Metabolite Cross-Feeding Enhances Virulence in a Model Polymicrobial Infection

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Abstract

Microbes within polymicrobial infections often display synergistic interactions resulting in enhanced pathogenesis; however, the molecular mechanisms governing these interactions are not well understood. Development of model systems that allow detailed mechanistic studies of polymicrobial synergy is a critical step towards a comprehensive understanding of these infections in vivo. In this study, we used a model polymicrobial infection including the opportunistic pathogen Aggregatibacter actinomycetemcomitans and the commensal Streptococcus gordonii to examine the importance of metabolite cross-feeding for establishing co-culture infections. Our results reveal that co-culture with S. gordonii enhances the pathogenesis of A. actinomycetemcomitans in a murine abscess model of infection. Interestingly, the ability of A. actinomycetemcomitans to utilize L-lactate as an energy source is essential for these co-culture benefits. Surprisingly, inactivation of L-lactate catabolism had no impact on mono-culture growth in vitro and in vivo suggesting that A. actinomycetemcomitans L-lactate catabolism is only critical for establishing co-culture infections. These results demonstrate that metabolite cross-feeding is critical for A. actinomycetemcomitans to persist in a polymicrobial infection with S. gordonii supporting the idea that the metabolic properties of commensal bacteria alter the course of pathogenesis in polymicrobial communities.

Introduction

The survival of pathogens in the human body has been rigorously studied for well over a century. The ability of bacteria to colonize, persist and thrive in vivo is due to an array of capabilities including the ability to attach to host tissues, produce extracellular virulence factors, and evade the immune system. Invading pathogens must also obtain carbon and energy from an infection site, and specific carbon sources are required for several pathogens to colonize and persist in the host [1]. Although mono-culture infections provide interesting insight into pathogenesis, many bacterial infections are not simply the result of colonization with a single species, but are instead a result of colonization with several [2,3,4,5]. The mammalian oral cavity is an excellent environment to study polymicrobial interactions as it is persistently colonized with diverse commensal bacteria as well as opportunistic pathogens. Our lab has utilized a two-species model system composed of the opportunistic pathogen Aggregatibacter actinomycetemcomitans and the commensal commensal Streptococcus gordonii to provide mechanistic insight into how specific carbon sources impact disease pathogenesis in polymicrobial infections [6,7].

A. actinomycetemcomitans is a Gram-negative facultative anaerobic bacterium that inhabits the human oral cavity and is a proposed causative agent of localized aggressive periodontitis [8]. A. actinomycetemcomitans is found between the gums and tooth surface in the subgingival crevice [9,10], an area restricted for O2 depending on tissue depth [11] and irrigated by a serum exudate called gingival crevicular fluid (GCF). GCF not only contains serum proteins such as complement and immunoglobulin [12], but also glucose from 10 to 500 μM in healthy patients [13] and as high as 3 mM in patients with periodontal infections [14]. L-lactate is produced by host lactate dehydrogenase in GCF [15,16] and resident oral streptococci. Together glucose and L-lactate represent two of the small number of carbon sources that A. actinomycetemcomitans is able to catabolize [17]. A. actinomycetemcomitans has been proposed to primarily inhabit the aerobic [9] “moderate” pockets (4 to 6 mm in depth) of the gingival crevice as opposed to deeper anaerobic subgingival pockets [18].

In addition to A. actinomycetemcomitans, the subgingival crevice is home to a diverse bacterial population, including numerous oral streptococci [19], that reside in surface-associated biofilm communities [20]. Oral streptococci, aside from Streptococcus mutans, are typically non-pathogenic and depending upon the human subject and method of sampling, comprise approximately 5% [21] to over 60% [22] of the recoverable oral flora. Through fermentation of carbohydrates to L-lactate and sometimes H2O2, acetate, and CO2, oral streptococci such as S. gordonii have been shown to influence the composition of oral biofilms [19,20,23,24]. Additionally, S. gordonii-produced H2O2 influences interactions between A. actinomycetemcomitans and the host by inducing production of ApoA, a factor H binding protein that inhibits complement-mediated lysis [7,25]. Thus, streptococcal metabolites
**Author Summary**

Many bacterial infections are not the result of colonization and persistence of a single pathogenic microbe in an infection site but instead the result of colonization by several. Although the importance of polymicrobial interactions and pathogenesis has been noted by many prominent microbiologists including Louis Pasteur, most studies of pathogenic microbes have focused on single organism infections. One of the primary reasons for this oversight is the lack of robust model systems for studying bacterial interactions in an infection site. Here, we use a model co-culture system composed of the opportunistic oral pathogen *Aggregatibacter actinomycetemcomitans* and the common oral commensal *Streptococcus gordonii* to assess the impact of polymicrobial growth on pathogenesis. We found that the abilities of *A. actinomycetemcomitans* to persist and cause disease are enhanced during co-culture with *S. gordonii*. Remarkably, this enhanced persistence requires *A. actinomycetemcomitans* catabolism of L-lactate, the primary metabolite produced by *S. gordonii*. These data demonstrate that during co-culture growth, *S. gordonii* provides a carbon source for *A. actinomycetemcomitans* that is necessary for establishing a robust polymicrobial infection. This study also demonstrates that virulence of an opportunistic pathogen is impacted by members of the commensal flora.

are important cues that influence the growth and population dynamics of oral biofilms and how oral bacteria interact with the host.

*A. actinomycetemcomitans* preferentially catabolizes L-lactate over high energy carbon sources such as glucose and fructose in multiple strains, despite the fact that this bacterium grows more slowly with L-lactate [6]. Given this preference for a presumably inferior carbon source and the observation that *A. actinomycetemcomitans* resides in close association with oral streptococci [26,27], we hypothesize an *in vivo* benefit exists for *A. actinomycetemcomitans* L-lactate preference. To test this hypothesis, we investigated the importance of *A. actinomycetemcomitans* L-lactate catabolism in polymicrobial communities.

**Results**

*A. actinomycetemcomitans* metabolism of glucose and L-lactate

Within the gingival crevice, host-produced glucose and L-lactate are present [13,14,15,16,28] and likely serve as *in vivo* carbon sources for *A. actinomycetemcomitans*. However in contrast to glucose, L-lactate is also produced by the oral microbial flora, primarily oral streptococci [20]. Indeed, the ability of *A. actinomycetemcomitans* to catabolize streptococcal-produced L-lactate has been demonstrated previously [6], and it was proposed that *A. actinomycetemcomitans* consumes streptococcal-produced L-lactate during co-culture. To assess the importance of *A. actinomycetemcomitans* L-lactate catabolism in polymicrobial communities *in vitro*, we examined the metabolic profile during catabolism of L-lactate and glucose under aerobic and anaerobic conditions. Aerobically, *A. actinomycetemcomitans* primarily produced lactate and acetate from glucose (Fig. 1A) while acetate was the sole metabolite produced by L-lactate-grown bacteria (Fig. 1C). It was intriguing that lactate was produced, but not consumed, by *A. actinomycetemcomitans* during aerobic catabolism of glucose. We hypothesized that the lactate produced by *A. actinomycetemcomitans* was likely D-lactate, which is not catabolized by *A. actinomycetemcomitans* [29]. Using an enzymatic assay [39], we were able to verify that >99% of the lactate produced by *A. actinomycetemcomitans* was indeed D-lactate.

Anaerobically from glucose, *A. actinomycetemcomitans* primarily produced the mixed acid fermentation products formate and acetate along with lactate, succinate, and trace amounts of ethanol (Fig. 1B). Surprisingly, *A. actinomycetemcomitans* was unable to...
catabolize L-lactate anaerobically (Fig. 1C), even if the potential alternative electron acceptors nitrate or dimethyl sulfoxide were added, suggesting that L-lactate oxidation was O₂ dependent. This is distinct from other oral bacteria including members of the genus Veillonella [24,31], in which L-lactate is an important anaerobic carbon and energy source. If O₂ respiration was indeed required for A. actinomycetemcomitans growth with L-lactate, we hypothesized that elimination of the terminal respiratory oxidase, which is required for aerobic respiration, would abolish L-lactate utilization by A. actinomycetemcomitans aerobically. To test this hypothesis, cydB, which encodes a component of the sole putative A. actinomycetemcomitans respiratory oxidase, was insertionally inactivated. The cydB mutant was unable to catabolize L-lactate aerobically supporting the hypothesis that L-lactate oxidation requires O₂ respiration (Fig. 1C). Interestingly when grown with glucose aerobically, the cydB mutant doubled much slower (6.6 hr) than the wt (1.9 hr) and cell suspensions produced a metabolite profile that differed from the wt (Fig. 1A) indicating that while not required for aerobic growth on glucose, O₂ respiration is the primary means by which glucose is catabolized by wt A. actinomycetemcomitans. As expected, the cydB mutant exhibited identical growth rates anaerobically on glucose (not shown) and produced similar metabolites as the wt (Fig. 1B). Collectively, these data indicate that O₂ respiration is required for L-lactate oxidation in A. actinomycetemcomitans.

As the ultimate goal of this study is to assess the importance of A. actinomycetemcomitans L-lactate catabolism for establishing co-culture with oral streptococci, it was important to assess whether eliminating the ability of A. actinomycetemcomitans to utilize L-lactate affected growth with glucose. To examine this, we examined growth and metabolic production in an A. actinomycetemcomitans strain in which the catabolic L-Lactate dehydrogenase LctD, which is present in all strains sequenced to date [32,33], was insertionally inactivated [29]. LctD oxidizes L-lactate to pyruvate and is required for A. actinomycetemcomitans growth with L-lactate as the sole energy source [29]. As expected, the lctD mutant was unable to catabolize L-lactate aerobically or anaerobically (Fig. 1C); however, metabolic production from glucose was not affected (Fig. 1A&B) nor was the growth rate with glucose (not shown). These data indicate that L-lactate catabolism can be eliminated in A. actinomycetemcomitans without affecting growth and metabolic production with glucose.

Utilization of L-lactate enhances co-culture growth

Because A. actinomycetemcomitans preferentially catabolizes L-lactate in lieu of hexose sugars [6], we hypothesized that L-lactate cross-feeding was important for establishing co-culture with oral streptococci grown on glucose. To test this hypothesis, we examined growth of glucose-grown A. actinomycetemcomitans and S. gordonii during in vitro co-culture aerobically and anaerobically. Aerobically, wt A. actinomycetemcomitans co-culture cell numbers were similar to those observed in mono-culture while the A. actinomycetemcomitans lctD mutant exhibited an approximate 25-fold decrease in cell number during co-culture with S. gordonii (Fig. 2). Anaerobically, both wt A. actinomycetemcomitans and A. actinomycetemcomitans lctD cell numbers diminished nearly 10-fold in co-culture compared to mono-culture (Fig. 2), likely due to the inability to catabolize S. gordonii-produced L-lactate.

Examination of aerobic metabolic end products of the A. actinomycetemcomitans lctD−/S. gordonii co-culture revealed high levels of lactate, reminiscent of S. gordonii mono-cultures, indicating that as expected, the A. actinomycetemcomitans lctD mutant is unable to catabolize L-lactate in co-culture (Fig. 3A). Additionally, metabolite concentrations in anaerobic co-cultures were similar to S. gordonii mono-culture (Fig. 3B). It should be noted that these metabolites were measured from growing cells, not cell suspensions as in Fig. 1. These data provide strong evidence that the inability...
to use L-lactate, even when glucose is present, significantly inhibits *A. actinomycetemcomitans* growth and survival in co-culture.

Interestingly, an approximate 7-fold increase in *S. gordonii* cell numbers were observed in the presence of *A. actinomycetemcomitans* aerobically, indicating that *A. actinomycetemcomitans* enhances *S. gordonii* proliferation under these co-culture conditions even when *A. actinomycetemcomitans* is unable to utilize L-lactate (Fig. S1 in Text S1). Importantly, the pH of the medium used in these experiments remained at neutrality; thus changes in cell numbers were not due to alterations in pH.

L-lactate consumption is required for co-culture growth of *A. actinomycetemcomitans* in vivo

The observation that L-lactate catabolism is critical for *A. actinomycetemcomitans* to establish co-culture with *S. gordonii* in vitro provides new insight into this model polymicrobial community; however, whether the requirement for this catabolic pathway extended to *in vivo* co-culture was not known. To examine the role of *A. actinomycetemcomitans* L-lactate catabolism for *in vivo* growth in mono- and co-culture, we used a mouse thigh abscess model. This model has relevance as *A. actinomycetemcomitans* causes abscess infections outside of the oral cavity in close association with other bacteria [34] and has been used as a model system to examine pathogenesis of several oral bacteria [35,36]. Using this model, bacterial survival and abscess formation was assessed for wt *A. actinomycetemcomitans* and *A. actinomycetemcomitans* *lctD* during mono- and co-culture with *S. gordonii* (Fig. 4).

Unexpectedly, wt *A. actinomycetemcomitans* and the *lctD* mutant established similar infections in terms of cell number (Fig. 4A) and in abscess weight (Fig. 4B), indicating that host-derived L-lactate is not an important nutrient source during mono-culture infection. Interestingly, wt *A. actinomycetemcomitans* displayed a 10-fold increase in cell number when co-cultured with *S. gordonii*, while cell number of the *lctD* mutant declined >100-fold compared to the wild-type providing evidence that the ability to catabolize L-lactate is crucial for *A. actinomycetemcomitans* co-culture survival *in vivo*. These data also indicate that while not critical for mono-culture growth, L-lactate is an important energy source during co-culture infection. Unlike the *in vitro* experiments (Fig. S1 in Text S1), *S. gordonii* numbers were not statistically different in monoculture or in co-culture abscesses (2.7×10^7 and 1.3×10^7 CFU/ml respectively; p = 0.15 via Mann-Whitney test) indicating that *S. gordonii* does not receive a benefit, at least in regard to cell number, from co-culture with *A. actinomycetemcomitans*. As a control, *in vivo* growth of the *A. actinomycetemcomitans apiA* mutant, which is hypersusceptible to killing by innate immunity, was examined. As expected, the *apiA* mutant exhibited a >250-fold decrease in mono-culture *in vivo* survival, which was unchanged in the presence of *S. gordonii* (Fig. 4A).

Discussion

Microbes within polymicrobial infections often display synergistic interactions that result in enhanced colonization and persistence in the infection site [3,34,36,37,38,39,40]. Such interactions have been particularly noted in oral polymicrobial infections, although the molecular processes controlling these synergistic interactions are not well defined. Detailed mechanistic studies of the interactions required for enhanced persistence in *in vivo* is a critical step towards a more comprehensive understanding of natural polymicrobial infections. In this study, we used a model polymicrobial infection [6,7] to determine the importance of metabolic cross-feeding for establishing co-culture infections. Cross-feeding in polymicrobial populations has been reported in numerous studies [24,41,42], but its importance for establishing co-culture infections has not been investigated in depth. The methodology used in this study began with detailed studies of the metabolic pathways required for growth with the *in vivo* carbon sources glucose and L-lactate, followed by examination of the importance of specific catabolic pathways for establishing co-culture infections.

It is relevant to discuss the rationale for two *in vivo* experimental parameters: using a ‘smooth’ strain of *A. actinomycetemcomitans* in lieu of a ‘rough’ strain; and using a murine abscess model in lieu of a rat periodontal infection model [43,44]. A ‘smooth’ strain of *A. actinomycetemcomitans*, which displays impaired surface attachment, was used in this study [45,46]. As we were not investigating attachment or biofilm development, we opted to utilize a smooth strain that had undergone robust metabolic characterization, and feel this decision is justified as this bacterium clearly causes abscess infections in this model (Fig. 4). The murine abscess model was used for several reasons. First, in addition to periodontal infections, *A. actinomycetemcomitans* causes abscess infections outside of the oral cavity that resemble, from a gross morphological standpoint, the abscess model infection [34]; thus the abscess model has clinical relevance. Second, the abscess model avoids complications arising from the normal flora, which are not completely eradicated in the periodontal rat infection models, and whose presence would make

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**Figure 4. Persistence of *A. actinomycetemcomitans*, *A. actinomy-
cetemcomitans* *lctD* and *A. actinomycetemcomitans* *apiA* in mono-
and co-culture in a murine abscess model.** A. Bacterial colony forming units per abscess. Wilcoxon signed-rank test values are: * p<0.02, ** p<0.01, *** p<0.008. B. Abscess weights 6 days post-
inoculation. Error bars represent 1 standard error of the mean, n = 9. p = 0.05 for wt *A. actinomycetemcomitans* in mono- and co-culture via Student’s t-test. doi:10.1371/journal.ppat.1002012.g004
interpretation of metabolic interactions extraordinarily complex.

Third, the abscess model allows direct, controlled inoculation with a finite number of cells that can be quantified throughout the infection by assessing colony forming units after removal of the entire abscess [37,47,48]. Finally, although the abscess model has primarily been used to study anaerobic pathogens [35,36], it is also relevant for studying aerobic pathogens, demonstrated by the large abscesses [48] formed by the strict aerobe Acinetobacter baumannii [17,49]. The presence of aerobic microenvironmets in the abscess is also supported by our observations that the S. gordonii spxB mutant is significantly impaired for abscess formation (Fig. S2 in Text S1). The spxB gene encodes pyruvate oxidase which utilizes O2 for biosynthesis of the virulence factor H2O2 [50]; thus its importance is limited to aerobic infections.

The observation that A. actinomycetemcomitans requires O2 to catabolize L-lactate was surprising, as many oral bacteria grow on L-lactate anaerobically [24,31]. These results also solve an apparent contradiction in the literature. It was reported by L-lactate anaerobically [24,31]. These results also solve an apparent contradiction in the literature. It was reported by

Figure 5. Model for electron transport during L-lactate oxidation in A. actinomycetemcomitans. A. actinomycetemcomitans requires O2 for oxidation of L-lactate. LctD may donate electrons from L-lactate directly to the quinone pool or utilize an unknown intermediate electron carrier represented by the dotted arrow. The cytochrome oxidase CydAD ultimately donates the electrons to O2.

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Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of Texas Tech University Health Sciences Center (Protocol Number: 09039).

Cross-Feeding in a Polymicrobial Infection

Periplasm

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Figure 6. Model for enhanced persistence of A. actinomyctemcomitans during aerobic co-culture with S. gordonii. During co-culture aerobic growth with glucose, S. gordonii produces L-lactate and H$_2$O$_2$ which inhibit A. actinomyctemcomitans glucose uptake (red line) and induce apiA expression (dotted line) respectively. The production of L-lactate provides S. gordonii for glucose during aerobic co-culture. During anaerobic co-culture, S. gordonii also produces L-lactate but A. actinomyctemcomitans is unable to catabolize this carbon source due to the absence of O$_2$; thus requiring A. actinomyctemcomitans to compete directly with S. gordonii for glucose (dashed line).

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DNA and plasmid manipulations

DNA and plasmid isolations were performed using standard methods [34]. Restriction endonucleases and DNA modification enzymes were purchased from New England Biolabs. Chromosomal DNA from A. actinomyctemcomitans was isolated using DNeasy tissue kits (Qiagen), and plasmid isolations were performed using QIAprep spin miniprep kits (Qiagen). DNA fragments were purified using QIAquick mini-elute PCR purification kits (Qiagen), and PCR was performed using the Expand Long Template PCR system (Roche). DNA sequencing was performed by automated sequencing technology using the University of Texas Institute for Cell and Molecular Biology sequencing core facility.

A. actinomyctemcomitans apiA mutant construction

Allelic replacement of apiA (AA2485) was carried out by double homologous recombination. For construction of the knockout construct, 856 bp and 842 bp DNA fragments flanking apiA were amplified and combined with the apiA gene (encoding kanamycin resistance) from pBBR1-MCS2 [55] by overlap extension PCR [56]. The construct was prepared so that apiA was positioned between the upstream and downstream regions. Primers used were: Kan-5’ (ATGTACACCTACTGGGCTATCTG) and Kan-3’ (ATTTCCTTGTTTGCCG) for the 1074 bp apiA-containing fragment; ApiA-UF [CCGATAACGTAAGATCTTCTAC] and ApiA-UR [CAGATGACGAGTACGATCTTTTTCG-GCCTGAATTTAC] for the upstream apiA fragment; and ApiA-DF [GCGGAGCCTTCGGGTTCCAAATGGGTCAGAATTT-TAGGTGT] and ApiA-DR [GAACACAGACTTCTTTAC] for the downstream apiA fragment. Underlined sequences indicate overlapping DNA sequences between the apiA fragments and apiA. The overlap extension product was TA-cloned into the pGEM-T Easy vector (Promega, USA) and excised by EcoRI digestion. The EcoRI fragment containing the overlap extension product was ligated into the unique EcoRI site within the 5’-dependent suicide vector pVT1461 [57]. The cloned construct, pVT1461-apiA-KO, was first transformed into E. coli DH5α-λpir then into E. coli SM10-λpir for conjugation into A. actinomyctemcomitans. Conjugal transfer was performed as described [53]. Recombinant and potential mutants were plated onto TSBYE agar plates containing kanamycin to select for recombinant A. actinomyctemcomitans and nalidixic acid to kill the E. coli donors. Kanamycin resistant, spectinomycin sensitive double recombinants were selected and verified by PCR. Enhanced susceptibility of the apiA mutant to serum was verified as described previously [7].

A. actinomyctemcomitans cydB mutant construction

Insertional mutagenesis of the cydB gene was performed by single homologous recombination using a 543 bp internal piece of the cydB (AA2843) gene amplified using the primers cydB-KO5 (GAAGATCTTTATGATTTAACAATCAGGCGCCG) and cydB-KO3 (GAAGATCTCCCAAAACCATCTTTAAGAATACCA). Underlined sequences represent BglII restriction sites. The cloned construct, pMRKO-1 [see below] to generate pMRKO-cydB. pMRKO-cydB was transformed into E. coli SM10-λpir and conjugated into A. actinomyctemcomitans. A. actinomyctemcomitans recombinants were grown anaerobically on TSBYE agar containing spectinomycin and nalidixic acid. Colonies were

50 μg/ml for selection and 10 μg/ml for maintenance for A. actinomyctemcomitans and E. coli and 100 μg/ml for selection and maintenance for S. gordonii spaB; kanamycin, 40 μg/ml for selection and 10 μg/ml for maintenance; naladixic acid, 25 μg/ml; streptomycin, 50 μg/ml for selection and 20 μg/ml for maintenance. For quantifying CFU/ml in co-culture assays, S. gordonii spxB was transfected with culture for glucose (dashed line).
subcultured anaerobically on liquid medium at the same antibiotic concentrations and insertion into cydB was verified by PCR.

**pMRKO-1 suicide vector construction**

The spectinomycin resistance gene from pDMG4 [58] was amplified by PCR using the primers: 5’Sspec-cass-NotI (ATTAAGATTGCAGCCGCGCACATTCGTTTGGAGGATG) and 3’Sspec-cass-EcoRI (CGGAATTCGATATGCAAGGGTTTATTTGTTT) digested with NotI-EcoRI and ligated into NotI-EcoRI digested pmCherry (Clontech) underlined sequences indicate NotI and EcoRI restriction sites. The 3105 bp region containing the pUC origin of replication, placmCherry and the spectinomycin resistance gene were PCR amplified using the primers: 5’pMcher-trunc (GAAGATCTGGCAGAACATTTAAATCATATGCAAGGGTTTATTTGTTT) and 3’Speccass-EcoRI (CGGAATTCGATATGCAAGGGTTTATTTGTTT). Underlined sequences indicate BglII and EcoRI restriction sites. This fragment was digested with BglII and EcoRI and ligated into the 2780 bp fragment from BglII-EcoRI digested pVT1461. The resulting plasmid (pMRKO-1, submitted to Genbank) is a suicide vector for *A. actinomycetemcomitans* and contains oriT, mob, and tra genes from pVT1461 along with the pUC origin of replication, mCherry expressed from placmCherry and a spectinomycin resistance cassette.

**Resting cell suspensions**

*A. actinomycetemcomitans* was grown in CDM overnight either aerobically or anaerobically in the presence of 20 mM glucose or 40 mM L-lactate. Bacteria were then subcultured in 30 ml of medium and exponential phase cells (OD$_{600}$ = 0.4) were collected by centrifugation (5,000 x g for 15 minutes) at 25°C. Cell pellets were resuspended in an equal volume of CDM lacking nucleotides, amino acids and any carbon source. Cells were incubated at 37°C aerobically or anaerobically depending on the test conditions for 1 h. Cells were collected again by centrifugation as described above and resuspended to an OD$_{600}$ of 2 in 3 ml of CDM without nucleotides, amino acids, pimelate and thiocic acid containing either 20 mM glucose or 40 mM lactate. Cells were incubated for 4 h at 37°C either aerobically or anaerobically. After incubation samples were stored at −20°C for HPLC analysis.

**D-Lactate assay**

D-lactate assays were performed as described [30] with modifications. Glycylglycine buffer was replaced with an equal concentration of Bicine (Fisher, USA) buffer and enzymatic assays were monitored by spectrophotometry at 340 nm for 4 hours.

**Co-culture experiments**

*A. actinomycetemcomitans* and *S. gordonii* were grown overnight in CDM containing 3 mM glucose. 3 mM glucose was used to ensure that the medium was limited for catabolizable carbon. Cells were diluted 1:50 in the same medium and allowed to grow to exponential phase (OD$_{600}$ of 0.2). Cells were then diluted 1:100 (2 x 10^8 S. gordonii/ml and 1 x 10^7 A. actinomycetemcomitans/ml) as mono-cultures or co-cultures in 3 ml CDM containing 3 mM glucose. Cultures were allowed to grow for 10 h aerobically or 12 h anaerobically, after which cells were serially diluted, plated on either TSBYE agar + vancomycin for *A. actinomycetemcomitans* enumeration or TSBYE agar + streptomycin for *S. gordonii* enumeration. Colonies were counted after incubation at 37°C for 48 h. An aliquot of the culture was also stored at −20°C for HPLC metabolite analysis.

**HPLC analysis**

Metabolite levels were quantified using a Varian HPLC with a Varian Metacarb 87H 300 x 6.5 mm column at 35°C. Samples were eluted using isocratic conditions with 0.025 N H$_2$SO$_4$ elution buffer and a flow rate of 0.5 ml/minute. A Varian refractive index (RI) detector at 35°C was used for metabolic enumeration by comparison with acetate, ethanol, formate, glucose, L-lactate, D-lactate, pyruvate and succinate standards.

**In vivo murine abscess growth**

Murine abscesses were generated essentially as described previously [37]. Briefly, 6–8 week-old, female, Swiss Webster mice were anesthetized with an intraperitoneal injection of Nembutal (50 mg/kg). The hair on the left inner thigh of each mouse was shaved, and the skin was disinfected with 70% alcohol. Mice were injected subcutaneously in the inner thigh with 10^7 CFU *A. actinomycetemcomitans*, *S. gordonii* or both. At 6 days post-infection, mice were euthanized and intact abscesses were harvested, weighed and placed into 2 ml of sterile PBS (or water for pH measurements). Tissues were homogenized, serially diluted and plated on Brain Heart Infusion (BHI) agar + 20 µg/ml Na$_2$CO$_3$ + vancomycin for *A. actinomycetemcomitans* enumeration or BHI agar + 20 µg/ml Na$_2$CO$_3$ + streptomycin for *S. gordonii* enumeration, to determine bacterial CFU/abscess. Experimental protocols involving mice were examined and approved by the Texas Tech University HSC Institutional Animal Care and Use Committee.

**Supporting Information**

**Text S1** Figure S1: Growth of *S. gordonii* in mono- or co-culture with *A. actinomycetemcomitans* or *A. actinomycetemcomitans lctD* in aerobic and anaerobic co-cultures. Strains were grown as mono- or co-cultures in 3 mM glucose aerobically or anaerobically for 10 or 12 h respectively, serially diluted, and plated on selective media to determine colony forming units per ml (CFU/ml). *S. gordonii* mono-cultures numbers are represented by black bars, co-culture numbers with *A. actinomycetemcomitans* are represented by white bars, and co-culture numbers with *A. actinomycetemcomitans lctD* are represented by grey bars. Error bars represent 1 standard error of the mean, n = 3. Figure S2: Survival of *S. gordonii* and *S. gordonii* spoB in a murine abscess model. A. Number of bacteria recovered from each abscess expressed as colony forming units per abscess (CFU/abscess). Wilcoxon signed-rank test value, p < 0.03. B. Abscess weights 6 days post-inoculation. Error bars represent 1 standard error of the mean, n = 4.

(LOC)

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**Author Contributions**

Conceived and designed the experiments: MMR KPR MW. Performed the experiments: MMR KPR MW. Analyzed the data: MMR KPR MW. Contributed reagents/materials/analysis tools: MMR KPR MW. Wrote the paper: MMR KPR MW.
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