Critical Roles of Arginine in Growth and Biofilm Development by *Streptococcus gordonii*

Nicholas S. Jakubovics¹, Jill C. Robinson¹, Derek S. Samarian², Ethan Kolderman², Sufian A. Yassin¹, Deepti Bettampadi², Matthew Bashton³, Alexander H. Rickard,²

1. School of Dental Sciences, Newcastle University, Newcastle upon Tyne, UK
2. School of Public Health, Department of Epidemiology, University of Michigan, Ann Arbor, MI, USA
3. Bioinformatics Support Unit, Newcastle University, Newcastle upon Tyne, UK

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Address for correspondence:

Nicholas S. Jakubovics
Oral Biology
School of Dental Sciences
Newcastle University
Framlington Place

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Summary

*Streptococcus gordonii* is an oral commensal and an early coloniser of dental plaque. *In vitro*, *S. gordonii* is conditionally auxotrophic for arginine in monoculture, but biosynthesises arginine when coaggregated with *Actinomyces oris*. Here, we investigated the arginine-responsive regulatory network of *S. gordonii* and the basis for conditional arginine auxotrophy. ArcB, the catabolic ornithine carbamoyltransferase involved in arginine degradation, was also essential for arginine biosynthesis. However, *arcB* was poorly expressed following arginine depletion, indicating that *arcB* levels may limit *S. gordonii* arginine biosynthesis. Arginine metabolism gene expression was tightly co-ordinated by three ArgR/AhrC family regulators, encoded by *argR*, *ahrC* and *arcR* genes. Microarray analysis revealed that >450 genes were regulated in response to rapid shifts in arginine concentration, including many genes involved in adhesion and biofilm formation. In a microfluidic salivary biofilm model, low concentrations of arginine promoted *S. gordonii* growth, whereas high concentrations (>5 mM arginine) resulted in dramatic reductions in biofilm biomass and changes to biofilm architecture. Collectively, these data indicate that arginine metabolism is tightly regulated in *S. gordonii* and that arginine is critical for gene regulation, cellular growth, and biofilm formation. Manipulating exogenous arginine concentrations may be an attractive approach for oral biofilm control.
Introduction

Oral streptococci including *S. sanguinis*, *S. mitis*, *S. oralis*, and *S. gordonii* are pioneer colonisers of tooth surfaces, and provide the foundations for the formation of mixed-species dental plaque biofilms (Kolenbrander *et al.*, 2010). These primary-colonizing streptococci produce multiple cell surface protein adhesins that promote attachment to the salivary pellicle, and aid the recruitment of other bacteria (Nobbs *et al.*, 2011). Shifts in the microbial population in dental plaque are responsible for the development of dental caries or periodontitis (Jakubovics & Kolenbrander, 2010). For the successful colonization of tooth surfaces, streptococci must obtain key nutrients for growth and survival from the extracellular environment. Saliva provides the major source of nutrients for bacteria in nascent dental plaque. *In vitro*, however, many streptococci isolated from dental plaque grow poorly in human saliva (Kolenbrander, 2011). Growth may be enhanced by the presence of microbial consortia, which together provide a pool of extracellular enzymes that efficiently degrade DNA, proteins, and complex salivary carbohydrates such as host mucins (Bradshaw *et al.*, 1994). Streptococci also benefit from the presence of lactate-utilising bacteria, which remove the waste products of metabolism (Johnson *et al.*, 2009, Ramsey *et al.*, 2011). Thus, in order to form biofilms, streptococci must be able to maximize the use of nutrients provided by saliva and partner species in the human oral cavity.

Following the development of chemically defined media, in the 1970’s, it became clear that oral streptococci lack the biosynthetic machinery for several amino acids. For example, cysteine is broadly required by strains of *S. sanguinis* and the cariogenic species *S. mutans* (Cowman *et al.*, 1974, Cowman *et al.*, 1975, Terleckyj & Shockman, 1975). Most strains of *S. sanguinis* also required arginine, tyrosine, and at least one branched chain amino
acid for growth (Cowman et al., 1975). In mutans streptococci, requirements for several
amino acids are dependent upon the growth conditions employed. Indeed, Streptococcus
criceti AHT, a member of the mutans group, required arginine when cultured aerobically, but
grew without arginine under strictly anaerobic conditions (Terleckyj & Shockman, 1975).
More recently, we have found that S. gordonii DL1 (Challis) also requires arginine for
aerobic, but not anaerobic, growth (Jakubovics et al., 2008a). It is not clear why exogenous
arginine is essential only under aerobic conditions. It seems unlikely that oxygen directly
inhibits arginine biosynthesis since, to the best of our knowledge, oxygen does not directly
inhibit any enzymes in the arginine biosynthesis pathway. Further, S. gordonii biosynthesises
arginine aerobically if a low concentration of arginine is provided initially (Jakubovics et al.,
2008a). It is possible that oxygen has an indirect effect, for example by increasing protein
damage which in turn places an increased demand on arginine for cell growth. In line with
this, cell-cell contact (coaggregation) with another pioneer coloniser of dental plaque,
Actinomyces oris MG1, triggers the up-regulation of S. gordonii arginine biosynthesis genes,
protects S. gordonii from protein carbonylation, and enables aerobic growth of S. gordonii in
arginine-restricted conditions (Jakubovics et al., 2008a, Jakubovics et al., 2008b). A. oris
MG1 produces catalase and degrades hydrogen peroxide produced by S. gordonii. Addition
of catalase alone enhances S. gordonii growth in low arginine but is not sufficient to allow
aerobic growth following a rapid shift to medium lacking arginine (Jakubovics et al., 2008b).
Therefore, the observed growth arrest when S. gordonii is rapidly shifted to aerobic media
without arginine may be due to a combination of (i) a lack of sufficient arginine to synthesise
essential biosynthetic enzymes and initiate de novo arginine biosynthesis and (ii) additional
requirements for arginine imposed by oxidative stress.

At present, the full pathway for arginine biosynthesis by S. gordonii is not entirely
clear. The conventional arginine biosynthesis genes argCJBD and argGH for conversion of
L-glutamate to L-ornithine and L-citrulline to L-arginine, respectively, are present. However, there is no clear argF gene encoding anabolic ornithine carbamoyltransferase (OTCase) to convert L-ornithine to L-citrulline (Fig. S1). Two genes, pyrB and arcB, encode proteins with significant homology to ArgF (Jakubovics et al., 2008a). By analogy with S. aureus (Nuxoll et al., 2012), it is likely that arcB fulfils the role of the anabolic OTCase in S. gordonii. It has been proposed that arcB may be co-transcribed with arcA, encoding arginine deiminase, and controlled by the P_{arcA} promoter which in turn is most active under high arginine and low oxygen (Dong et al., 2002, Zeng et al., 2006, Liu et al., 2008). Therefore, it may be predicted that the expression of arcB is low under in vitro aerobic arginine-restricted conditions. This in turn may contribute to the conditional arginine auxotrophy phenotype of S. gordonii.

A recent analysis of the regulation of amino acid influx and efflux pathways in E. coli identified just three different logical circuitries connecting transport, biosynthesis and utilisation (Cho et al., 2012). The response to arginine sensing by the ArgR regulator involves repression of transport and biosynthesis genes and activation of the arginine utilisation pathway. This circuitry is indicative of a primary role for arginine as a signal or cue rather than as a key nutrient or substrate (Cho et al., 2012). Genomes of bacteria of the order Lactobacillales typically encode two or more orthologues of E. coli ArgR or the related regulator AhrC of Bacillus subtilis. For example, Streptococcus pneumoniae has three ArgR/AhrC family regulators even though it is apparently auxotrophic for arginine (Kloosterman & Kuipers, 2011). The presence of multiple ArgR/AhrC family regulators potentially enables a wide range of responses to different arginine concentrations, and is consistent with a role for arginine as an important chemical cue for gene regulation.

Here, we aimed to investigate the roles of arginine in gene regulation, growth and biofilm formation by S. gordonii. Specifically, we set out to (i) assess the role of arcB in
arginine biosynthesis and conditional arginine auxotrophy, (ii) determine the functions of ArgR/AhrC family regulators in the expression of L-arginine uptake, biosynthesis and catabolism genes, (iii) identify global gene regulation responses to arginine in S. gordonii and (iv) investigate the impact of L-arginine on biofilm formation in an environmentally germane model system.

Results

ArcB is pivotal for arginine biosynthesis and catabolism in S. gordonii. In silico analysis had previously indicated that two genes in the S. gordonii genome have significant homology to the anabolic OTCase (ArgF) of Lactococcus lactis (Jakubovics et al., 2008a). On the basis of homology and genome context, the products of these genes have been annotated PyrB (gene locus SGO_1109) and ArcB (SGO_1592). The product of the arcB gene has relatively strong homology to ArgF (66% identity), whereas the pyrB gene product is only 24% identical to ArgF. To determine whether either the pyrB or arcB gene plays a role in arginine biosynthesis, the pyrB or arcB genes of S. gordonii DL1 (Challis) were replaced with the non-polar aphA3 kanamycin resistance determinant. Under anaerobic conditions, S. gordonii DL1, S. gordonii arcB::aphA3 and S. gordonii pyrB::aphA3 grew well in chemically defined medium (CDM): in each case cultures reached a final turbidity of >200 Klett Units (KU) within 24 h after inoculation. In CDM lacking arginine, S. gordonii DL1 and S. gordonii pyrB::aphA3 grew to >200 KU. However, no growth of S. gordonii arcB::aphA3 was observed in this medium. Therefore, arcB appears to be essential for arginine biosynthesis, in addition to its previously identified role in arginine catabolism (Dong et al., 2002). S. gordonii pyrB::aphA3 did not grow in CDM without uracil, indicating that pyrB likely encodes an aspartate carbamoyltransferase for pyrimidine biosynthesis. To ensure that the presence of the aphA3 cassette did not affect growth of strains, mutants were also constructed.
in which pyrB or arcB were replaced with the non-polar ermAM erythromycin resistance determinant and similar patterns of growth were observed (data not shown).

**The arcB gene is co-transcribed with arcA.** The *S. gordonii* arcB gene is located within a six-gene cluster comprising arcABCDTR. The expression of arcA is induced in low pH and high arginine (Liu et al., 2008). To assess whether arcB is cotranscribed with arcA, *S. gordonii* DL1 was cultured anaerobically in CDM supplemented with 5 mM arginine to mid-exponential phase (125-175 KU) and RNA was extracted. The presence of mRNA containing arcA-arcB was detected by RT-PCR using primers arcAF1/arcBR1 which span the arcA and arcB genes (Fig. 1). In the absence of reverse transcriptase, no products were detected with these primers. Therefore, it is evident that arcB is cotranscribed with arcA.

To determine whether arcA and arcB are subject to similar patterns of gene regulation, *S. gordonii* was cultured under conditions aimed to induce the expression of arginine biosynthesis gene expression (growth in CDM supplemented with 5 mM arginine, followed by a shift to no arginine), or conditions favouring induction of arginine catabolism genes (growth in CDM with 10 mM glucose, followed by addition of 50 mM arginine) (Zeng et al., 2006). For the arginine catabolism conditions, a small amount of extra glucose (approximately 10% higher than unamended CDM) was included to maintain a low background level of arcA and arcB expression prior to addition of arginine, since it has been shown that adding 10 mM glucose to complex medium represses expression from the *P_{arcA}* promoter (Dong et al., 2004, Zeng et al., 2006). Expression of arcA and arcB decreased steadily for 5 min under both sets of conditions (Fig. 2). Following a shift from 5 mM arginine to no arginine, arcA and arcB expression continued to decrease for 45 min. Conversely, following arginine addition to CDM supplemented with 10 mM glucose, the expression of arcA and arcB increased dramatically after 5 min and continued to increase for 45 min. In all samples, the changes in expression of arcA and arcB were similar, and this is
consistent with a shared regulatory mechanism for the two genes. However, it should be noted that gene regulation does not necessarily correlate with enzyme activity since ArcA and ArcB may be subject to post-transcriptional regulation (Liu et al., 2008).

Increasing arcB mRNA levels by genetic manipulation improves growth in low arginine.

We hypothesized that low levels of *arcB* expression during arginine-restrictive conditions may be responsible for the functional arginine auxotrophic phenotype of *S. gordonii* under aerobic laboratory conditions. To obtain increased *arcB* gene copies, plasmid pNJ-arcB was constructed in which the *arcB* gene was placed directly downstream of its native promoter $P_{arcA}$, and was introduced into *S. gordonii arcB::aphA3*. The replication regions of pNJ-arcB originate from pTRKL2, which is maintained at 6-9 copies per cell (O'Sullivan & Klaenhammer, 1993). The complemented strain grew anaerobically in CDM without arginine, and was able to grow aerobically at lower concentrations of arginine than *S. gordonii* DL1 (Table 1).

To further enhance the levels of *arcB* expression under arginine depletion, a copy of *arcB* was inserted into the *S. gordonii* chromosome downstream of *argD* and under control of the $P_{argC}$ promoter (Fig. 3). Initially, attempts were made to introduce the *arcB* gene directly into the *S. gordonii arcB::aphA3* mutant with selection for transformants that were able to grow anaerobically on CDM agar without arginine. No transformants were obtained using this approach, even after several attempts. However, in control reactions transformants were identified on CDM agar without arginine when the *arcB* complementation construct was introduced into *S. gordonii* DL1. The transformants contained a copy of *arcB* downstream of *argD* in addition to the native *arcB* gene within the *arcABC* operon, and this strain was labelled *S. gordonii* arcB++. To construct a strain with only one copy of *arcB*, located downstream of *argD*, the native copy of *arcB* in *S. gordonii* arcB++ was replaced with an *aphA3* kanamycin resistance cassette, generating *S. gordonii* arcBComp. The expression of
arcB was assessed in each strain by qRT-PCR following anaerobic culture to mid-exponential phase in CDM amended to 5 mM arginine, harvesting and re-suspension in either high (5 mM) arginine or no arginine (Fig. 3B). Expression of arcB in S. gordonii DL1 was 5-fold lower in no arginine than in high arginine ($P<0.001$). In 5 mM arginine, there were no significant differences in levels of arcB between S. gordonii arcB++ and the isogenic wild-type. However, arcB levels were 12.5-fold lower in S. gordonii arcBComp ($P<0.001$). In no arginine, arcB was elevated in S. gordonii arcB++ and S. gordonii arcBComp by 165-fold and 150-fold, respectively, in comparison to the wild-type under arginine restriction ($P<0.001$). Therefore, the relocation of arcB to a position immediately downstream of argD resulted in strong up-regulation of arcB in response to arginine depletion, and a second copy of arcB under control of the $P_{arcA}$ promoter in S. gordonii arcB++ prevented reduced arcB expression under high arginine.

The effects of relocating the arcB gene on growth of S. gordonii in low arginine were assessed by measuring the final growth yield of cells after culture in CDM amended to different arginine concentrations (Table 1). Anaerobically, all strains of S. gordonii except the arcB mutant grew strongly in the absence of arginine. In aerobic conditions, S. gordonii DL1 did not grow in CDM containing 16 µM arginine and grew moderately in 32 µM arginine. S. gordonii arcB::aphA3 did not grow at any tested concentrations below 64 µM arginine. Moderate growth of S. gordonii arcBComp was observed in CDM containing 16 µM arginine. Only S. gordonii arcB++ grew at very low arginine (8 µM), and none of the strains grew aerobically in medium without arginine. Therefore, the poor expression of arcB under low arginine conditions plays an important contribution to the lack of S. gordonii aerobic growth under low arginine.

Arginine biosynthesis and catabolism genes are co-ordinately regulated by three ArgR/AhrC family regulators. To identify the key regulators controlling arginine-dependent
gene regulation in *S. gordonii*, the genome sequence of *S. gordonii* was BLAST-searched for
genes encoding proteins with similarity to *Lactococcus lactis* ArgR or AhrC, *E. coli* ArgR or
*Bacillus subtilis* AhrC, and three sequences were identified (Fig. S2). ArcR (SGO_1588) has
previously been characterized as an activator of arginine catabolism genes (Zeng et al., 2006).
The closest match to *E. coli* ArgR was encoded by SGO_2057, and is termed here *S. gordonii*
ArgR. Searching with *Bacillus subtilis* AhrC identified an *S. gordonii* AhrC orthologue,
encoded by gene SGO_0697. Each of the predicted *S. gordonii* polypeptides include
conserved amino acids that have been shown to be important for arginine-dependent
transcriptional regulation (Fig. S2).

To investigate the function of *S. gordonii* ArgR/AhrC family regulators, each of the
three genes (*arcR*, *argR* and *ahrC*) was disrupted by allelic exchange mutagenesis using a
non-polar antibiotic insertion cassette, and double and triple mutants were produced as
described in the Materials and Methods. To ensure that the observed effects of gene
disruptions were not due to the introduction of antibiotic resistance cassettes, *argR*, *ahrC* and
*arcR* were each disrupted with two different antibiotics, and patterns of regulation were
shown to be similar in each mutant. Predicted Rho-independent terminators were identified
downstream of *arcR* and *argR*. However, the *ahrC* gene is predicted to be in an operon with
the DNA repair protein gene *recN* and SGO_0699 (Table S1). To ensure that the knockouts
of *argR*, *ahrC* and *arcR* did not have polar effects on downstream genes, *S. gordonii* DL1
(wild-type), *argR* and *ahrC* mutants were cultured in THB medium supplemented with
5 g L⁻¹ yeast extract to mid-exponential phase (OD₆₀₀=0.5-0.7), RNA was extracted and
expression of *recN* and *mutS* (the gene downstream of *argR*) were assessed by qRT-PCR,
normalised to levels of 16S rDNA expression. There were no significant differences in
expression of *recN* or *mutS* in any of the strains (<1.5 fold change between all strains). For *S.
gordonii* *arcR*, the expression of the downstream gene *arcT* was assessed in high (5 mM) and
no arginine as part of an ongoing microarray analysis, and in each case there was no
difference in expression between the wild-type and mutant (data not shown). Therefore,
disruptions of argR, ahrC and arcR did not have polar effects on downstream genes. In
addition, the expression of argR, ahrC or arcR was not significantly altered in mutants
lacking one or more of the ArgR/AhrC family regulators (data not shown).

The role of each regulator in controlling the expression of arginine metabolism genes
in response to a shift in the arginine concentration was assessed by culturing strains
anaerobically in CDM supplemented with 5 mM arginine to mid-exponential phase,
harvesting and re-suspending in either CDM with 5 mM arginine or CDM without arginine.
Expression of arginine metabolism genes was assessed by qRT-PCR (Fig. 4).

In S. gordonii DL1 (wild-type), arginine biosynthesis genes argC, argG and pyrA
were strongly up-regulated in response to a shift to no arginine (400-fold, 210-fold and 11-
fold, respectively; P<0.001 in each case) (Fig. 4A-C). Disruption of argR or ahrC resulted in
strong expression of argC or pyrA under 5 mM arginine or no arginine, indicating that both
ArgR and AhrC are essential for down-regulation of argC and pyrA in response to arginine.
Disruption of arcR alone did not affect the expression of argC or pyrA under 5 mM arginine
and led to small but significant (P<0.01) decreases in no arginine. Therefore, ArcR appears to
play a minor role in promoting expression of argC or pyrA in response to arginine depletion.
By contrast, a clear role for ArcR was identified in regulation of argG. Disruption of arcR
resulted in 11-fold increased expression of argG under high arginine compared with S.
gordonii DL1 under the same conditions (P<0.001). The effects of arcR disruption were
independent of the presence or absence of ArgR or AhrC. Thus, the expression of argG in
mutants disrupted in either arcR or argR/ahrC was partially reduced in 5 mM arginine
compared with no arginine, whereas disruption of arcR in addition to argR and/or ahrC
resulted in strong expression of argG independent of the arginine concentration.
On the *S. gordonii* genome, the *arcD* gene encoding an arginine-ornithine antiporter is immediately downstream and in the same direction as the *arcABC* genes, and it has been suggested that *arcD* may be part of the same operon (Dong et al., 2002). However, in contrast to *arcA* or *arcB*, the expression of *arcD* was up-regulated in low-arginine compared with 5 mM arginine (Fig. 4D; *P*<0.001). In all mutants lacking *argR* or *ahrC*, expression of *arcD* was high, independent of arginine levels. Expression of *arcD* in the *arcR* single mutant was not significantly different from the wild-type under 5 mM arginine or no arginine, indicating that ArcR does not regulate *arcD*. Using the promoter finding algorithm within PePPER (http://genome2d.molgenrug.nl/), a putative promoter was identified immediately upstream of *arcD*. It is possible that *arcD* is also co-transcribed to some extent from the *P_{arcA}* promoter and that differences in mRNA stability across the transcript may also affect mRNA levels detected by qRT-PCR. Nevertheless, the above data strongly indicate that *arcD* expression is subject to different regulatory controls compared with *arcA* or *arcB*.

In *S. gordonii* DL1, expression of *arcA* and *arcB* was approximately 4- to 5-fold lower in CDM lacking arginine than in CDM containing 5 mM arginine (Fig. 4E-F; *P*<0.001). Disruption of *argR* and/or *ahrC* did not significantly affect the expression of *arcA* and *arcB*. By contrast, arginine-dependent regulation of *arcA* and *arcB* was abrogated in all strains in which *arcR* was disrupted. In these mutants, *arcA* and *arcB* expression was low regardless of the arginine concentration, indicating the ArcR is required for optimal expression of *arcA* and *arcB* under high arginine. Together, the above data demonstrate that (i) ArgR and AhrC are both required for down-regulation of arginine biosynthesis and transporter genes under high arginine, and (ii) ArcR acts independently of ArgR and AhrC to down-regulate *argG* expression under high arginine. Further, in agreement with previous observations (Dong et al., 2002), ArcR is needed for up-regulation of *arcA* and *arcB* genes under high arginine. This pattern of gene regulation, in which both the biosynthesis and
transporter genes are down-regulated in high arginine, and catabolism genes are up-regulated is similar to that identified in *E. coli* (Cho et al., 2012).

**Global gene regulation in response to arginine.** The above data indicate that *S. gordonii* mounts a robust response to a shift in the arginine concentration involving the co-ordinated regulation of arginine biosynthesis, transport and catabolism genes. These experiments were performed using CDM supplemented to 5 mM arginine. The unamended CDM contains approximately 0.5 mM arginine, and we have previously observed that *S. gordonii* arginine biosynthesis genes are up-regulated during batch growth in this medium, once arginine is depleted (Jakubovics et al., 2008a). In preliminary experiments (not shown), we observed that arginine biosynthesis genes were strongly regulated in exponentially growing *S. gordonii* cells harvested and re-suspended in CDM without arginine compared with cells re-suspended in unamended CDM. We therefore chose to focus on comparing responses to 0.5 mM arginine with no arginine for studies on global arginine-mediated gene regulation.

A DNA microarray containing 2,051 probes, covering >95% of predicted *S. gordonii* genes, was designed and employed to assess global *S. gordonii* gene expression patterns in response to a 30 min exposure of anaerobically grown cells to high (0.5 mM arginine) or no arginine (see Materials and Methods). Initially, the microarray was validated by comparing microarray data with qRT-PCR for seven genes that had different levels of regulation in response to a shift from 5 mM arginine to no arginine, and nine genes that were regulated by shifting from 0.5 mM arginine to no arginine (Fig. 5). All of the 16 genes analysed that were significantly regulated by microarray analysis were similarly regulated by qRT-PCR. By linear regression analysis there was a close correlation between data from microarrays and the combined data from qPCR ($R^2=0.98$). The slope of the regression line was 0.94, indicating that the magnitude of gene regulation was similar independent of whether qRT-
PCR or microarray was used and independent of whether cells were shifted to no arginine from 5 mM arginine or from 0.5 mM arginine.

In total, 464 genes were significantly regulated in response to arginine restriction, representing approximately 22.6% of all predicted *S. gordonii* genes. The complete list of regulated genes is presented in Table S1. Genes were assigned to clusters of orthologous groups based on predicted function (COGFun categories), and the number of genes in each group that were regulated in response to a shift in the arginine concentration are shown in Fig. S3. Overall, the COGFun group with the largest number of arginine-regulated genes was amino acid metabolism and transport (group E). In addition to arginine biosynthesis genes, a major group of genes encoding the histidine biosynthesis pathway was up-regulated between 5- and 17-fold in no arginine. Genes encoding enzymes for biosynthesis of aromatic amino acids (*aroCBED*) and isoleucine/leucine/valine (*ilvH*, *ilvA*, *ilvB*, *ilvC*) were 3- to 6-fold down-regulated by arginine depletion. The oligopeptide transport system genes *hppH* and *SGO_1716*, the glutamine transport gene *glnQ*, and an amino acid-binding permease gene (*SGO_1727*) were up-regulated 2- to 6-fold in no arginine, whereas genes encoding the polyamine transporter (*potABCD*), putative amino acid permease (*SGO_0985* and *SGO_1482*), and branched chain amino acid transport systems (*brnQ*, *SGO_1626*, *SGO_1627*, *braE*, *livH* and *SGO_1630*) were down-regulated between 2- to 10-fold.

Several COGFun groups contained more members that were down-regulated than up-regulated when cells were exposed to CDM without arginine. In general, these pathways cover a diverse range of metabolic and biosynthetic pathways that are involved in cell maintenance and growth. Apart from genes with function unknown, only COGFun groups energy production and conversion (C), nucleotide metabolism and transport (F) and transcription (K) contained more members that were up-regulated in no arginine than down-regulated. Many of the genes involved in transcription encoded predicted transcription
regulators, and it is possible that these were involved in co-ordinating the wider gene regulation response to arginine depletion. Overall, the effects of arginine depletion were consistent with an active reduction in cell growth.

Many of the genes that were most strongly regulated in response to a shift in arginine concentration were grouped in apparent operons. The structure of putative operons was predicted on the basis of gene location and orientation (Dehal et al., 2010). Predicted operons with at least one gene that was regulated >10-fold in response to arginine restriction are shown in Table 2. The most strongly regulated operons were those involved in arginine biosynthesis (argCJBD and argGH-SGO_0177) which were up-regulated >200-fold following a shift to no arginine. The arginine biosynthesis genes pyrA\textsubscript{a} and pyrA\textsubscript{b} were also strongly up-regulated (~24-fold) following arginine restriction. The histidine biosynthesis operon, SGO_1401-1411, was co-ordinately up-regulated ~9-fold in low arginine. The SGO_1656 (ppc) gene was up-regulated 44-fold in low arginine, and several single genes and putative multi-gene operons encoding hypothetical proteins were also strongly up-regulated in response to arginine depletion. The most strongly down-regulated operon in low arginine was the bfb gene locus encoding the cellobiose phosphotransferase system, which is also involved in biofilm formation and was down-regulated ~30-fold. Other major multi-gene operons that were strongly down-regulated in response to arginine depletion included the fatty acid biosynthesis operon (SGO_1686-SGO_1700), receptor polysaccharide biosynthesis (SGO_2015-SGO_2028) and the hsa gene locus encoding the Hsa adhesin and the secondary secretion apparatus (SGO_0966-SGO_0978). Single genes SGO_0831 and SGO_0832 encoding hypothetical proteins, rpsD encoding ribosomal protein S4 and ileS encoding isoleucyl tRNA synthetase were down-regulated 11- to 14-fold in low arginine.

**Gene regulation responses to arginine compared with other stimuli.** To determine whether the observed regulatory responses were specific to arginine, or whether they were indicative
of a more general stress response to amino acid depletion and growth arrest, cells were
cultured in amino acid-replete CDM, and switched to CDM lacking L-arginine, L-histidine or
branched chain amino acids (BCAA) L-leucine, L-isoleucine and L-valine. In each case
amino acid depletion resulted in a rapid growth arrest (Fig. 6A), even though the S. gordonii
genome encodes genes for biosynthesis of all these amino acids. After 30 min, the expression
of 14 different genes in each medium was determined by qRT-PCR (Fig. 6B). The genes
selected for this analysis included genes significantly up-regulated by arginine depletion,
genes down-regulated and genes that were unchanged. The expression of two of the tested
genes (argC and asp5) were significantly different between arginine depletion and depletion
of either histidine or BCAA (P<0.005). In addition, the expression of SGO_1686 was
significantly different between CDM without arginine and CDM without histidine (P<0.05).
Several other genes appeared to be expressed at different levels following arginine depletion
compared with depletion of BCAA, though the differences were not statistically significant.
Therefore, the response to arginine depletion appears to involve a combination of stimulus-
specific gene regulatory responses and a more general amino acid starvation stress response.

We have previously identified 23 genes in S. gordonii that were regulated in response
to coaggregation with A. oris, including nine gene involved in arginine biosynthesis
(Jakubovics et al., 2008a). Since arginine biosynthesis genes are regulated in response to
changes in arginine concentration, we hypothesized that arginine may be a key signal for
coaaggregation sensing by S. gordonii. The effects of arginine depletion on the expression of
the 23 coaggregation-regulated genes are shown in Fig. 7. In general, there was a strong
correlation between the regulation of this set of genes under the two different conditions
(R^2=0.87). The magnitude of the regulation was stronger in response to arginine restriction
than to coaggregation (slope of the line = 0.44). Twenty-one of the 23 coaggregation-
regulated genes were significantly changed under arginine restriction, as determined by the
significance criteria outlined above. Only two genes (*spxB* and SGO_1308) that were regulated by coaggregation were not significantly regulated by arginine. Of these, *spxB* encodes pyruvate oxidase that is involved in the generation of hydrogen peroxide, and may be important specifically in interbacterial interactions (Jakubovics et al., 2008b). Overall, these data indicate that *S. gordonii* coaggregation-responsive genes are a subset of the genes regulated by arginine.

**Effects of arginine on biofilm formation by *S. gordonii***. To assess the impact of L-arginine on biofilm formation by *S. gordonii*, cells were initially cultured for 24 h in a plastic 96-well microplate anaerobically in CDM adjusted to different concentrations of L-arginine. However, in this model *S. gordonii* growth was reduced in low arginine concentrations, and the extent of biofilm formation, measured by staining with crystal violet, closely correlated with the amount of growth (linear regression, $R^2=0.94$; data not shown). This model was somewhat artificial since the mouth is an open system where nutrients are constantly replenished.

In order to assess the impact of L-arginine on *S. gordonii* biofilms grown under conditions representative of the oral cavity, a 24-channel Bioflux microfluidic system coupled to a Leica SPE CLSM was used. This system benefits from the requirement for only small volumes (>1ml) of saliva and carefully controlled flow and temperature conditions. Recently, oral care products have been developed that incorporate up to 8% (460 mM) L-arginine (Sullivan et al., 2014), and it was of interest to determine whether either high or low L-arginine concentrations would affect *S. gordonii* biofilm formation. The high-throughput nature of the system made it possible to test a range (0.5 µM-500 mM) of arginine concentrations. Quantification of biofilm biomass and cell viability was enabled by Live/Dead stain and allowed arginine-dependent biofilm development to be characterized. An
optimal range for enhanced biofilm development was observed when the 25% saliva was supplemented with between 0.5 µM and 500 µM arginine (Fig. 8).

A pre-treatment of the glass surfaces in the Bioflux microfluidic device with L-arginine at concentration up to 500 mM did not appear to affect initial adhesion of *S. gordonii* cells to the substratum (Fig. S4). Three dimensional rendering showed that *S. gordonii* biofilms grown for 22 h in 25% saliva formed thin, patchy biofilms (Fig. 8A) with an average biovolume of 0.89 µm$^3$/µm$^2$. Supplementing 25% saliva with between 0.5 µM and 500 µM arginine resulted in significant ($P<0.05$) increases in biofilm biovolume by up to 3-fold. This was coincident with an increase in average biofilm thickness, although only biofilms developed in 500 µM arginine were significantly thicker than biofilms grown without added arginine ($P<0.05$). Biofilms were structured in heterogeneous stack-like micro-colonies and there was a great deal of variation in thickness within individual samples, resulting in high error bars for this parameter (Fig. 8B-8E). Based upon biofilm biovolume, average biofilm thickness, and biofilm roughness, *S. gordonii* biofilms developed in 25% saliva supplemented with 5 mM arginine were not statistically different ($P>0.05$) from those developed in non-supplemented 25% saliva. However, biofilms developed in 50 mM-500 mM arginine were drastically altered in biofilm architecture and biomass. Architecturally, the biofilms were increasingly patchy as the arginine concentration increased and the likelihood of detecting the presence of aggregated micro-colonies was reduced. When developed in saliva containing 500 mM arginine, the biomass was significantly ($P<0.05$) reduced by 15-fold, as compared with no added arginine, and possessed significantly reduced average thickness (35-fold decrease, $P<0.05$). Roughness, which is a description of the variation in biofilm thickness, was also significantly different ($P<0.05$). High concentrations of L-arginine, up to 500 mM, did not affect the growth of *S. gordonii* in planktonic cultures in CDM (data not shown). In addition, viable counts of *S. gordonii* in unamended saliva or in saliva adjusted to 500 mM L-arginine...
arginine remained stable over 24 h, indicating that high concentrations of L-arginine were not toxic to *S. gordonii* in saliva (data not shown). The addition of L-arginine (for all experiments, as HCl salt) had little effect on the pH in the growth medium. The pH of saliva without arginine or with different L-arginine concentrations varied between 7.1-7.9. In general, the pH of the effluent was slightly higher, and ranged between 7.9-8.3. The above data indicate that arginine stimulates *S. gordonii* biofilm development at lower concentrations (0.5 µM-500 µM) and retards biofilm development at higher concentrations (50 mM-500 mM).

In addition to the architectural changes that were caused by the supplementation of L-arginine, subtle effects on biofilm viability were observed (Fig. 8). As inferred from pixel intensity analysis (red/green) of Live/Dead stained biofilms, low (0.5 µM-5 µM) and high (500 mM) concentrations of arginine caused significantly more cell-death/damage, when compared with the unsupplemented saliva. While significant (*P*<0.05), these might be a little misleading as they might be caused in-part by the architectural changes of the biofilms (e.g. Fig. 8A versus 8B) or loss of the majority of the viable biofilm cells in the flowing saliva, due to dispersive or de-adhesive effects of arginine, leaving damaged/dead cells behind (e.g. Fig. 8A versus 8E). In order to further investigate the viability of *S. gordonii* downstream of the biofilm model, cells in the effluent were visualised (Fig. S5). Images clearly showed that there were abundant cell masses in both unsupplemented saliva and in saliva supplemented with 500 mM L-arginine, indicating that *S. gordonii* had grown in both media, and that the vast majority of cells were viable.

**Discussion**

The work presented here demonstrates that arginine has a concentration-dependent effect on *S. gordonii* gene expression and can alter the ability of this oral bacterium to form
biofilms. In other bacterial species such as *E. coli*, high levels of exogenous arginine lead to repression of arginine biosynthesis and transport genes by the arginine-dependent regulator ArgR, and to increased expression of the arginine catabolism operon *astCADBE* (Cho et al., 2012). This regulatory circuitry is consistent with a proposed role for arginine in signalling, rather than simply functioning as an exogenous nutrient (Cho et al., 2012). Here, we have demonstrated that *S. gordonii* has a similar regulatory logic, since arginine biosynthesis genes (*argCJBD, pyrA, pyrAb, argGH*) and arginine transport (*arcD*) are repressed in high arginine, whereas arginine catabolism (*arcABC*) is up-regulated. However, the regulatory circuitry is more complex in *S. gordonii* since (i) *arcB* appears to have dual roles in biosynthesis and catabolism and (ii) arginine-dependent gene regulation in *S. gordonii* involves the concerted actions of three ArgR/AhrC family regulators.

It appears that ArcB, an ornithine carbamoyltransferase (OTCase), is essential for arginine biosynthesis in *S. gordonii* since strains disrupted in *arcB* were unable to grow anaerobically in the absence of arginine. This enzyme catalyses the carbamoylation of the δ-amino group of ornithine by carbamoylphosphate to produce citrulline and inorganic phosphate. The production of citrulline is thermodynamically favoured and *in vitro* ArcB enzymes are always assayed in the anabolic direction (Sainz et al., 1998). However, studies on the catabolic OTCase from *P. aeruginosa* (ArcB) have shown that it is essentially unidirectional *in vivo* due to poor affinity for carbamoyl phosphate and high co-operativity for this substrate (Tricot et al., 1993). It is not clear whether catabolic OTCase’s from other bacteria are also subject to allosteric regulation or whether they direct catalysis towards citrulline catabolism by coupling with carbamate kinase, the next enzyme in the catabolic pathway. The *P. aeruginosa* genome also contains an *argF* gene encoding an anabolic OTCase. Mutants lacking a functional *argF* grew on minimal medium without arginine only after prolonged incubation, indicating that ArcB was either unable to function in the anabolic
direction, or that its anabolic OTCase activity was very weak (Haas et al., 1977). Our data indicate that *S. gordonii* ArcB can function for arginine biosynthesis in *S. gordonii*, but biosynthesis is only sufficient to sustain rapid growth under certain conditions, such as the gradual depletion of arginine during exponential growth in CDM (Jakubovics et al., 2008a).

*S. gordonii* DL1 does not grow aerobically after a rapid shift to no arginine, possibly due to a lack of time to accumulate a pool of carbamoyl phosphate as a substrate for ArcB or due to increased demand for arginine in the presence of oxygen. Other strains of *S. gordonii*, appear to have similar phenotypes since *S. gordonii* Blackburn, Channon, FSS2 FSS3, M5 and PK488, also failed to grow in CDM without arginine (data not shown). Growth of all strains except *S. gordonii* Channon, was restored in CDM containing high (8 mM) arginine.

The ability of ArcB to function in an anabolic direction may also be limited by poor gene expression following arginine depletion. The *arcB* gene is part of a six gene *arcABCDTR* cluster, in which *arcR* is present in reverse orientation compared with the other genes (Dong et al., 2002). The promoter upstream of *arcA* (*P*<sub>arcA</sub>) has been mapped and shown to contain two CRE box consensus elements that are recognised by the carbon catabolite protein CcpA and a 27 bp element that is bound by ArcR (Dong et al., 2002, Zeng et al., 2006). The expression of *arcA* is repressed by glucose in the presence of CcpA, and is induced under anaerobic conditions by the Fnr-like protein Flp and the two-component system VicRK, and in low pH by the two-component systems CiaRH and ComDE (Dong et al., 2002, Dong et al., 2004, Liu et al., 2008, Liu & Burne, 2009). Further, in glucose-grown cells, arginine sensing by ArcR results in approximately four-fold induction of expression from *P*<sub>arcA</sub> (Zeng et al., 2006). We have now demonstrated that *arcB* is co-transcribed with *arcA* and that the expression of *arcB* is also decreased in low arginine conditions. Relocating the *arcB* gene to a location downstream of *argD* and up-regulated following arginine restriction significantly improved growth in low arginine, suggesting that poor expression of
arcB is a major restriction on arginine biosynthesis in S. gordonii in vitro. However, even though relocation of arcB increased the levels of arcB transcripts 150-fold in low arginine, it did not enable aerobic growth in the absence of arginine.

The presence of multiple ArgR/AhrC family regulators is common in the Lactobacillales, perhaps reflecting a critical role for arginine sensing in this group of organisms. For example, Lactobacillus plantarum and Lactococcus lactis each have two paralogues of ArgR and AhrC, S. pneumoniae has three, and the E. faecalis genome encodes four ArgR/AhrC family proteins (Paulsen et al., 2003, Larsen et al., 2004, Nicoloff et al., 2004, Kloosterman & Kuipers, 2011). In L. plantarum, ArgR1 and ArgR2 are both required for repression of arginine biosynthesis genes under high arginine, and mutations in the DNA binding or oligomerization domains of either argR1 or argR2 genes abolish arginine-dependent repression (Nicoloff et al., 2004). Similarly, in L. lactis ArgR and AhrC act interdependently to control arginine biosynthesis and catabolism gene expression, and it has been proposed that these may combine in the presence of arginine to form a heterohexameric complex that is an active repressor (Larsen et al., 2004, Larsen et al., 2008). However, the DNA binding activities of AhrC and ArgR regulons are not completely equivalent and promoter binding assays indicate that AhrC interferes with ArgR binding to the promoter upstream of the arginine catabolic operon (Larsen et al., 2005). S. pneumoniae contains three ArgR/AhrC regulators, of which ArgR1 and AhrC have been shown to act cooperatively to repress the expression of at least five promoters in response to high arginine (Kloosterman & Kuipers, 2011). In contrast to L. lactis, the S. pneumoniae ArgR1 and AhrC proteins are not involved in the control of the arginine catabolism operon arcABC. The third S. pneumoniae ArgR paralogue has not been analysed to date.

To the best of our knowledge, our data represent the first holistic analysis of the roles of three ArgR/AhrC family regulators in any organism. As in other bacteria, S. gordonii
ArgR and AhrC act cooperatively to repress the expression of arginine biosynthesis and transport genes in high arginine. ArcR has already been shown to induce arginine catabolism genes under high arginine (Zeng et al., 2006). Here, we have shown that ArcR also strongly represses argGH under high arginine. This presumably reduces the conversion of citrulline to arginine, and channels citrulline to the arginine catabolism pathway under high arginine (see Fig. S1). Under low arginine, ArcR had a minor stimulatory effect on the expression of argCJBD and pyrA<sub>dp</sub>pyrA<sub>b</sub>. Therefore, all three ArgR/AhrC family regulators are required for the co-ordinated control of arginine metabolism gene expression in S. gordonii. A model for the functions of ArgR, AhrC and ArcR in the regulation of arginine metabolism genes is presented in Fig. 9. It is important to note that we have not investigated direct binding of ArgR/AhrC regulators to promoter regions and it is possible that some regulatory effects may occur through other transcriptional regulators or by differential mRNA degradation.

Predictions of transcription factor binding sites at RegPrecise (http://regprecise.lbl.gov/RegPrecise/) or PePPER (http://genome2d.molgenrug.nl/) databases identified putative ArgR/AhrC regulatory box elements upstream of a number of arginine-regulated genes, including argC, argG, arcD, pyrR, serS, asd, SGO_1716, SGO_1317, SGO_1656 and SGO_1716. However, these results must be interpreted with caution since searches also returned a number of ‘false positives’, where apparent regulatory elements were identified in genes that were not regulated in response to arginine depletion by microarray. It was not possible to search specifically for ArcR regulatory elements as the ArcR consensus element is not well established.

Global gene expression in response to arginine limitation or to disruption in ArgR/AhrC family regulators has been investigated in a number of bacteria. In L. lactis, disruption of argR and/or ahrC led to de-repression of arginine biosynthesis genes in high arginine (Larsen et al., 2008). Disruption of ahrC also led to down-regulation of arginine
catabolism genes, while argR knockout resulted in slight increases in pyrimidine biosynthesis genes. In S. pneumoniae, growth in low arginine resulted in the up-regulation of 13 genes including genes involved in amino acid or oligopeptide transport and arginine biosynthesis, and down-regulation of five genes including pyrD which is required for pyrimidine biosynthesis (Kloosterman & Kuipers, 2011). In E. coli, the ArgR regulon is extensive, and includes 423 genes (Cho et al., 2012). Many of these are controlled indirectly through the action of ArgR on other transcriptional regulators. Genes that are controlled directly by ArgR include those involved in arginine biosynthesis and transport, histidine biosynthesis and the biosynthesis of glutamate, aromatic amino acids and lysine. Our microarray analysis demonstrates that S. gordonii also mounts a major restructuring of gene expression in response to arginine restriction involving changes in expression of >450 genes. As with other organisms, amino acid metabolism and transport are among the functions most strongly regulated by arginine. In addition, arginine modulates expression of genes in the pyrimidine metabolism pathway, which is closely linked to arginine metabolism. However, in S. gordonii the overall impact of arginine restriction appears to be a reduction in processes associated with growth and metabolic activity such as protein synthesis, biosynthetic pathways and cell envelope biogenesis. In addition, among the most strongly regulated genes were those associated with adhesion and biofilm formation.

Selected genes were validated by qRT-PCR analysis, and we attempted to define the structure of operons based on in silico analyses combined with analysis of gene expression data. Several important operons were shown to be regulated by arginine. For example, Hsa, or its allelic variant GspB in certain strains of S. gordonii, is a critical adhesin for binding host glycoproteins and platelets (Takamatsu et al., 2006, Jakubovics et al., 2009, Pyburn et al., 2011). The function of Hsa is dependent of secretion by the SecA2-SecY2 system and five accessory secretory proteins (Asp proteins) encoded by genes present in the hsa locus.
Although the microarray data presented here indicated that all genes in the *hsa* locus were down-regulated in response to arginine, there were differences in the level of regulation across the locus (Fig. S6). Generally, genes further downstream of *hsa* were more strongly regulated in response to arginine restriction than genes closer to *hsa*. It is likely that there are several promoters in the *hsa* gene locus, and/or that there is selective degradation of mRNA from this region. Further studies will be required to identify the impact of fluctuations in extracellular arginine on Hsa function. Other major adhesion or biofilm formation loci were more consistently regulated in response to arginine. For example, all genes in the *bfb* (biofilm formation/cellobiose PTS) locus were downregulated >15-fold following arginine restriction by microarray analysis (Table 2 and Fig. S6) and, in the case of *bfbC* and *bfbF*, strong down-regulation was confirmed by qRT-PCR.

We have previously shown that *S. gordonii* responds to coaggregation with *A. oris* by up-regulating genes involved in arginine biosynthesis and biofilm formation (Jakubovics et al., 2008a). Here, we have demonstrated that arginine restriction influences the expression of 21 of the 23 genes that were shown to be responsive to coaggregation. The effects of coaggregation on gene expression were not seen in co-cultures of *S. gordonii* and *A. oris* in which cells were dispersed, indicating that physical contact with a surface is required for gene regulation (Jakubovics et al., 2008a). A number of previous studies have identified connections between arginine and biofilm formation in different bacteria. For example, at physiological concentrations found in cystic fibrosis sputum, arginine promotes *P. aeruginosa* biofilm formation and prevents swarming motility (Bernier et al., 2011a). In model *P. aeruginosa* biofilms, arginine catabolism genes are up-regulated compared with planktonic cells, leading to anaerobic metabolism and increased susceptibility to ciprofloxacin and tobramycin (Sauer et al., 2002, Borriello et al., 2004, Xu et al., 2013). Similarly, arginine deiminase activity is up-regulated in *S. aureus* and *S. pneumoniae*...
biofilms compared with planktonic cells (Zhu et al., 2007, Allan et al., 2014). In E. faecalis, the arginine-dependent regulators ArgR and AhrC are critical for biofilm formation in vitro (Kristich et al., 2008), and AhrC is also required in vivo in a mouse model of catheter-associated urinary tract infection (Frank et al., 2013). Interestingly, under static conditions in nutrient-rich media we have observed that S. gordonii strains disrupted in the arcR gene form approximately 50% reduced biofilms compared with the isogenic wild-type progenitor (data not shown). This does not appear to be directly associated with the role of ArcR in regulating arginine biosynthesis, transport or catabolism genes since mutants in argH, arcA, arcB or arcD are not impaired in biofilm formation. Therefore it is possible that a different target of ArcR gene regulation plays a key role in biofilm formation, and we are currently investigating this hypothesis.

Using a microfluidic system that facilitates the growth of biofilms in flowing pooled human cell-free saliva, we show that arginine has a major impact on biofilm formation in S. gordonii. Free arginine in whole saliva is generally very low, around 6 µM and increasing to approximately 8 µM following a protein-rich meal (Brand et al., 1997). It has been suggested that microbial proteases may play a key role in releasing amino acids from salivary proteins (Syrjänen et al., 1990), and therefore amino acid levels in the dental plaque microenvironment may depend upon the microbial species present. In the microfluidic model, arginine-supplemented saliva in the µM range enhanced biofilm development while the upper-mM range retarded biofilm development and altered biofilm architecture (Fig. 8). It is unclear why high concentrations of arginine cause reductions in the biomass of S. gordonii biofilms, though this observation is important since high concentrations of arginine are currently being incorporated into oral healthcare products (Sullivan et al., 2014). The catabolism of arginine produces ammonia, which is alkaline, and trials are currently underway to assess the potential for arginine to be used as an anti-caries agent on the basis
that the ammonia released by bacterial metabolism of arginine neutralises dental plaque acid (Nascimento et al., 2009, Nascimento et al., 2013a, Nascimento et al., 2013b). It is possible that excessive alkaline production in biofilm cells of *S. gordonii* may trigger the release of cells from surfaces, Ammonia itself has been shown to be a signalling molecule that modulates biofilm formation and resistance to antibiotics (Nijland & Burgess, 2010, Bernier et al., 2011b). However, in the microfluidics biofilm system, used in this study, high concentrations of arginine did not result in significantly greater increases in pH of the effluent during biofilm growth than low arginine concentrations. Thus, arginine may inhibit cell-cell interactions directly. It is already known that arginine inhibits or retards coaggregation (Kamaguchi et al., 1994, Levesque et al., 2003) and autoaggregation (Merritt et al., 2009). Changes in cell-cell interactions will likely be most apparent in biofilm models that incorporate fluid flow such as the microfluidic system employed in this study. Whether these effects extend to multi-species oral biofilms is currently being examined (manuscript in press).

In summary, our data strongly support the concept that arginine plays a major role in modulating key processes including growth and biofilm formation in *S. gordonii*. The regulatory response network for arginine is set up to allow arginine biosynthesis and growth when changes in external arginine are gradual, but to shut down cell growth in response to rapid depletion of arginine. We hypothesise that this regulatory architecture prevents *S. gordonii* from over-committing resources to cell growth when arginine transiently reaches high concentrations, such as during a meal. High concentrations of arginine trigger the dispersal of *S. gordonii* from biofilms, which could potentially enable *S. gordonii* to relocate to distant sites in the mouth. We are now undertaking investigations into the mechanisms underlying this process. Early colonisers such as *S. gordonii* are critical for the initiation of dental plaque development and for recruitment of potentially pathogenic microorganisms.
Ultimately, therefore, it may be possible to develop new strategies for oral biofilm control based on interfering with arginine sensing by oral bacteria.

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**Materials and Methods**

**Bacterial media and growth conditions.** *S. gordonii* was routinely cultured in Todd Hewitt Broth (THB) medium (Difco, Detroit, MI) without shaking at 37°C or on THB solidified by the addition of 15g/L Bacto-agar at 37°C in a candle jar. For some experiments, *S. gordonii* was cultured in chemically defined medium (CDM), prepared as described previously (Jakubovics et al., 2008a) and incubated either aerobically or in an anaerobic environment under 90% N₂/5% H₂/5% CO₂. For gene regulation studies, L-arginine.HCl (Sigma) was added to growth media as appropriate. Alternatively, when required, L-arginine was omitted from CDM (CDM-arg) or CDM was prepared without L-histidine or BCAA (L-leucine, L-isoleucine and L-valine). Alternatively, CDM was supplemented with L-arginine to a final concentration of 5 mM. Prior to growth in CDM, *S. gordonii* was cultured in TYEG medium containing (per L) 10 g Bacto tryptone, 5 g yeast extract, 3 g K₂HPO₄ and 2 g D-glucose, adjusted to pH 7.5 before autoclaving. For microfluidics biofilms, *S. gordonii* was initially cultured in Schaedler’s medium (Difco) at 37°C without shaking. *E. coli* was cultured in LB medium or on LB medium solidified by the addition of 15 g L⁻¹ Bacto-agar (Difco).
blue/white selection, 16 µL of 0.1 M isopropyl β-d-1-thiogalactopyranoside (IPTG) and
50 µL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were spread over
solidified LB medium before adding cells. When required antibiotics were included in growth
media at the following concentrations: erythromycin 2 µg ml\(^{-1}\) (for \textit{S. gordonii}) or 100 µg ml\(^{-1}\)
(for \textit{E. coli}), ampicillin 50 µg ml\(^{-1}\), spectinomycin 100 µg ml\(^{-1}\), kanamycin 250 µg ml\(^{-1}\).

**Genetic manipulation of \textit{S. gordonii}**. Routine genetic manipulations were conducted as
described by Sambrook & Russell (2001). All gene replacement constructs were generated
using PCR overlap extension mutagenesis either with or without a cloning step in vector
pGEM-T. Primers for mutagenesis are listed in Table S2. For disruption of \textit{arcR}, \textit{argR} or
\textit{ahrC} by insertion of an \textit{ermAM} cassette, or disruption of \textit{argR} by \textit{aphA3} insertion, primers
were designed to amplify approximately 500 bp regions upstream and downstream of the
target genes from \textit{S. gordonii} chromosomal DNA, with a central \textit{EcoRI} restriction site. The
‘F2’ primer (forward primer for the region downstream of the gene of interest) contained a 5’
extension designed to overlap with the ‘R1’ primer (reverse primer for the upstream region).
The upstream and downstream regions were PCR amplified, and the fragments were cleaned
with the QIAquick PCR clean-up kit (Qiagen). Equimolar ratios of the products were
combined and used as template for a second round of PCR. The product generated was
cloned in pGEM-T vector (Promega) to generate pGEM-\textit{arcR}, pGEM-\textit{argR} or pGEM-\textit{ahrC},
and used for transformation of \textit{E. coli} JM109. To insert antibiotic resistance cassettes, \textit{ermAM}
\textit{or \textit{aphA3}} genes were PCR-amplified from plasmids pCM18 (Hansen \textit{et al.}, 2001) or pSF151
(Tao \textit{et al.}, 1992) with primers \textit{ermF1/R1} or \textit{aphA3F1/R1} that contained \textit{EcoRI} restriction
sites (Table S2). Fragments were cleaned, digested with \textit{EcoRI} and ligated with pGEM-based
plasmids to generate pGEM-\textit{arcR::ermAM}, pGEM-\textit{argR::ermAM}, pGEM-\textit{argR::aphA3} or
pGEM-\textit{ahrC::ermAM} and used to transform \textit{E. coli} JM109. Plasmids were screened for those
that contained the antibiotic resistance cassette in the same orientation as the gene that it was
replacing. Plasmid inserts were amplified with arcRF1/R2, argRF1/R2 or ahrCF1/R2 as appropriate, and products were used to transform *S. gordonii* DL1 (Challis) as previously described (Jakubovics *et al.*, 2005). All mutants were checked by PCR amplification and sequencing.

For disruption of *arcB* or *pyrB*, or replacement of *arcR* with the *aad9* spectinomycin resistance cassette amplified from plasmid pDL278 (LeBlanc *et al.*, 1992), mutagenesis was employed without a cloning step. Primers were designed to amplify approximately 500 bp upstream or downstream of the gene of interest. Extensions were added to the 5’ end of the ‘R1’ and ‘F2’ primers to overlap primers for amplification of the antibiotic resistance cassette. Following PCR amplification of the regions upstream and downstream of the gene of interest and the antibiotic resistance cassette, the three fragments were combined in equimolar quantities and used as template for a second round of PCR. For replacement of *arcB* with *aphA3*, the upstream and downstream regions of *arcB* were amplified with *arcBF1/R1* and *arcBF2/R2*, respectively, and the *aphA3* cassette was amplified from plasmid pSF151 with primers *aphA3F2/R2*. To replace *arcB* with *ermAM*, *arcB* was amplified from *S. gordonii* chromosomal DNA using primers *arcBF1/R3* and *arcBF3/R2*, in which 5’ overlap extensions were included in the ‘R3’ and ‘F3’ primers. The *ermAM* cassette was amplified from pCM18 with *ermF1/R1*. Similarly, for *pyrB* mutagenesis, regions around the *pyrB* gene were amplified with *pyrBF1/R1* and *pyrBF2/R2* for disruption with *aphA3*, or with *pyrBF1/R3* and *pyrBF3/R2* for disruption with *ermAM*. Overall, these reactions generated products *arcB::ermAM*, *arcB::aphA3*, *pyrB::ermAM*, *pyrB::aphA3* or *arcB::aad9*. Fragments were cleaned and used for transformation of *S. gordonii* DL1 (Challis). A similar approach was employed to generate the *argD-arcB* complementation strain. Approximately 500 bp regions surrounding a predicted Rho-independent terminator (5’- AAAAGGATTCAGTTTGAGCTGGATTCTTTTT-3’) downstream of *argD* were amplified.
with primers argDF1/R1 and argDF2/R2 (Table 3). The arcB gene was amplified with primers arcBF4/R4. Following PCR amplification, the three products were mixed and used as a template for a second round of PCR with primers argDF1/R2. The argD-arcB fragment generated was cleaned and used for transformation of S. gordonii DL1. Transformants were selected on solidified CDM-arg medium. All transformants were checked by PCR amplification and DNA sequencing. For complementation of arcB mutants with arcB gene immediately downstream of the promoter P_\text{arcA}, the arcB gene region, P_{arcA} promoter and an approximately 5 kb region of plasmid pPE1010 (Egland et al., 2004) were PCR amplified using primer pairs arcBF5/R5, ParcAF1/R1 and pPE_F1/R1, respectively, fused to generate plasmid pNJ-arcB and used for transformation of E. coli Stellar competent cells using the In-Fusion HD PCR ligation cloning kit (Clontech, Mountain View, CA). Plasmids were extracted, checked by DNA sequencing and used for transformation of S. gordonii arcB::aphA3.

**Growth in chemically defined media.** For assessing growth in CDM amended to different concentrations of L-arginine, cells were initially cultured on solidified TYEG medium for 96 h at 37°C and 5% CO₂. Individual colonies were subcultured to CDM and incubated for 24 h at 37°C, 5% CO₂. Cultures were diluted 1:100 in CDM and incubated for a further 24 h at 37°C, 5% CO₂. Cells were harvested by centrifugation, washed twice in CDM-arg and resuspended in CDM-arg. Cultures were used to inoculate CDM amended to various concentrations of arginine, to achieve an initial turbidity of between 25-30 Klett Units (KU), measured using a Klett-Summerson colorimeter with a 660 nm filter (Klett Manufacturing Co., New York). Cultures were incubated at 37°C anaerobically (90% N₂/5% H₂/5% CO₂) or aerobically (atmospheric CO₂) for 96-120 h and the final growth yields were determined. Growth experiments were repeated three times independently and converted to a four point semi-quantitative scale [<51 KU (-), 51-150 KU (+), 151-250 KU (++) or >250 KU (+++)].
In most cases at least two of the three cultures had the same growth yield on the four point scale, and this value was reported. Occasionally, three different values were obtained for the same strain at one arginine concentration, in which case the median value was given.

For experiments investigating transcriptional regulation in response to shifts in arginine, histidine or BCAA concentration, *S. gordonii* DL1 or isogenic ArgR-family regulator mutants were cultured in TYEG at 37°C for 24 h, with antibiotics as appropriate. Cells were subcultured to CDM and incubated at 37°C for 24 h. Cells were further subcultured and grown at 37°C in CDM to mid-exponential phase (140-160 KU). Cultures were split into two 4 ml portions, and each was harvested at 3,800 g, 20°C for 10 min in a swing-out rotor. Cell pellets were resuspended in CDM-arg, CDM, CDM supplemented to 5 mM L-arginine or CDM without either L-histidine or BCAA, and incubated at 37°C for up to a further 45 min.

**RNA extraction and RT-PCR/qRT-PCR.** Intracellular RNA was stabilised by the addition of 2 volumes of RNAProtect (Qiagen, Valencia, CA) and vortex mixing for 5 s, and RNA was extracted as previously described (Jakubovics et al., 2008a). Briefly, cells were pelleted by centrifugation, the supernatant was removed and cells were stored at -70°C for up to 72 h. Cells were re-suspended in 1 ml Trizol reagent (Invitrogen, Carlsbad, CA), mixed with lysing matrix B (Qiogene, Morgan, Irvine, CA) and disrupted in a FastPrep bead beater (Qiogene). Subsequently, RNA was extracted using the Trizol manufacturer’s protocol. Extracted RNA was treated for 1 h at 37°C with RQ1 DNase I (Promega, Madison, WI) and purified using RNeasy MinElute columns (Qiagen). A sample of RNA was analysed on a 0.8% (wt/vol) agarose gel containing 3% (vol/vol) formaldehyde to check for degradation. The concentration of RNA in each sample was estimated with a NanoDrop ND-1000 spectrophotometer (Labtech, Uckfield, East Sussex).
For RT-PCR and qRT-PCR analysis, samples were reverse transcribed with Superscript III reverse transcriptase (Invitrogen) and cleaned using MinElute columns (Qiagen). Primers 1446F/R and 1447F/R for RT-PCR analysis of arcA/arcB are described in Table S2. Reactions were carried out using REDTaq polymerase (Sigma-Aldrich, St Louis, MO) with the following thermocycle protocol: denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 90 s, and a final elongation at 72°C for 5 min. Primers 1446F/R and 1447F/R were also used for qRT-PCR analysis of arcA and arcB. Other qRT-PCR primers are shown in Table S2, except the following which have previously been reported: 16SSgF1/R1 (16S rDNA), 0175F/R (argG), 1590F/R (arcD), 1569F/R (argC), 1104F/R (pyrA b) and 1075F/R (amyB) (Jakubovics et al., 2008a). Reactions (25 µL total volume) contained 0 to 10 ng cDNA template, 12.5 µL Power SyBr Green PCR mix (Applied Biosystems, Foster City, CA) and forward/reverse primers each at 300 nM, with the exception of 16SSgF1/R1 reactions, which contained primers at 100 nM. An MX3005P thermocycler (Stratagene, La Jolla, CA) was employed for qRT-PCR using the protocol: 95°C for 10 min, 40 cycles of 95°C for 30 s, 56°C for 1 min and 72°C for 30 s, and a dissociation curve consisting of 95°C for 1 min, 56°C for 30 s, and incremental increases in temperature up to 95°C. Fluorescence readings were collected following the 56°C primer annealing step and throughout the dissociation curve. Specific amplification of the desired fragments was assessed by the presence of a single sharp fluorescence decrease during the dissociation phase, and by analysis of representative samples on agarose gels. Reaction efficiencies were estimated by performing three independent reactions for each set of primers using dilutions of one S. gordonii cDNA sample as template over a 6-log range of concentrations. All primers gave reaction efficiencies >80%. Relative quantities of transcripts were calculated from three independent experiments by normalising against the 16S rDNA.
gene as described previously (Jakubovics et al., 2008a). The heatmap was drawn in R (R Core Team, 2014).

**DNA microarray analysis.** A microarray containing 2,051 probes for *S. gordonii* genes was designed using the Agilent eArray platform (Agilent Technologies, Wokingham, Berkshire, UK). Custom settings were employed to design probes optimised for hybridization at 65°C. Probe sequences and microarray data have been deposited in the Gene Expression Omnibus (GEO) database under accession numbers GSE51346 and GPL17786. An annotation file for the array was produced by aligning the probe sequences to the *S. gordonii* genome using Bowtie2 (Langmead & Salzberg, 2012). BEDTools (Quinlan & Hall, 2010) and custom Perl scripts were then used to produce the annotation for each probe using the GenBank file for the *S. gordonii* genome as a source of annotation. Samples of RNA from four independent experiments were sent to the Functional Genomics Unit, Birmingham University, UK for reverse transcription, labelling and hybridization. Data were analysed using GeneSpring software (Agilent). All data were normalised using the 75th percentile normalisation with baseline to median. Samples were taken from four independent experiments, and significant differences between expression levels in high or no arginine were assessed using T-tests with p-values corrected for multiple testing using Benjamini-Hochberg false discovery rate (FDR) correction (Reiner et al., 2003) within Genespring GX 11 (Agilent) in conjunction with the custom annotation file. Genes were considered significantly regulated if they had FDR corrected p-value of ≤0.05 and the fold change was >2. Functional Clusters of Orthologous Gene (COGFun) designations were taken from the MicrobesOnline database (http://meta.microbesonline.org/operons/gnc467705.html).

**Saliva and inoculum preparation for biofilm experiments.** Human saliva was collected from volunteers in accordance with the University of Michigan Institutional Review Board evaluated protocol (HUM00042954) described by Nance et al. (2013). Cell-free saliva (CFS)
was used as the lone nutrient source. This was prepared using a similar protocol to that
described by Rao et al. (2011). Briefly, saliva was gathered from five healthy adults who had
not consumed anything but water for at least two hours prior to collection. All donors had not
taken any antibiotics for at least 3 months and did not smoke. The saliva from each donor was
pooled and 2.5 mM DTT was added before standing on ice for 10 min. The pooled saliva was
then centrifuged at 20,000 g for 30 min. The supernatant was collected and diluted with
distilled water to a final concentration of 25%. The 25% saliva was then filter sterilized using
a 0.22 µm pore-size filter (Nalgene) to yield CFS. For long-term storage, CFS was separated
into 30 ml aliquots and stored at -80°C. Prior to use, arginine was added to CFS to final
concentrations of 0.5 µM, 5 µM, 50 µM, 500 µM, 5 mM, 50 mM, or 500 mM. A control with
CFS containing no arginine was used for all experiments. Inocula were prepared by growing
S. gordonii DL1 in 5 ml of Schaedler’s broth that had been pre-reduced in and atmosphere
containing 5% CO₂. The culture was grown under an atmosphere containing 5% CO₂ at 37°C
until an OD₆₀₀ of 0.4 was reached, whereupon it was used as an inoculum for microfluidics
experiments.

Microfluidics system. Biofilms were developed using a Bioflux 200 microfluidics system
(Fluxion, San Francisco, CA) with attached 48-well Bioflux microfluidics plates. Each
Bioflux plate was conditioned with CFS prior to use. One hundred µl of each concentration
of arginine-supplemented CFS were added in triplicate to each inlet well and flowed at 1.0
dyne/cm² for 2-3 min at 20°C, followed by 20 min at 20°C with no flow in order to condition
the channels for cell attachment. Exponentially growing S. gordonii DL1 cell suspensions in
Schaedler’s broth at an OD₆₀₀ of 0.4 were flowed into the Bioflux system for 6 s from the
outlet port at a speed of 1.0 dyne/cm², to facilitate inoculation of the viewing area and not
further upstream (ie preventing contamination of the inlet reservoir). The plate was then
incubated at 37°C for 45 min with no flow. The outlet wells containing the inoculum were
aspirated and 900 µl of each of the respective arginine concentrations of CFS were added to each inlet well to bring the total inlet volume to approximately 1 ml. The CFS then flowed from the inlet to outlet wells for 20 h at 0.2 dyne/cm$^2$ at 37°C.

Following incubation, all wells were aspirated and the biofilms were washed with 100 µl of PBS for 20 min at 0.2 dyne/cm$^2$ at 20°C. Biofilms were stained with BacLight LIVE/DEAD viability kit (Invitrogen, Grand Island, NY), prepared using 3 µl of each component (Sty-9 and propidium iodide) per 1 ml of PBS. After aspirating all PBS from the wells, 100 µl of the stain were added to each inlet well and flowed at 0.2 dyne/cm$^2$ for 40 min at 20°C. All stain was then aspirated from the wells and 100 µl of PBS were added to each inlet well and run at 0.2 dyne/cm$^2$ for 20 min at room temperature to remove any excess stain.

**Imaging and analysis of microfluidics biofilms.** A Leica SPE confocal laser scanning microscope (CLSM) (Leica, Exon, PA) equipped with a 40× 1.25 NA HCX PL APO infinity-corrected oil objective lens was used to image the biofilms. Images were obtained using a 488 nm laser set at 15% of maximum power, which allowed the excitation of both components of the BacLight LIVE/DEAD stain (Syto-9 and propidium iodide). The excitation capture range for Syto-9 was 510-540 nm while the range for propidium iodide was 620-650 nm. A negative control of arginine-free CFS was used to calibrate the offset and gain for the microscope, which was then kept constant for image capturing of all biofilms within that experiment.

After image collection, IMARIS software (Bitplane, Zurich, Switzerland) was used to visualize the biofilms in 2D and 3D. Additionally, IMARIS was used to prepare the images for analysis using COMSTAT software (Heydorn *et al.*, 2000) and IMAGEJ (Schneider *et al.*, 2012). COMSTAT was used to quantify the biomass of the biofilm, as well as its average thickness and roughness. Thickness was measured from where one surface of the biofilm
contacts the glass on the bottom of the plate to the top of the opposite surface in the channel in the field of view, while roughness was a measurement of thickness variability in a single field of view. For the LIVE/DEAD quantification, IMAGEJ was used. Green represented viable cells, while red represented dead or damaged cells and the pixel intensity of red and green was measured in throughout the image stacks using the Histogram function for each channel. The percentage of green and red, and therefore the percentage of viable and non-viable cells, was subsequently determined for each image stack. This was performed using Excel (Microsoft, Redmond, WA) to multiply the total pixels by the intensity (8 bit images, 0-255 in intensity levels). All software programs were run on a computer containing an Intel i5 processor (Intel, Santa Clara, CA) and a Radeon 5850 graphics card with 1 GB of RAM.

Statistical tests. Testing for significant differences was performed by one-way ANOVA using the Tukey’s post-hoc test for pairwise comparisons.
References


R Core Team (2014) R: A language and environment for statistical computing.


### Tables

**Table 1.** Growth yield of *S. gordonii* strains in CDM amended to different arginine concentrations.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Anaerobic</th>
<th>Aerobic</th>
<th>0</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>128</th>
<th>256</th>
<th>512</th>
<th>µM arginine</th>
</tr>
</thead>
<tbody>
<tr>
<td>w-t</td>
<td>+++(^a)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><em>arcB::aphA3</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>pNJ-<em>arcB</em></td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><em>arcB</em>(_{comp})</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><em>arcB</em>++</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^a\)Values represent final growth yields after incubation in CDM amended to different concentrations of arginine for 96 h. Semi-quantitative assessment of growth from three independent experiments was as follows: <51 KU (-), 51-150 KU (+), 151-250 KU (++) or >250 KU (+++).
Table 2. Predicted operons containing genes that were strongly (>10-fold) regulated by a shift from 0.5 mM arginine to no arginine in microarrays.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Description</th>
<th>Fold Change (range)³</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upregulated (no arginine vs 0.5 mM arginine)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGO_1566-1569</td>
<td>ArgD/ArgB/ArgJ/ArgC, arginine biosynthesis</td>
<td>339.6 (260.9, 520.1)</td>
</tr>
<tr>
<td>SGO_0175-0177</td>
<td>ArgG/ArgH, arginine biosynthesis</td>
<td>267.6 (207.9, 342.4)</td>
</tr>
<tr>
<td>SGO_1656</td>
<td>Phosphoenolpyruvate carboxykinase</td>
<td>44.4</td>
</tr>
<tr>
<td>SGO_0645-0648</td>
<td>Hypothetical proteins</td>
<td>40.5 (32.4, 54.1)</td>
</tr>
<tr>
<td>SGO_1102-1106</td>
<td>PyrAa/PyrAb, arginine/pyrimidine biosynthesis</td>
<td>23.9 (18.7, 28.9)</td>
</tr>
<tr>
<td>SGO_0091-0094</td>
<td>Hypothetical proteins</td>
<td>12.1 (4.5, 36.6)</td>
</tr>
<tr>
<td>SGO_0021</td>
<td>Hypothetical protein</td>
<td>10.1</td>
</tr>
<tr>
<td>SGO_0681</td>
<td>IleS, isoleucyl tRNA-synthetase</td>
<td>-11.1</td>
</tr>
<tr>
<td>SGO_0966-0978</td>
<td>Hsa, secondary secretion and glycosylation systems</td>
<td>-4.9 (-1.9, -10.8)</td>
</tr>
</tbody>
</table>

| SGO_1102-1106  | Hypothetical proteins                                                       | 13.7                 |
| SGO_0831       | Hypothetical protein                                                        | 13.3                 |
| SGO_2098       | RpsD, ribosomal protein S4                                                  | 12.5                 |
| SGO_1686-1700  | Fab/acc locus, fatty acid biosynthesis                                      | -11.4 (-6.4, -30.8)  |
| SGO_0681       | IleS, isoleucyl tRNA-synthetase                                             | -11.1                |
| SGO_2015-2028  | Receptor polysaccharide biosynthesis                                       | -8.6 (-4.0, -12.9)   |
| SGO_0966-0978  | Hsa, secondary secretion and glycosylation systems                          | -4.9 (-1.9, -10.8)   |

³Fold increase (positive numbers) or decrease (negative numbers) in no arginine compared with 0.5 mM arginine. Where genes appear to be part of operons, the expression levels of the most strongly and most weakly expressed genes in the operon are shown (range).
**Figure legends**

**Figure 1. Analysis of arcA-arcB gene transcript by RT-PCR.** Combinations of arcAF1, arcAR1, arcBF1 and arcBR1 primers were used to amplify fragments of the arcB gene (111 bp; lanes 1, 4 and 7), arcA gene (173 bp; lanes 3, 6 and 9), or a region spanning arcA-arcB (1,438 bp; lanes 2, 5 and 8). Positive control reactions (lanes 1-3) employed chromosomal DNA as a template for PCR. Alternatively, DNase I-treated RNA preparations were used as PCR templates either without reverse transcriptase (RT) (lanes 4-6) or with RT (lanes 7-9). The presence of a band at 1,438 bp from cDNA template (lane 8) indicates that arcA and arcB are co-transcribed.

**Figure 2. Regulation of arcA and arcB genes in response to shifts in arginine concentrations.** Cells were cultured in CDM supplemented with 10 mM glucose to mid-exponential phase and arginine was added to a final concentration of 50 mM at time = 0 min (dashed lines). Alternatively, cells were cultured in CDM supplemented with 5 mM arginine to early exponential phase and, at time = 0 min, cells were harvested and resuspended in CDM lacking arginine. At intervals, aliquots were removed and expression of arcA (closed symbols) and arcB (open symbols) was determined by qRT-PCR. Total levels of RNA were normalised by comparison with 16S rRNA levels and relative levels compared with time = 0 min are shown.

**Figure 3. Constructs for mutagenesis and complementation of arcB.** A. The native position of arcB in the arcABC gene cluster is shown, along with the site of insertion of the aphA3 cassette in the arcB::aphA3 gene knockout construct. An additional strain was constructed in which arcB was replaced with the ermAM cassette at the same location (not
shown). In the complementation strain, \(arcB_{\text{Comp}}\), the \(arcB\) gene was inserted immediately downstream of \(argD\) and upstream of the predicted Rho-independent transcription terminator. An additional construct containing two copies of \(arcB\), one downstream of \(arcA\) and one downstream of \(argD\) was also produced (\(arcB++\)). Predicted gene promoters are indicated by bent lines with arrows. Under arginine restriction, \(arcA\) promoter activity is reduced whereas \(argC\) promoter activity is increased. Expression of \(arcB\) in the different strains after a shift from 5 mM arginine to either 5 mM arginine (black bars) or no arginine (grey bars) was determined by qRT-PCR (B). Values are means and SDs of \(\log_2\) fold change compared with \(S.\ gordonii\) DL1 in 0.5 mM arginine (marked as ‘C’ for comparator).

**Figure 4. Effects of disrupting ArgR/AhrC family regulators on the expression of arginine biosynthesis, transport and catabolism genes under high and no arginine.** Anaerobically growing cells of \(S.\ gordonii\) DL1 (w-t) and isogenic \(argR, ahrC\) and \(arcR\) single mutants, \(argR ahrC\), \(argR arcR\) and \(ahrC arcR\) double mutants and an \(argR ahrC arcR\) triple mutant were exposed to 5 mM arginine or no arginine for 30 min, and RNA was extracted. Expression of arginine biosynthesis genes (\(argC, pyrA_b\) and \(argG\)), the gene encoding an arginine-ornithine antiporter (\(arcD\)) and arginine catabolism genes (\(arcA\) and \(arcB\)) was quantified by qRT-PCR. In each case, expression levels were compared with \(S.\ gordonii\) DL1 in 5 mM arginine (marked as ‘C’ for comparator). Bars represent means, and SDs from three independent experiments are shown. Note that different scales have been used for the y-axes.

**Figure 5. Comparison between microarray data and qRT-PCR.** Total RNA was extracted from \(S.\ gordonii\) following 30 min exposure to CDM either without or with arginine. The
relative levels of gene expression in the absence of arginine compared with arginine-containing medium determined by microarray were plotted against levels assessed by qRT-PCR. The relative levels of expression of six arginine metabolism/transport genes (argC, argG, pyrAb, arcD, arcB and arcA) and one control gene (amyB) in 0.5 mM arginine versus no arginine were assessed by microarray and compared with expression levels in 5 mM arginine versus no arginine, determined by qRT-PCR (closed circles). In addition, qRT-PCR was used to confirm the levels of expression of several genes (SGO_0846, hsa, asp5, hisC, bfbC, bfbF, SGO_1686, wefE and wzg) in the same RNA samples as those used for the microarray (open triangles). A linear regression line was drawn based on all the comparisons of qRT-PCR data with microarray data.

**Figure 6. Growth and gene expression in S. gordonii DL1 following depletion of arginine, histidine or BCAA.** A. Cells were cultured anaerobically in CDM to mid-exponential phase (OD$_{600}$ ~ 0.5), harvested and resuspended in CDM (filled circles) or CDM lacking arginine (open circles), histidine (closed triangles) or BCAA (open triangles), indicated by an arrow, and growth was monitored until stationary phase. B. 30 minutes after resuspension in different media, aliquots of cells were removed and gene expression was monitored by qRT-PCR. Expression of 14 different genes is shown as a heatmap, and each colour represents the mean fold change compared with cells resuspended in CDM from 4 independent experiments.

**Figure 7. Comparison between regulation of S. gordonii genes by coaggregation and by arginine depletion.** The arginine-dependent expression of genes that had previously been identified as being regulated by coaggregation with A. oris was assessed using DNA
microarrays. All genes that were significantly up-regulated in monocultures compared with coaggregates were also up-regulated in low arginine compared with high arginine. Most genes that were down-regulated in monocultures were also down-regulated in low arginine, with the exception of spxB (pyruvate oxidase) and SGO_1308 (hypothetical protein), which were not regulated by arginine (circled).

Figure 8. CLSM micrographs showing *S. gordonii* biofilms developed in different concentrations of L-arginine in 25% human saliva. Biofilm images are rendered in the XY dimension (A-H), the XZ dimension (A1-H1), and XYZ dimension (A2-H2). Images are ordered by increasing arginine concentration: control/no added arginine, 0.5 µM arginine, 5 µM arginine, 50 µM arginine, 500 µM arginine, 5 mM arginine, 50 mM arginine, 500 mM arginine. Bar represents 20 µm. Associated table shows biofilm characteristics after development in different arginine concentrations. Values represent an average of at least nine images from three different microfluidic channels.

Figure 9. A model of regulation of arginine metabolism genes by ArcR, ArgR and AhrC. ArgR and AhrC are dependent on each other for activity and here they are represented as a functional protein complex. A. In the presence of arginine, ArcR, ArgR and AhrC are activated. This is shown as direct binding by 6 arginine residues (Arg$_6$). Activated ArgR/AhrC represses transcription of genes involved in arginine biosynthesis (shaded arrows) or accessory arginine-related functions (black arrows), indicated by lines with capped ends. In the presence of arginine, ArcR positively regulates expression of arginine catabolism genes (white arrows), shown by a line with an arrowhead, and negatively regulates argGH expression. Elements upstream of these genes that have consensus ARG box signatures are indicated by shaded boxes. Predicted promoters are indicated by thin right-facing arrows, and
terminators are shown as loops and vertical lines. B. In very low or no arginine, ArcR weakly up-regulates (dashed lines) promoters upstream of \textit{argC} and \textit{pyrR}.
Figure 2

MMI_13023_F2
A

Optical Density (OD/gpp)

Time (minutes)

B

Color Key

Fold change

-30 -20 -10 0 10 20 30

haC
1401
gueA
arcB
hse
1699
wzg
0846
argC
aspS
1686
bhc
bhe
wreE

No arginine No histidine No BCAA

MMI_13023_F6
<table>
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<th>Biofilm Parameter</th>
<th>Control</th>
<th>0.5 µM</th>
<th>5 µM</th>
<th>50 µM</th>
<th>500 µM</th>
<th>5 mM</th>
<th>50 mM</th>
<th>500 mM</th>
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<tr>
<td>Viability (%)</td>
<td>84.00</td>
<td>69.99</td>
<td>76.78</td>
<td>82.34</td>
<td>81.24</td>
<td>86.30</td>
<td>81.65</td>
<td>75.37</td>
</tr>
<tr>
<td>(SEM)</td>
<td>(4.96)</td>
<td>(6.69)**</td>
<td>(5.37)**</td>
<td>(4.42)</td>
<td>(4.05)</td>
<td>(3.34)</td>
<td>(4.99)</td>
<td>(7.99)**</td>
</tr>
<tr>
<td>Biomass (µm²/µm²)</td>
<td>0.89</td>
<td>1.12</td>
<td>2.53</td>
<td>1.78</td>
<td>2.30</td>
<td>0.99</td>
<td>0.21</td>
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</tr>
<tr>
<td>(SEM)</td>
<td>(0.09)</td>
<td>(1.11)*</td>
<td>(1.66)*</td>
<td>(0.64)*</td>
<td>(1.32)*</td>
<td>(1.11)</td>
<td>(0.22)</td>
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<td>Thickness (µm)</td>
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<td>2.10</td>
<td>4.91</td>
<td>3.71</td>
<td>5.04</td>
<td>1.77</td>
<td>0.31</td>
<td>0.06</td>
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<tr>
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<td>(2.31)</td>
<td>(2.20)</td>
<td>(3.45)</td>
<td>(1.39)</td>
<td>(2.67)*</td>
<td>(2.21)</td>
<td>(0.38)*</td>
<td>(0.04)*</td>
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<tr>
<td>Roughness</td>
<td>1.74</td>
<td>1.80</td>
<td>1.72</td>
<td>1.66</td>
<td>1.65</td>
<td>1.76</td>
<td>1.90</td>
<td>1.98</td>
</tr>
<tr>
<td>(SEM)</td>
<td>(0.13)</td>
<td>(0.06)</td>
<td>(0.07)</td>
<td>(0.06)</td>
<td>(0.08)</td>
<td>(0.16)</td>
<td>(0.08)**</td>
<td>(0.01)**</td>
</tr>
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</table>

Data were derived from at least three separate microfluidics channels.
P<0.05 and **P<0.01: significant differences from the control.

MMI_13023_F8
A. High arginine

B. Low/no arginine

MMI_13023_F9