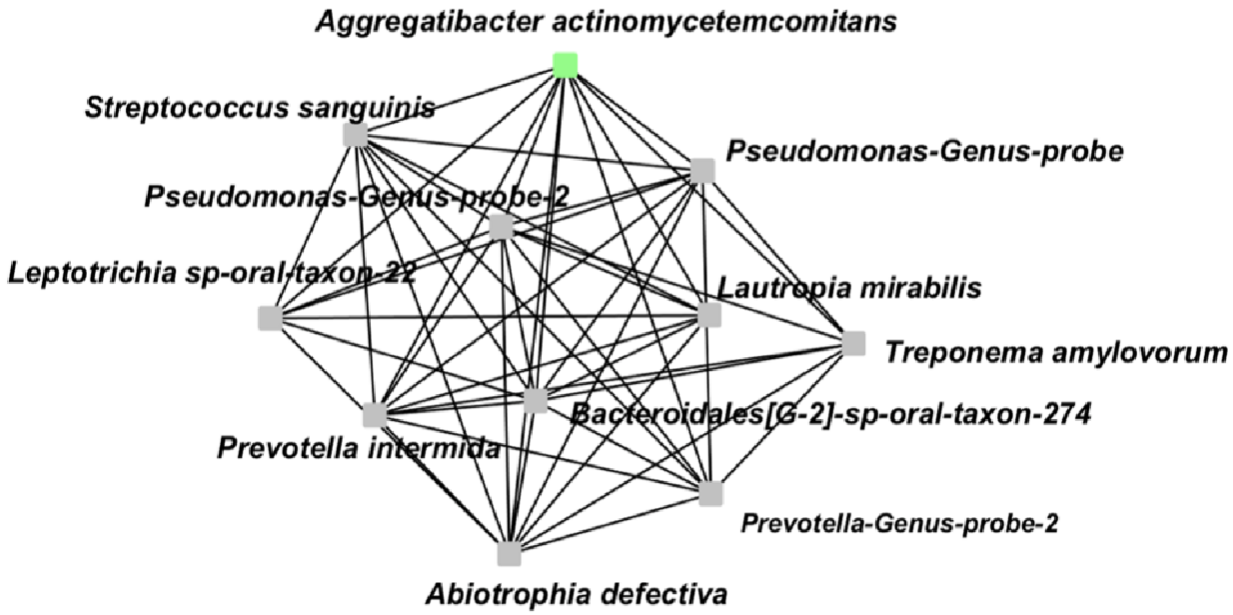

Fig. S2. CoNet analysis illustrating *A. actinomycetemcomitans* colonization partners at baseline

In this analytical tool *A. actinomycetemcomitans* was also associated with *Abiotrophia defectiva*, Leptotrichia and *S. sanguinis* all lactate producers and Pseudomonas, a lactate utilizer. See page 2 of supplementary information (See the section “Estimation of patterns of association of *A. actinomycetemcomitans* and other members of the Rh monkeys oral microbiota by means of WGCNA and CoNET” in appendix page 2) for more detailed explanation.

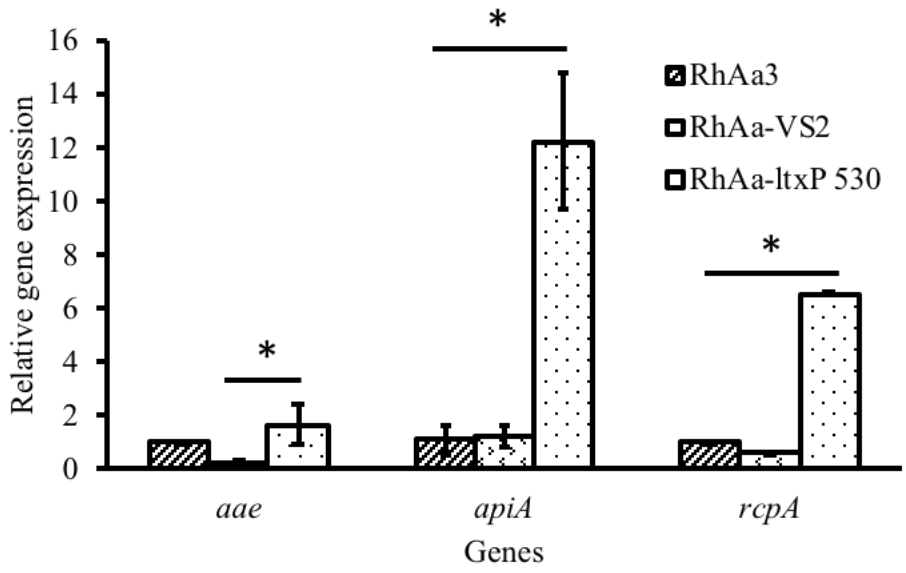

Fig. S3. qRT-PCR analysis of attachment related genes

There was a significant increase in the expression of *aae*, *api*A and *rcp*A genes n RhAa-ltxP530 strain compared to RhAa3 and RhAa-VS2 strains. Significant differences in gene expression levels were calculated by one-way ANOVA with Tukey's post-hoc multiple comparison test. *P<0.05 was considered as significance.

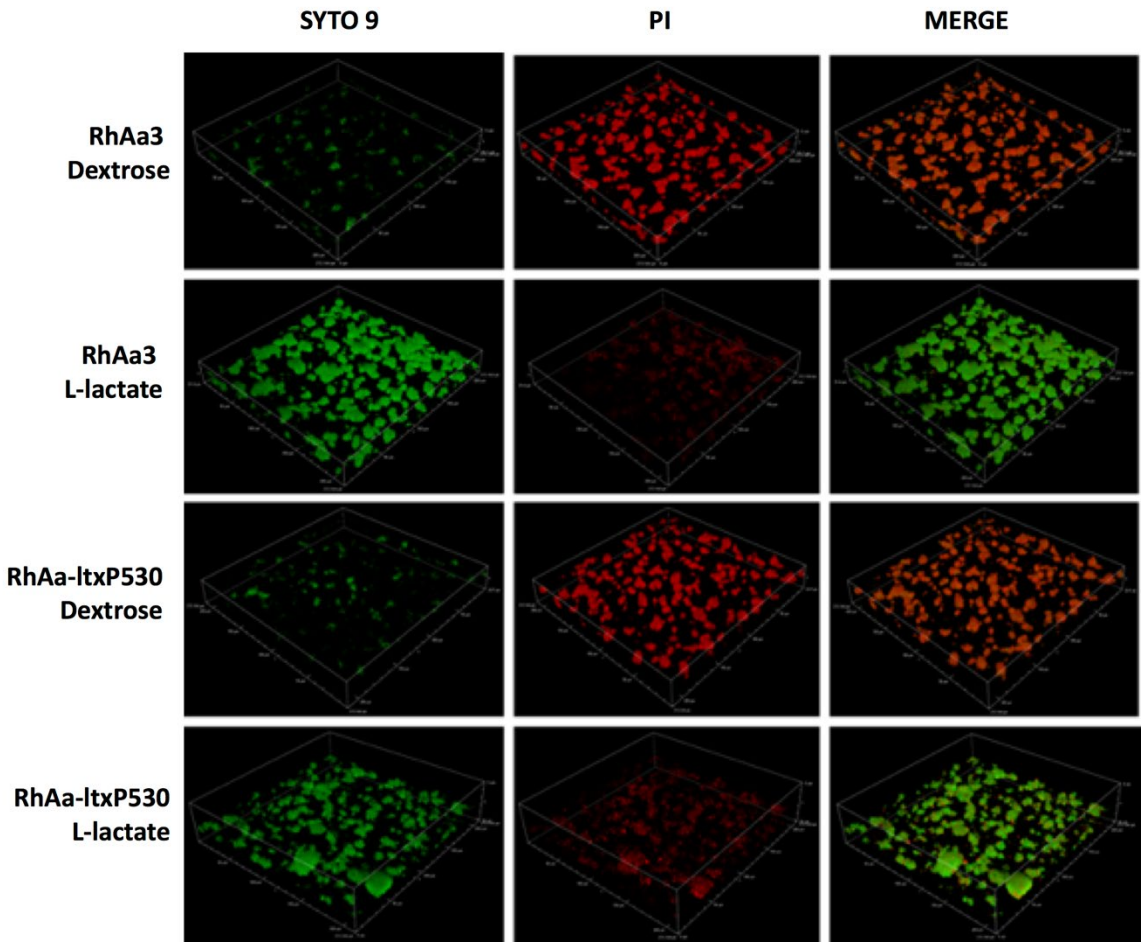

Fig. S4. Comparison of growth of RhAa3 and RhAa-ltxP530 in chemically defined media supplemented with either L-lactate or dextrose

Film tracer Live (syto 9)/dead (PI) stained confocal images show that there were a greater number of dead cells (PI) in strains grown on dextrose supplemented media as compared to L-lactate supplemented media for both RhAa3 and RhAa-ltxP530 strains. Both strains of *A. actinomycetemcomitans* are seen growing better in the presence of L-lactate as compared to dextrose in a chemically defined media.

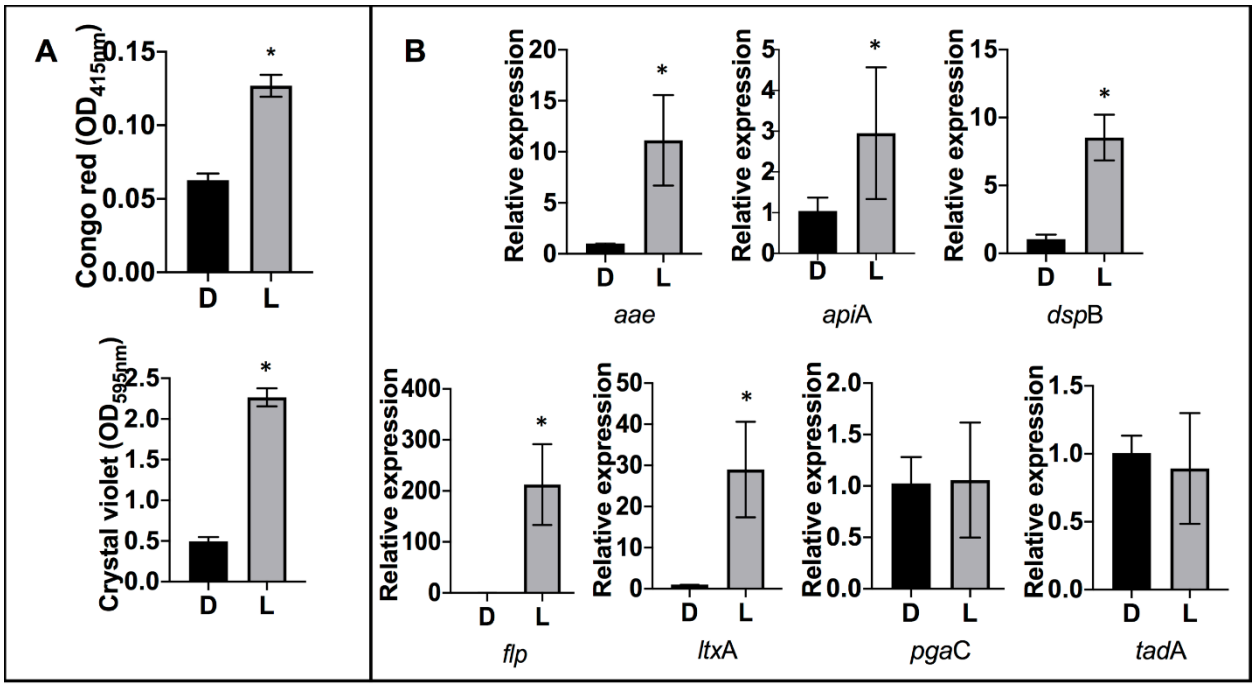

Fig. S5. Effect of L-lactate on exopolysaccharide (EPS) production, biofilm formation and virulence gene expression by wild type RhAa3 strain

Biofilms were grown on dextrose (D) and L-lactate (L) for 72 h was stained with congo red (biofilm) and crystal violet (EPS) solutions. The bound congo red was suspended in DMSO and the intensity of stain was read at OD_{419nm} representing the exopolysaccharide production. (*P<0.05). The bound crystal violet was suspended in distilled water and the intensity of stain was read at OD_{595nm} representing the biofilm forming ability (*P<0.05). All experiments were conducted in at least in quadruplicates (A). The qRT-PCR analysis shows the significantly high level (*P<0.05) expression of gene related to virulence such as *aae*, *api*A, *dsp*B, flp and *ltx*A in the media supplemented with L-lactate (L) as compared to dextrose (D) in RhAa3. However, the level of *pga*C and *tad*A are unchanged (B). All the experiments performed in triplicates. The significant high-level expression was calculated by the Student's *t*-test (* *P*<0.05).

Fig. S6. Western blot showing leukotoxin expression in *A. actinomycetemcomitans* strains

The culture supernatant total protein western blot image showed that the RhAa-ltxP530 strain produced more leukotoxin than RhAa3. Blots are not sensitive enough to show the minimal amount of toxin produced by RhAa3 hence we the use of the more sensitive THP1 cell killing assay. Western blot was used to show that RhAa-ltxP530 did indeed produce perceptible levels of leukotoxin and not to show lack of leukotoxin by the other two strains.

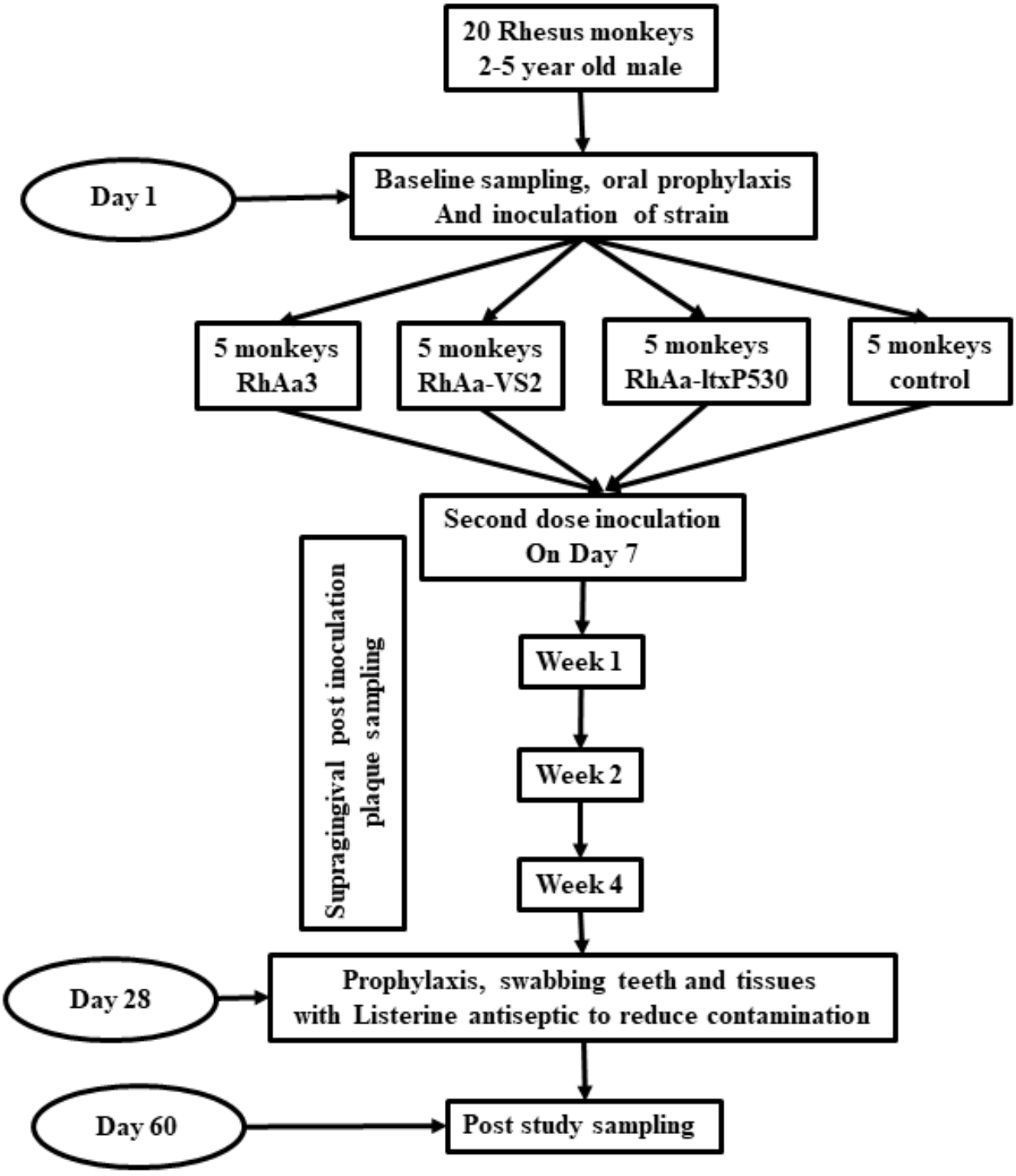
20 Rhesus monkeys
2-5 year old male
Day 1
Baseline sampling, oral prophylaxis
And inoculation of strain
5 monkeys
RhAa3
5 monkeys
RhAa-VS2
5 monkeys
RhAa-ltxP530
5 monkeys
control
Second dose inoculation
On Day 7
Supragingival post inoculation
plaque sampling
Week 1
Week 2
Week 4
Day 28
Prophylaxis, swabbing teeth and tissues
with Listerine antiseptic to reduce contamination
Day 60
Post study sampling

Fig. S7. Flow diagram illustrating the inoculation and colonization protocol
Baseline plaque samples were collected to assess indigenous *A. actinomycetemcomitans* and to check for spectinomycin resistant ($Spec^R$) strains. A thorough debridement and Listerine was applied to all hard and soft tissue surfaces to reduce contamination in the oral cavity. The monkeys were divided into 4 groups. After debridement, the three treatment groups of monkeys were inoculated with $Spec^R$ strains (RhAa3, RhAa-VS2 or RhAa-ltxP530) and then again 7 days later. The un-inoculated control group was not swabbed with bacteria. Subsequent samples were collected on week 1, 2 and 4. On day 28, Listerine antiseptic was applied to remove any residual *A. actinomycetemcomitans* and on day 60 samples were collected by buccal brush and shipped for analysis of remaining labeled *A. actinomycetemcomitans* in Rh monkeys oral cavity. Sampling thereafter showed that SpecR *A. actinomycetemcomitans* was no longer detectable by cultural means in the mouths of the previously inoculated monkeys.

Table S1. *A. actinomycetemcomitans* strains used in this study

**To develop the Spec^R spontaneous mutant strains, *A. actinomycetemcomitans* strains were grown in BHI broth supplemented with spectinomycin from 1μg to 50 μg/ml gradually over several subcultures. The Spec^R resistant RhAa3 and RhAa-ltxP530 strains were tested for morphology and LtxA activity. There was no change due to the accruement of spectinomycin resistant (see papers below). All experiments excluding Rh monkey inoculation experiment was conducted without addition of spectinomycin.

S. No	Strains	Relevant characteristics/genotype	Source/reference
1	RhAa3-Spec^{R*}	RhAa3, spontaneous spectinomycin resistance strain	1
2	RhAa-VS2	Δ*ltxA* mutant strain	2
3	RhAa-ltxP530	530 bp of *ltx*A promoter deleted strain	3
4	RhAa-ltxP530-Spec^{R*}	530 bp of *ltx*A promoter deleted spontaneous spectinomycin resistance strain	This study

Table S2. *A. actinomycetemcomitans* and total bacteria at differing time points for Rh monkeys plaque collections

*Percentage of inoculated *A. actinomycetemcomitans* to total *A. actinomycetemcomitans* varies from 7.4% to 16.5% in this study.

Time period	Total *A. actinomycetemcomitans* (CFU)	Total bacteria (CFU)	%of *A. actinomycetemcomitans* to total bacteria*
Baseline	62100±7189	48700000±3568815	0.13
Week 4	178030±6238	324200000±10487982	0.05

References:

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