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Critical roles of arginine in growth and biofilm development by *Streptococcus gordonii*

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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 *Streptococcus 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-colonising 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 and Kolenbrander, 2010). For the successful colonisation 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 maximise the use of nutrients provided by saliva and partner species in the human oral cavity.

Following the development of chemically defined media, in the 1970s, 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; 1975; Terleckyj and 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, *S. criceti* AHT, a member of the mutans group, required arginine when cultured aerobically, but grew without arginine under

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strictly anaerobic conditions (Terleckyj and 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 as, to the best of our knowledge, oxygen does not directly inhibit any enzymes in the arginine biosynthesis pathway. Furthermore, *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 that 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 upregulation 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,b). *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 *Escherichia 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, *S. pneumoniae* has three ArgR/AhrC family regulators even though it is apparently auxotrophic for arginine (Kloosterman and 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 anaerobically 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* was 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 co-transcribed 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 reverse transcription polymerase chain reaction (RT-PCR) using primers 1446F/1447R, 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 co-transcribed 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, as 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 as ArcA and ArcB may be subject to post-transcriptional regulation (Liu *et al*., 2008).

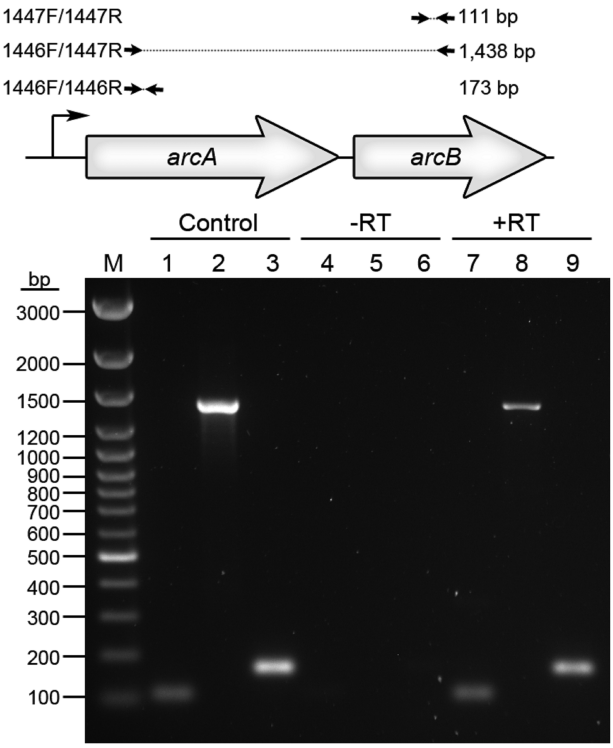


Fig. 1. Analysis of *arcA-arcB* gene transcript by RT-PCR. Combinations of 1446F, 1446R, 1447F and 1447R primers were used to amplify fragments of the *arcB* gene (111 bp; lanes 1, 4 and 7), *arcA* gene (173 bp; lanes 3, 6 and 9), or a region spanning *arcA-arcB* (1438 bp; lanes 2, 5 and 8). Positive 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 1438 bp from cDNA template (lane 8) indicates that *arcA* and *arcB* are co-transcribed.

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

We hypothesised 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 and 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. gordo-*

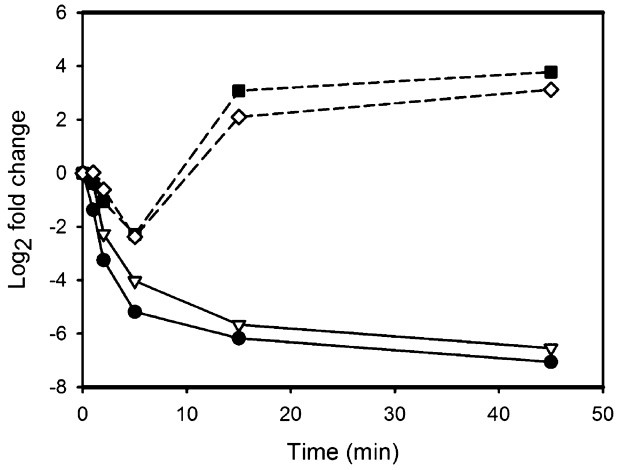


Fig. 2. Regulation of *arcA* and *arcB* genes in response to shifts in arginine concentrations. Cells were cultured in CDM supplemented with 10 mM glucose to mid-exponential phase, and arginine was added to a final concentration of 50 mM at time = 0 min (dashed lines). Alternatively, cells were cultured 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.

nii 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* $arcB_{Comp}$. The expression of *arcB* was assessed in each strain by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) following anaerobic culture to mid-exponential phase in CDM

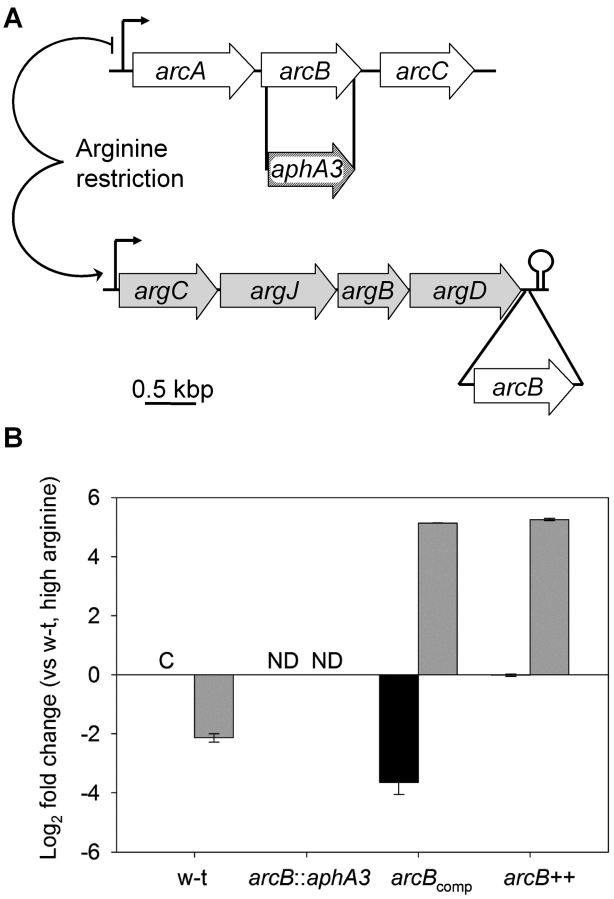


Fig. 3. Constructs for mutagenesis and complementation of *arcB*. A. The native position of *arcB* in the *arcABC* gene cluster is shown, along with the site of insertion of the *aphA3* cassette in the *arcB*::*aphA3* gene knockout construct. An additional strain was constructed in which *arcB* was replaced with the *ermAM* cassette at the same location (not shown). In the complementation strain, $arcB_{Comp}$, 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 .5 mM arginine (marked as 'C' for comparator).

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

	Anaerobic (μM arginine)	Aerobic (μM arginine)							
Strain	0	0	8	16	32	64	128	256	512
w-t	+++[a]	–	–	–	++	+++	+++	+++	+++
arcB::*aphA3*	–	–	–	–	–	++	+++	+++	+++
pNJ-*arcB*	+++	–	+	++	+++	+++	+++	+++	+++
$arcB_{Comp}$	+++	–	–	++	+++	+++	+++	+++	+++
arcB ++	+++	–	+++	+++	+++	+++	+++	+++	+++

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 (+++).

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 fivefold 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* $arcB_{Comp}$ ($P < 0.001$). In no arginine, *arcB* was elevated in *S. gordonii arcB*++ and *S. gordonii* $arcB_{Comp}$ by 165-fold and 150-fold, respectively, in comparison with the wild type under arginine restriction ($P < 0.001$). Therefore, the relocation of *arcB* to a position immediately downstream of *argD* resulted in strong upregulation 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* $arcB_{Comp}$ 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 *Lactococcis lactis* ArgR or AhrC, *E. coli* ArgR or *B. subtilis* AhrC, and three sequences were identified (Fig. S2). ArcR (SGO_1588) has previously been characterised 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 *B. 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 *Experimental procedures*. 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 (OD_{600} = 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 (0.5 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 $pyrA_b$ were strongly upregulated 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 $pyrA_b$ under 5 mM arginine or no arginine, indicating that both ArgR and AhrC are essential for down-regulation of *argC* and $pyrA_b$ in response to arginine. Disruption of *arcR* alone did not affect the expression of *argC* or $pyrA_b$ 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 $pyrA_b$ 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

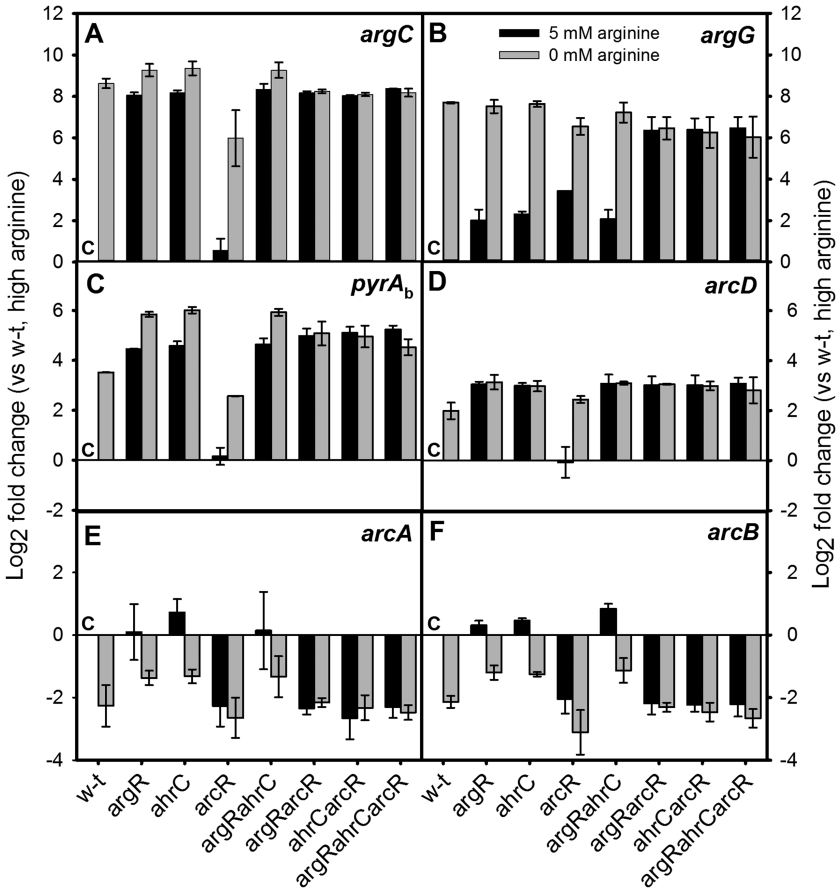


Fig. 4. Effects of disrupting ArgR/AhrC family regulators on the expression of arginine biosynthesis, transport and catabolism genes under high and no arginine. Anaerobically growing cells of *S. gordonii* DL1 (w-t) and isogenic *argR*, *ahrC* and *arcR* single mutants, *argR ahrC*, *argR arcR* and *ahrC 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.

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 upregulated 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 Genome2D (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 four- to fivefold lower in CDM lacking arginine than in CDM containing 5 mM arginine (Fig. 4E and 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 downregulation of arginine biosynthesis and transporter genes under high arginine, and (ii) ArcR acts independently of ArgR and AhrC to downregulate *argG* expression under high arginine. Furthermore, in agreement with previous observations (Dong *et al.*, 2002), ArcR is needed for upregulation of *arcA* and *arcB* genes under high arginine. This pattern of gene

regulation, in which both the biosynthesis and transporter genes are downregulated in high arginine, and catabolism genes are upregulated 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 upregulated 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 2051 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 *Experimental procedures*). 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 qRT-PCR ($R^2 = 0.98$). 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

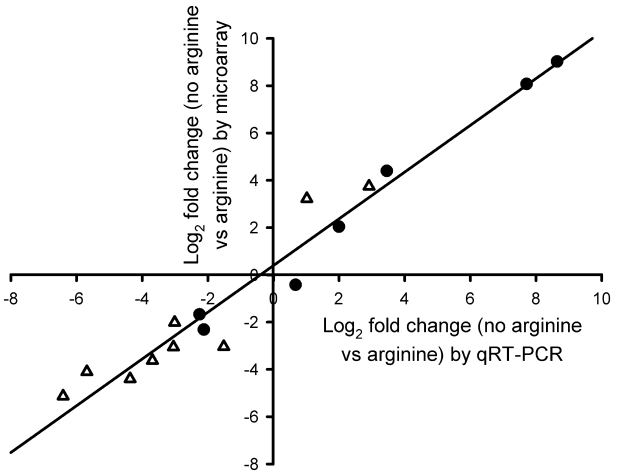


Fig. 5. Comparison between microarray data and qRT-PCR. Total RNA was extracted from *S. gordonii* following 30 min exposure to CDM either without or with arginine. The relative levels of gene expression in the absence of arginine compared with arginine-containing medium determined by microarray were plotted against levels assessed by qRT-PCR. The relative levels of expression of six arginine metabolism/transport genes (*argC*, *argG*, $pyrA_b$, *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.

acid metabolism and transport (group E). In addition to arginine biosynthesis genes, a major group of genes encoding the histidine biosynthesis pathway was upregulated 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 threefold to sixfold downregulated 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 upregulated two- to sixfold 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 downregulated between 2- and 10-fold.

Several COGFun groups contained more members that were downregulated than upregulated 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

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.

Locus	Description	Fold change (range)[a]
Upregulated (no arginine vs 0.5 mM arginine)		
SGO_1566–1569	ArgD/ArgB/ArgJ/ArgC, arginine biosynthesis	339.6 (260.9, 520.1)
SGO_0175–0177	ArgG/ArgH, arginine biosynthesis	267.6 (207.9, 342.4)
SGO_1656	Phosphoenolpyruvate carboxykinase	44.4
SGO_0645–0648	Hypothetical proteins	40.5 (32.4, 54.1)
SGO_0021	Hypothetical protein	26.7
SGO_1102–1106	PyrAa/PyrAb, arginine/pyrimidine biosynthesis	23.9 (18.7, 28.9)
SGO_0091–0094	Hypothetical proteins	12.1 (4.5, 36.6)
SGO_0874	Hypothetical protein	10.1
SGO_1401–1411	Histidine biosynthesis	9.4 (4.4, 17.0)
SGO_1831–1835	Hypothetical proteins	8.7 (5.5, 10.6)
Downregulated (no arginine vs 0.5 mM arginine)		
SGO_1575–1582	Bfb locus, biofilm formation and cellobiose PTS	–30.2 (–15.6, –81.9)
SGO_0831	Hypothetical protein	–13.7
SGO_0832	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)

a. Fold 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).

(K) contained more members that were upregulated in no arginine than downregulated. 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) that were upregulated > 200-fold following a shift to no arginine. The arginine biosynthesis genes $pyrA_a$ and $pyrA_b$ were also strongly upregulated (~ 24-fold) following arginine restriction. The histidine biosynthesis operon, SGO_1401–1411, was co-ordinately upregulated ~ 9-fold in low arginine. The SGO_1656 (*ppc*) gene was upregulated 44-fold in low arginine, and several single genes and putative multi-gene operons encoding hypothetical proteins were also strongly upregulated in response to arginine depletion. The most strongly downregulated operon in low arginine was the *bfb* gene locus encoding the cellobiose phosphotransferase system, which is also involved in biofilm formation and was downregulated ~ 30-fold. Other major multi-gene operons that were strongly downregulated 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 downregulated 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 upregulated by arginine depletion, genes downregulated 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

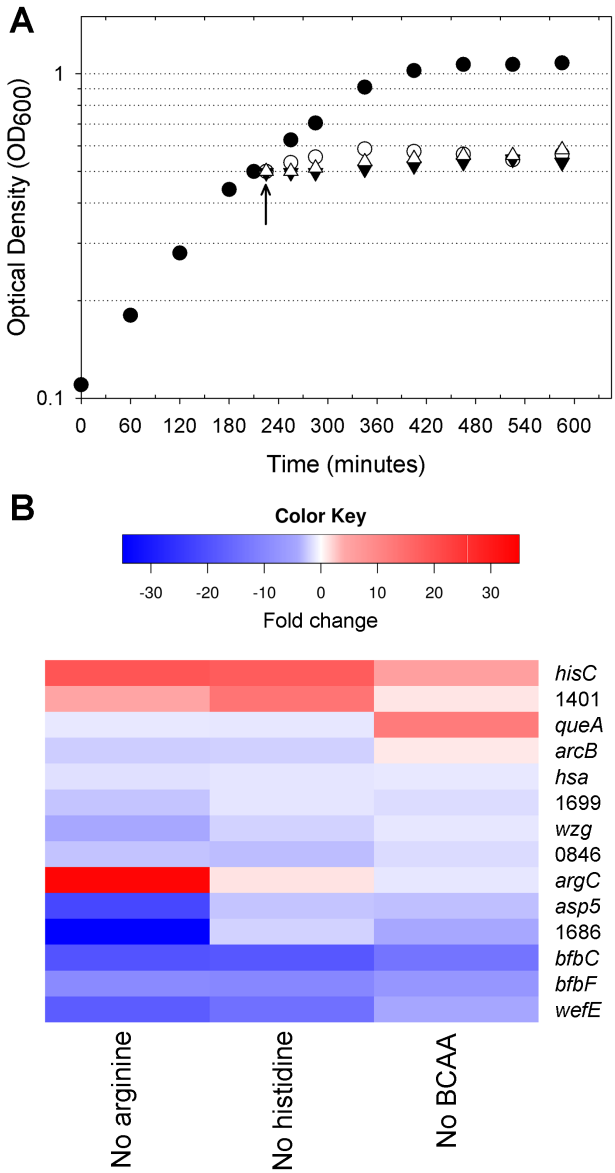


Fig. 6. Growth and gene expression in *S. gordonii* DL1 following depletion of arginine, histidine or BCAA.
A. Cells were cultured anaerobically in CDM to mid-exponential phase (OD_{600} ~ 0.5), harvested and resuspended in CDM (filled circles) or CDM lacking arginine (open circles), histidine (closed 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 four independent experiments.

($P < 0.005$). 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). As arginine biosynthesis genes are regulated in response to changes in arginine concentration, we hypothesised that arginine may be a key signal for coaggregation 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.

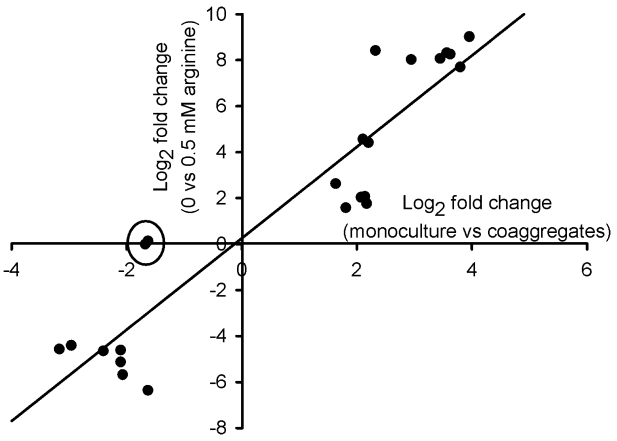


Fig. 7. Comparison between regulation of *S. gordonii* genes by coaggregation and by arginine depletion. The arginine-dependent expression of genes that had previously been identified as being regulated by coaggregation with *A. oris* was assessed using DNA microarrays. All genes that were significantly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

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 as the mouth is an open system where nutrients are constantly replenished.

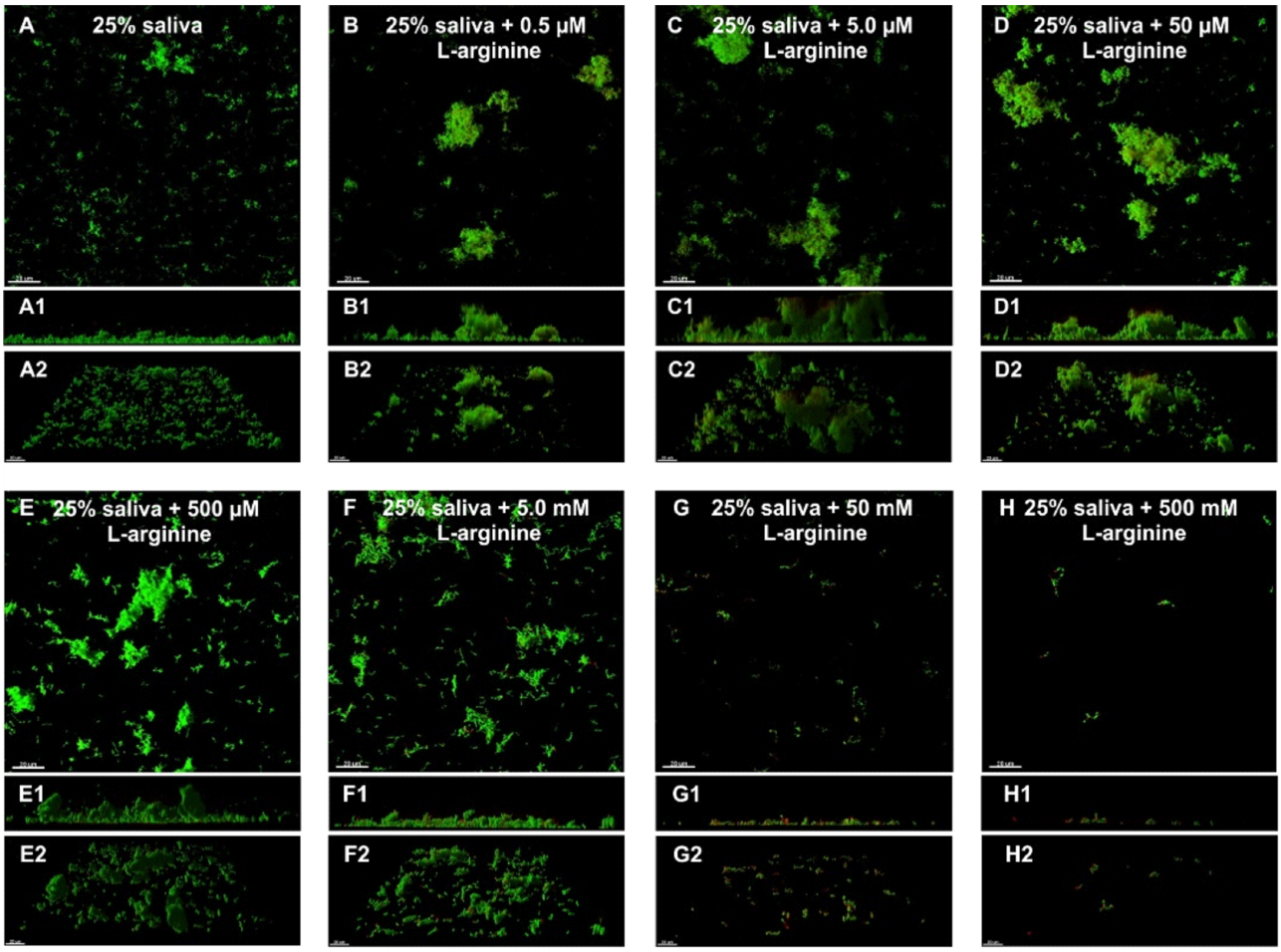
In order to assess the impact of L-arginine on *S. gordonii* biofilms grown under conditions representative of the oral cavity, a 24 channel Bioflux microfluidic system (Fluxion, San Francisco, CA) coupled to a Leica SPE CLSM (Leica, Exon, PA) was used. This system benefits from the requirement for only small volumes (< 1 ml) 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 characterised. 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 concentrations 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 $\mu m^3/\mu m^2$. 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 threefold. 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 microcolonies, and there was a great deal of variation in thickness within individual samples, resulting in high error bars for this parameter (Fig. 8B–E). 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 50–500 mM arginine were substantially altered in biofilm architecture, thickness 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 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 and 7.9. In general, the pH of the effluent was slightly higher and ranged between 7.9 and 8.3. The above data indicate that arginine stimulates *S. gordonii* biofilm development at lower concentrations (0.5–500 μM) and retards biofilm development at higher concentrations (50–500 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 (0.5–5 μM) and high (500 mM) concentrations of arginine caused significantly more cell-death/damage, when compared with the unsupplemented saliva. Although 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



Biofilm Parameter	Control	0.5 µM	5 µM	50 µM	500 µM	5 mM	50 mM	500 mM
Viability (%)	**84.00** (4.56)	**69.99** (8.69)**	**76.78** (5.37)**	**82.34** (4.42)	**81.24** (4.05)	**86.30** (3.34)	**81.65** (4.99)	**75.37** (7.09)**
Biomass ($\mu m^3/\mu m^2$)	**0.89** (0.89)	**1.12** (1.11)*	**2.53** (1.68)*	**1.78** (0.64)*	**2.30** (1.32)*	**0.90** (1.11)	**0.21** (0.22)	**0.06** (0.02)*
Thickness (µm)	**2.15** (2.31)	**2.10** (2.20)	**4.91** (3.46)	**3.71** (1.39)	**5.04** (2.67)*	**1.77** (2.21)	**0.31** (0.38)*	**0.06** (0.04)*
Roughness	**1.74** (0.13)	**1.80** (0.06)	**1.72** (0.07)	**1.66** (0.05)	**1.65** (0.08)	**1.76** (0.16)	**1.90** (0.08)**	**1.98** (0.01)**

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

Fig. 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.

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, as arginine biosynthesis genes (*argCJBD*, *pyrA*$_a$*pyrA*$_b$, *argGH*) and arginine transport (*arcD*) are repressed in high arginine, whereas arginine catabolism (*arcABC*) is upregulated. However, the regulatory circuitry is more complex in *S. gordonii* as (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* as 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 *Pseudomonas aeruginosa* (ArcB) have shown that it is essentially unidirectional *in vivo* due to poor affinity for carbamoyl phosphate and high cooperativity for this substrate (Tricot *et al*., 1993). It is not clear whether catabolic OTCases 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 as *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_{arcA}) 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; 2004; Liu *et al*., 2008; Liu and Burne, 2009). Furthermore, in glucose-grown cells, arginine sensing by ArcR results in approximately fourfold induction of expression from P_{arcA} (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 upregulated 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 *Enterococcus faecalis* genome encodes four ArgR/AhrC family proteins (Paulsen *et al*., 2003; Larsen *et al*., 2004; Nicoloff *et al*., 2004; Kloosterman and 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 oligomerisation 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; 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 and 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

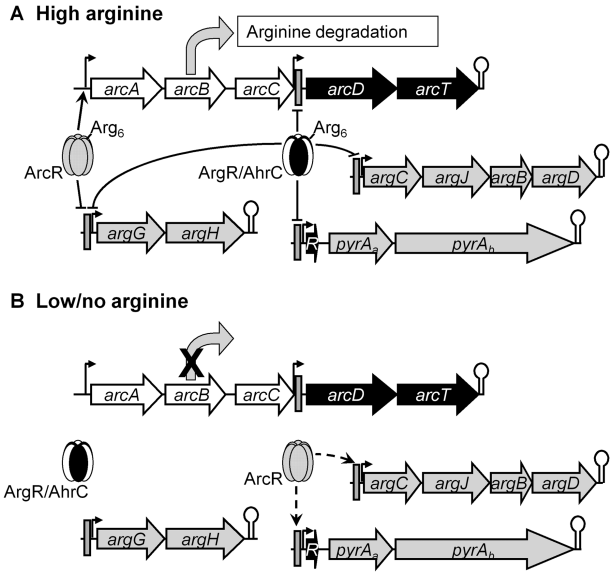


Fig. 9. A model of regulation of arginine metabolism genes by ArcR, ArgR and AhrC. ArgR and AhrC are dependent on each other for activity and here they are represented as a functional protein complex.
A. In the presence of arginine, ArcR, ArgR and AhrC are activated. This is shown as direct binding by six 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 upregulates (dashed lines) promoters upstream of *argC* and *pyrR*.

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 $pyrA_apyrA_b$. 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 Genome2D (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 as 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 downregulation of arginine catabolism genes, whereas *argR* knockout resulted in slight increases in pyrimidine biosynthesis genes. In *S. pneumoniae*, growth in low arginine resulted in the upregulation of 13 genes including genes involved in amino acid or oligopeptide transport and arginine biosynthesis, and downregulation of five genes including *pyrD*, which is required for pyrimidine biosynthesis (Kloosterman and 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 (Yen *et al*., 2013). Although the microarray data presented here indicated that all genes in the *hsa* locus were downregulated 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 downregulation was confirmed by qRT-PCR.

We have previously shown that *S. gordonii* responds to coaggregation with *Actinomyces oris* by upregulating 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 between cells 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 upregulated 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 upregulated 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 as 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-fee 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 as 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; 2013; 2014). 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 and 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.

Experimental procedures

Bacterial media and growth conditions

Streptococcus 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 15 g l^{-1} Bacto-agar at 37°C in a candle jar. For some experiments, *S. gordonii* was cultured in CDM, prepared as described previously (Jakubovics *et al*., 2008a) and incubated either aerobically or in an anaerobic environment under 90% N_2/5% H_2/5% CO_2. 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 K_2HPO_4 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 Luria–Bertani (LB) medium or on LB medium solidified by the addition of 15 g l^{-1} Bacto-agar (Difco). For 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 *S. gordonii*) or 100 μg ml^{-1} (for *E. coli*), ampicillin 50 μg ml^{-1}, spectinomycin 100 μg ml^{-1}, kanamycin 250 μg ml^{-1}.

Genetic manipulation of S. gordonii

Routine genetic manipulations were conducted as described by Sambrook and 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 *arcR*, *argR* or *ahrC* by insertion of an *ermAM* cassette, or disruption of *argR* by *aphA3* insertion, primers were designed to amplify approximately 500 bp regions upstream and downstream of the target genes from *S. gordonii* chromosomal DNA, with a central *Eco*RI 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-*arcR*, pGEM-*argR* or pGEM-*ahrC*, and used for transformation of *E. coli* JM109. To insert antibiotic resistance cassettes, *ermAM* or *aphA3* genes were PCR-amplified from plasmids pCM18 (Hansen *et al*., 2001) or pSF151 (Tao *et al*., 1992) with primers ermF1/R1 or aphA3F1/R1 that contained *Eco*RI restriction sites (Table S2). Fragments were cleaned, digested with *Eco*RI and ligated with pGEM-based plasmids to generate pGEM-*arcR::ermAM*, pGEM-*argR::ermAM*, pGEM-*argR::aphA3* or pGEM-*ahrC::ermAM* and used to transform *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 S2). 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_{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% CO_2. Individual colonies were subcultured to CDM and incubated for 24 h at 37°C, 5% CO_2. Cultures were diluted 1:100 in CDM and incubated for a further 24 h at 37°C, 5% CO_2. 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 and 30 Klett Units (KU), measured using a Klett-Summerson colorimeter with a 660 nm filter (Klett Manufacturing, New York). Cultures were incubated at 37°C anaerobically (90% N_2/5% H_2/5% CO_2) or aerobically (atmospheric CO_2) 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 3800 *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-histine 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 (Qbiogene, Morgan, Irvine, CA) and disrupted in a FastPrep bead beater (Qbiogene). 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 that 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 0–10 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 2051 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 hybridisation 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 and Salzberg, 2012). BEDTools (Quinlan and 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 hybridisation. 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 2 h 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 sterilised 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, L-arginine HCl 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 an atmosphere containing 5% CO_2. The culture was grown under an atmosphere containing 5% CO_2 at 37°C until an OD_{600} 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 microlitres of each concentration of L-arginine-supplemented CFS was added in triplicate to each inlet well and flowed at 1.0 dyne cm^{-2} 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 OD_{600} of 0.4 were flowed into the Bioflux system for 6 s from the outlet port at a speed of 1.0 dyne cm^{-2}, 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 L-arginine concentrations of CFS

was 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 (Styo-9 and propidium iodide) per 1 ml of PBS. After aspirating all PBS from the wells, 100 µl of the stain was 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 was 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, whereas 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 visualise 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, whereas 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 (AMD, Sunnyvale, CA).

Statistical tests

Testing for significant differences was performed by one-way analysis of variance using the Tukey's post-hoc test for pairwise comparisons.

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References

Allan, R.N., Skipp, P., Jefferies, J., Clarke, S.C., Faust, S.N., Hall-Stoodley, L., *et al*. (2014) Pronounced metabolic changes in adaptation to biofilm growth by *Streptococcus pneumoniae*. *PLoS ONE* **9:** e107015.

Bernier, S.P., Ha, D.G., Khan, W., Merritt, J.H., and O'Toole, G.A. (2011a) Modulation of *Pseudomonas aeruginosa* surface-associated group behaviors by individual amino acids through c-di-GMP signaling. *Res Microbiol* **162:** 680–688.

Bernier, S.P., Letoffe, S., Delepierre, M., and Ghigo, J.M. (2011b) Biogenic ammonia modifies antibiotic resistance at a distance in physically separated bacteria. *Mol Microbiol* **81:** 705–716.

Borriello, G., Werner, E., Roe, F., Kim, A.M., Ehrlich, G.D., and Stewart, P.S. (2004) Oxygen limitation contributes to antibiotic tolerance of *Pseudomonas aeruginosa* in biofilms. *Antimicrob Agents Chemother* **48:** 2659–2664.

Bradshaw, D.J., Homer, K.A., Marsh, P.D., and Beighton, D. (1994) Metabolic cooperation in oral microbial communities during growth on mucin. *Microbiology* **140:** 3407–3412.

Brand, H.S., Jorning, G.G., Chamuleau, R.A., and Abraham-Inpijn, L. (1997) Effect of a protein-rich meal on urinary and salivary free amino acid concentrations in human subjects. *Clin Chim Acta* **264:** 37–47.

Cho, B.K., Federowicz, S., Park, Y.S., Zengler, K., and Palsson, B.O. (2012) Deciphering the transcriptional regulatory logic of amino acid metabolism. *Nat Chem Biol* **8:** 65–71.

Cowman, R.A., Perrella, M.M., and Fitzgerald, R.J. (1974) Influence of incubation atmosphere on growth and amino acid requirements of *Streptococcus mutans*. *Appl Microbiol* **27:** 86–92.

Cowman, R.A., Perrella, M.M., Adams, B.O., and Fitzgerald, R.J. (1975) Amino acid requirements and proteolytic activity of *Streptococcus sanguis*. *Appl Microbiol* **30:** 374–380.

Dehal, P.S., Joachimiak, M.P., Price, M.N., Bates, J.T., Baumohl, J.K., Chivian, D., *et al*. (2010) MicrobesOnline: an integrated portal for comparative and functional genomics. *Nucleic Acids Res* **38:** D396–D400.

Dong, Y., Chen, Y.Y., Snyder, J.A., and Burne, R.A. (2002) Isolation and molecular analysis of the gene cluster for the

arginine deiminase system from *Streptococcus gordonii* DL1. *Appl Environ Microbiol* **68:** 5549–5553.
Dong, Y., Chen, Y.Y., and Burne, R.A. (2004) Control of expression of the arginine deiminase operon of *Streptococcus gordonii* by CcpA and Flp. *J Bacteriol* **186:** 2511–2514.
Egland, P.G., Palmer, R.J., Jr, and Kolenbrander, P.E. (2004) Interspecies communication in *Streptococcus gordonii–Veillonella atypica* biofilms: signaling in flow conditions requires juxtaposition. *Proc Natl Acad Sci USA* **101:** 16917–16922.
Frank, K.L., Guiton, P.S., Barnes, A.M., Manias, D.A., Chuang-Smith, O.N., Kohler, P.L., *et al*. (2013) AhrC and Eep are biofilm infection-associated virulence factors in *Enterococcus faecalis*. *Infect Immun* **81:** 1696–1708.
Haas, D., Holloway, B.W., Schambock, A., and Leisinger, T. (1977) The genetic organization of arginine biosynthesis in *Pseudomonas aeruginosa*. *Mol Gen Genet* **154:** 7–22.
Hansen, M.C., Palmer, R.J., Jr, Udsen, C., White, D.C., and Molin, S. (2001) Assessment of GFP fluorescence in cells of *Streptococcus gordonii* under conditions of low pH and low oxygen concentration. *Microbiology* **147:** 1383–1391.
Heydorn, A., Nielsen, A.T., Hentzer, M., Sternberg, C., Givskov, M., Ersboll, B.K., *et al*. (2000) Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* **146:** 2395–2407.
Jakubovics, N.S., and Kolenbrander, P.E. (2010) The road to ruin: the formation of disease-associated oral biofilms. *Oral Dis* **16:** 729–739.
Jakubovics, N.S., Kerrigan, S.W., Nobbs, A.H., Stromberg, N., van Dolleweerd, C.J., Cox, D.M., *et al*. (2005) Functions of cell surface-anchored antigen I/II family and Hsa polypeptides in interactions of *Streptococcus gordonii* with host receptors. *Infect Immun* **73:** 6629–6638.
Jakubovics, N.S., Gill, S.R., Iobst, S.E., Vickerman, M.M., and Kolenbrander, P.E. (2008a) Regulation of gene expression in a mixed-genus community: stabilized arginine biosynthesis in *Streptococcus gordonii* by coaggregation with *Actinomyces naeslundii*. *J Bacteriol* **190:** 3646–3657.
Jakubovics, N.S., Gill, S.R., Vickerman, M.M., and Kolenbrander, P.E. (2008b) Role of hydrogen peroxide in competition and cooperation between *Streptococcus gordonii* and *Actinomyces naeslundii*. *FEMS Microbiol Ecol* **66:** 637–644.
Jakubovics, N.S., Brittan, J.L., Dutton, L.C., and Jenkinson, H.F. (2009) Multiple adhesin proteins on the cell surface of *Streptococcus gordonii* are involved in adhesion to human fibronectin. *Microbiology* **155:** 3572–3580.
Johnson, B.P., Jensen, B.J., Ransom, E.M., Heinemann, K.A., Vannatta, K.M., Egland, K.A., *et al*. (2009) Interspecies signaling between *Veillonella atypica* and *Streptococcus gordonii* requires the transcription factor CcpA. *J Bacteriol* **191:** 5563–5565.
Kamaguchi, A., Baba, H., Hoshi, M., and Inomata, K. (1994) Coaggregation between *Porphyromonas gingivalis* and mutans streptococci. *Microbiol Immunol* **38:** 457–460.
Kloosterman, T.G., and Kuipers, O.P. (2011) Regulation of arginine acquisition and virulence gene expression in the human pathogen *Streptococcus pneumoniae* by transcription regulators ArgR1 and AhrC. *J Biol Chem* **286:** 44594–44605.
Kolenbrander, P.E. (2011) Multispecies communities: interspecies interactions influence growth on saliva as sole nutritional source. *Int J Oral Sci* **3:** 49–54.
Kolenbrander, P.E., Palmer, R.J., Jr, Periasamy, S., and Jakubovics, N.S. (2010) Oral multispecies biofilm development and the key role of cell-cell distance. *Nat Rev Microbiol* **8:** 471–480.
Kristich, C.J., Nguyen, V.T., Le, T., Barnes, A.M., Grindle, S., and Dunny, G.M. (2008) Development and use of an efficient system for random mariner transposon mutagenesis to identify novel genetic determinants of biofilm formation in the core *Enterococcus faecalis* genome. *Appl Environ Microbiol* **74:** 3377–3386.
Langmead, B., and Salzberg, S.L. (2012) Fast gapped-read alignment with Bowtie 2. *Nat Methods* **9:** 357–359.
Larsen, R., Buist, G., Kuipers, O.P., and Kok, J. (2004) ArgR and AhrC are both required for regulation of arginine metabolism in *Lactococcus lactis*. *J Bacteriol* **186:** 1147–1157.
Larsen, R., Kok, J., and Kuipers, O.P. (2005) Interaction between ArgR and AhrC controls regulation of arginine metabolism in *Lactococcus lactis*. *J Biol Chem* **280:** 19319–19330.
Larsen, R., van Hijum, S.A., Martinussen, J., Kuipers, O.P., and Kok, J. (2008) Transcriptome analysis of the *Lactococcus lactis* ArgR and AhrC regulons. *Appl Environ Microbiol* **74:** 4768–4771.
LeBlanc, D.J., Lee, L.N., and Abu-Al-Jaibat, A. (1992) Molecular, genetic, and functional analysis of the basic replicon of pVA380-1, a plasmid of oral streptococcal origin. *Plasmid* **28:** 130–145.
Levesque, C., Lamothe, J., and Frenette, M. (2003) Coaggregation of *Streptococcus salivarius* with periodontopathogens: evidence for involvement of fimbriae in the interaction with *Prevotella intermedia*. *Oral Microbiol Immunol* **18:** 333–337.
Liu, Y., and Burne, R.A. (2009) Multiple two-component systems modulate alkali generation in *Streptococcus gordonii* in response to environmental stresses. *J Bacteriol* **191:** 7353–7362.
Liu, Y., Dong, Y., Chen, Y.Y., and Burne, R.A. (2008) Environmental and growth phase regulation of the *Streptococcus gordonii* arginine deiminase genes. *Appl Environ Microbiol* **74:** 5023–5030.
Merritt, J., Niu, G., Okinaga, T., and Qi, F. (2009) Autoaggregation response of *Fusobacterium nucleatum*. *Appl Environ Microbiol* **75:** 7725–7733.
Nance, W.C., Dowd, S.E., Samarian, D., Chludzinski, J., Delli, J., Battista, J., *et al*. (2013) A high-throughput microfluidic dental plaque biofilm system to visualize and quantify the effect of antimicrobials. *J Antimicrob Chemother* **68:** 2550–2560.
Nascimento, M.M., Gordan, V.V., Garvan, C.W., Browngardt, C.M., and Burne, R.A. (2009) Correlations of oral bacterial arginine and urea catabolism with caries experience. *Oral Microbiol Immunol* **24:** 89–95.
Nascimento, M.M., Liu, Y., Kalra, R., Perry, S., Adewumi, A., Xu, X., *et al*. (2013) Oral arginine metabolism may decrease the risk for dental caries in children. *J Dent Res* **92:** 604–608.
Nascimento, M.M., Browngardt, C., Xiaohui, X., Klepac-

Ceraj, V., Paster, B.J., and Burne, R.A. (2014) The effect of arginine on oral biofilm communities. *Mol Oral Microbiol* **29:** 45–54.
Nicoloff, H., Arsene-Ploetze, F., Malandain, C., Kleerebezem, M., and Bringel, F. (2004) Two arginine repressors regulate arginine biosynthesis in *Lactobacillus plantarum*. *J Bacteriol* **186:** 6059–6069.
Nijland, R., and Burgess, J.G. (2010) Bacterial olfaction. *Biotechnol J* **5:** 974–977.
Nobbs, A.H., Jenkinson, H.F., and Jakubovics, N.S. (2011) Stick to your gums: mechanisms of oral microbial adherence. *J Dent Res* **90:** 1271–1278.
Nuxoll, A.S., Halouska, S.M., Sadykov, M.R., Hanke, M.L., Bayles, K.W., Kielian, T., *et al*. (2012) CcpA regulates arginine biosynthesis in *Staphylococcus aureus* through repression of proline catabolism. *PLoS Pathog* **8:** e1003033.
O'Sullivan, D.J., and Klaenhammer, T.R. (1993) High- and low-copy-number *Lactococcus* shuttle cloning vectors with features for clone screening. *Gene* **137:** 227–231.
Paulsen, I.T., Banerjei, L., Myers, G.S., Nelson, K.E., Seshadri, R., Read, T.D., *et al*. (2003) Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. *Science* **299:** 2071–2074.
Pyburn, T.M., Bensing, B.A., Xiong, Y.Q., Melancon, B.J., Tomasiak, T.M., Ward, N.J., *et al*. (2011) A structural model for binding of the serine-rich repeat adhesin GspB to host carbohydrate receptors. *PLoS Pathog* **7:** e1002112.
Quinlan, A.R., and Hall, I.M. (2010) BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* **26:** 841–842.
R Core Team. (2014) R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from http://www.R-project.org.
Ramsey, M.M., Rumbaugh, K.P., and Whiteley, M. (2011) Metabolite cross-feeding enhances virulence in a model polymicrobial infection. *PLoS Pathog* **7:** e1002012.
Rao, D., Arvanitidou, E., Du-Thumm, L., and Rickard, A.H. (2011) Efficacy of an alcohol-free CPC-containing mouthwash against oral multispecies biofilms. *J Clin Dent* **22:** 187–194.
Reiner, A., Yekutieli, D., and Benjamini, Y. (2003) Identifying differentially expressed genes using false discovery rate controlling procedures. *Bioinformatics* **19:** 368–375.
Sainz, G., Tricot, C., Foray, M.F., Marion, D., Dideberg, O., and Stalon, V. (1998) Kinetic studies of allosteric catabolic ornithine carbamoyltransferase from *Pseudomonas aeruginosa*. *Eur J Biochem* **251:** 528–533.
Sambrook, J., and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
Sauer, K., Camper, A.K., Ehrlich, G.D., Costerton, J.W., and Davies, D.G. (2002) *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J Bacteriol* **184:** 1140–1154.
Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* **9:** 671–675.
Sullivan, R., Rege, A., Corby, P., Klaczany, G., Allen, K., Hershkowitz, D., *et al*. (2014) Evaluation of a dentifrice containing 8% arginine, calcium carbonate, and sodium monofluorophosphate to prevent enamel loss after erosive challenges using an intra-oral erosion model. *J Clin Dent* **25:** A7–A13.
Syrjänen, S.M., Alakuijala, L., Alakuijala, P., Markkanen, S.O., and Markkanen, H. (1990) Free amino acid levels in oral fluids of normal subjects and patients with periodontal disease. *Arch Oral Biol* **35:** 189–193.
Takamatsu, D., Bensing, B.A., Prakobphol, A., Fisher, S.J., and Sullam, P.M. (2006) Binding of the streptococcal surface glycoproteins GspB and Hsa to human salivary proteins. *Infect Immun* **74:** 1933–1940.
Tao, L., LeBlanc, D.J., and Ferretti, J.J. (1992) Novel streptococcal-integration shuttle vectors for gene cloning and inactivation. *Gene* **120:** 105–110.
Terleckyj, B., and Shockman, G.D. (1975) Amino acid requirements of *Streptococcus mutans* and other oral streptococci. *Infect Immun* **11:** 656–664.
Tricot, C., Nguyen, V.T., and Stalon, V. (1993) Steady-state kinetics and analysis of pH dependence on wild-type and a modified allosteric *Pseudomonas aeruginosa* ornithine carbamoyltransferase containing the replacement of glutamate 105 by alanine. *Eur J Biochem* **215:** 833–839.
Xu, Z., Fang, X., Wood, T.K., and Huang, Z.J. (2013) A systems-level approach for investigating *Pseudomonas aeruginosa* biofilm formation. *PLoS ONE* **8:** e57050.
Yen, Y.T., Cameron, T.A., Bensing, B.A., Seepersaud, R., Zambryski, P.C., and Sullam, P.M. (2013) Differential localization of the streptococcal accessory Sec components and implications for substrate export. *J Bacteriol* **195:** 682–695.
Zeng, L., Dong, Y., and Burne, R.A. (2006) Characterization of cis-acting sites controlling arginine deiminase gene expression in *Streptococcus gordonii*. *J Bacteriol* **188:** 941–949.
Zhu, Y., Weiss, E.C., Otto, M., Fey, P.D., Smeltzer, M.S., and Somerville, G.A. (2007) *Staphylococcus aureus* biofilm metabolism and the influence of arginine on polysaccharide intercellular adhesin synthesis, biofilm formation, and pathogenesis. *Infect Immun* **75:** 4219–4226.

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