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

Nicholas S. Jakubovics[1*], Jill C. Robinson[1], Derek S. Samarian[2], Ethan Kolderman[2], Sufian A. Yassin[1], Deepti Bettampadi[2], Matthew Bashton[3], Alexander H. Rickard,[2]

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

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*Address for correspondence:

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

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Newcastle upon Tyne
NE2 4BW
UK
Tel: +191 208 6796
Fax: +191 208 8807
e-mail: nick.jakubovics@ncl.ac.uk

Summary

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

Introduction

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

Following the development of chemically defined media, in the 1970's, it became clear that oral streptococci lack the biosynthetic machinery for several amino acids. For example, cysteine is broadly required by strains of *S. sanguinis* and the cariogenic species *S. mutans* (Cowman *et al.*, 1974, Cowman *et al.*, 1975, Terleckyj & Shockman, 1975). Most strains of *S. sanguinis* also required arginine, tyrosine, and at least one branched chain amino

43 acid for growth (Cowman et al., 1975). In mutans streptococci, requirements for several
44 amino acids are dependent upon the growth conditions employed. Indeed, *Streptococcus*
45 *criceti* AHT, a member of the mutans group, required arginine when cultured aerobically, but
46 grew without arginine under strictly anaerobic conditions (Terleckyj & Shockman, 1975).
47 More recently, we have found that *S. gordonii* DL1 (Challis) also requires arginine for
48 aerobic, but not anaerobic, growth (Jakubovics *et al.*, 2008a). It is not clear why exogenous
49 arginine is essential only under aerobic conditions. It seems unlikely that oxygen directly
50 inhibits arginine biosynthesis since, to the best of our knowledge, oxygen does not directly
51 inhibit any enzymes in the arginine biosynthesis pathway. Further, *S. gordonii* biosynthesises
52 arginine aerobically if a low concentration of arginine is provided initially (Jakubovics et al.,
53 2008a). It is possible that oxygen has an indirect effect, for example by increasing protein
54 damage which in turn places an increased demand on arginine for cell growth. In line with
55 this, cell-cell contact (coaggregation) with another pioneer coloniser of dental plaque,
56 *Actinomyces oris* MG1, triggers the up-regulation of *S. gordonii* arginine biosynthesis genes,
57 protects *S. gordonii* from protein carbonylation, and enables aerobic growth of *S. gordonii* in
58 arginine-restricted conditions (Jakubovics et al., 2008a, Jakubovics *et al.*, 2008b). *A. oris*
59 MG1 produces catalase and degrades hydrogen peroxide produced by *S. gordonii*. Addition
60 of catalase alone enhances *S. gordonii* growth in low arginine but is not sufficient to allow
61 aerobic growth following a rapid shift to medium lacking arginine (Jakubovics et al., 2008b).
62 Therefore, the observed growth arrest when *S. gordonii* is rapidly shifted to aerobic media
63 without arginine may be due to a combination of (i) a lack of sufficient arginine to synthesise
64 essential biosynthetic enzymes and initiate *de novo* arginine biosynthesis and (ii) additional
65 requirements for arginine imposed by oxidative stress.

66 At present, the full pathway for arginine biosynthesis by *S. gordonii* is not entirely
67 clear. The conventional arginine biosynthesis genes *argCJBD* and *argGH* for conversion of

L-glutamate to L-ornithine and L-citrulline to L-arginine, respectively, are present. However, there is no clear *argF* gene encoding anabolic ornithine carbamoyltransferase (OTCase) to convert L-ornithine to L-citrulline (Fig. S1). Two genes, *pyrB* and *arcB*, encode proteins with significant homology to ArgF (Jakubovics et al., 2008a). By analogy with *S. aureus* (Nuxoll et al., 2012), it is likely that *arcB* fulfils the role of the anabolic OTCase in *S. gordonii*. It has been proposed that *arcB* may be co-transcribed with *arcA*, encoding arginine deiminase, and controlled by the P_{arcA} promoter which in turn is most active under high arginine and low oxygen (Dong *et al.*, 2002, Zeng *et al.*, 2006, Liu *et al.*, 2008). Therefore, it may be predicted that the expression of *arcB* is low under *in vitro* aerobic arginine-restricted conditions. This in turn may contribute to the conditional arginine auxotrophy phenotype of *S. gordonii*.

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

Here, we aimed to investigate the roles of arginine in gene regulation, growth and biofilm formation by *S. gordonii*. Specifically, we set out to (**i**) assess the role of *arcB* in

arginine biosynthesis and conditional arginine auxotrophy, (**ii**) determine the functions of ArgR/AhrC family regulators in the expression of L-arginine uptake, biosynthesis and catabolism genes, (**iii**) identify global gene regulation responses to arginine in *S. gordonii* and (**iv**) investigate the impact of L-arginine on biofilm formation in an environmentally germane model system.

Results

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

in which *pyrB* or *arcB* were replaced with the non-polar *ermAM* erythromycin resistance determinant and similar patterns of growth were observed (data not shown).

***The* arcB *gene is co-transcribed with* arcA.** The *S. gordonii arcB* gene is located within a six-gene cluster comprising *arcABCDTR*. The expression of *arcA* is induced in low pH and high arginine (Liu et al., 2008). To assess whether *arcB* is cotranscribed with *arcA*, *S. gordonii* DL1 was cultured anaerobically in CDM supplemented with 5 mM arginine to mid-exponential phase (125-175 KU) and RNA was extracted. The presence of mRNA containing *arcA-arcB* was detected by RT-PCR using primers *arcA*F1/*arcB*R1 which span the *arcA* and *arcB* genes (Fig. 1). In the absence of reverse transcriptase, no products were detected with these primers. Therefore, it is evident that *arcB* is cotranscribed with *arcA*.

To determine whether *arcA* and *arcB* are subject to similar patterns of gene regulation, *S. gordonii* was cultured under conditions aimed to induce the expression of arginine biosynthesis gene expression (growth in CDM supplemented with 5 mM arginine, followed by a shift to no arginine), or conditions favouring induction of arginine catabolism genes (growth in CDM with 10 mM glucose, followed by addition of 50 mM arginine) (Zeng et al., 2006). For the arginine catabolism conditions, a small amount of extra glucose (approximately 10% higher than unamended CDM) was included to maintain a low background level of *arcA* and *arcB* expression prior to addition of arginine, since it has been shown that adding 10 mM glucose to complex medium represses expression from the P_{arcA} promoter (Dong *et al.*, 2004, Zeng et al., 2006). Expression of *arcA* and *arcB* decreased steadily for 5 min under both sets of conditions (Fig. 2). Following a shift from 5 mM arginine to no arginine, *arcA* and *arcB* expression continued to decrease for 45 min. Conversely, following arginine addition to CDM supplemented with 10 mM glucose, the expression of *arcA* and *arcB* increased dramatically after 5 min and continued to increase for 45 min. In all samples, the changes in expression of *arcA* and *arcB* were similar, and this is

consistent with a shared regulatory mechanism for the two genes. However, it should be noted that gene regulation does not necessarily correlate with enzyme activity since ArcA and ArcB may be subject to post-transcriptional regulation (Liu et al., 2008).

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

To further enhance the levels of *arcB* expression under arginine depletion, a copy of *arcB* was inserted into the *S. gordonii* chromosome downstream of *argD* and under control of the P_{argC} promoter (Fig. 3). Initially, attempts were made to introduce the *arcB* gene directly into the *S. gordonii arcB*::*aphA3* mutant with selection for transformants that were able to grow anaerobically on CDM agar without arginine. No transformants were obtained using this approach, even after several attempts. However, in control reactions transformants were identified on CDM agar without arginine when the *arcB* complementation construct was introduced into *S. gordonii* DL1. The transformants contained a copy of *arcB* downstream of *argD* in addition to the native *arcB* gene within the *arcABC* operon, and this strain was labelled *S. gordonii arcB*++. To construct a strain with only one copy of *arcB*, located downstream of *argD*, the native copy of *arcB* in *S. gordonii arcB*++ was replaced with an *aphA3* kanamycin resistance cassette, generating *S. gordonii* $arcB_{Comp}$. The expression of

arcB was assessed in each strain by qRT-PCR following anaerobic culture to mid-exponential phase in CDM amended to 5 mM arginine, harvesting and re-suspension in either high (5 mM) arginine or no arginine (Fig. 3B). Expression of *arcB* in *S. gordonii* DL1 was 5-fold lower in no arginine than in high arginine ($P<0.001$). In 5 mM arginine, there were no significant differences in levels of *arcB* between *S. gordonii arcB*++ and the isogenic wild-type. However, *arcB* levels were 12.5-fold lower in *S. gordonii* $arcB_{Comp}$ ($P<0.001$). In no arginine, *arcB* was elevated in *S. gordonii arcB*++ and *S. gordonii* $arcB_{Comp}$ by 165-fold and 150-fold, respectively, in comparison to the wild-type under arginine restriction ($P<0.001$). Therefore, the relocation of *arcB* to a position immediately downstream of *argD* resulted in strong up-regulation of *arcB* in response to arginine depletion, and a second copy of *arcB* under control of the P_{arcA} promoter in *S. gordonii arcB*++ prevented reduced *arcB* expression under high arginine.

The effects of relocating the *arcB* gene on growth of *S. gordonii* in low arginine were assessed by measuring the final growth yield of cells after culture in CDM amended to different arginine concentrations (Table 1). Anaerobically, all strains of *S. gordonii* except the *arcB* mutant grew strongly in the absence of arginine. In aerobic conditions, *S. gordonii* DL1 did not grow in CDM containing 16 μM arginine and grew moderately in 32 μM arginine. *S. gordonii arcB*::*aphA3* did not grow at any tested concentrations below 64 μM arginine. Moderate growth of *S. gordonii* $arcB_{Comp}$ 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

192 gene regulation in *S. gordonii*, the genome sequence of *S. gordonii* was BLAST-searched for
193 genes encoding proteins with similarity to *Lactococcis lactis* ArgR or AhrC, *E. coli* ArgR or
194 *Bacillus subtilis* AhrC, and three sequences were identified (Fig. S2). ArcR (SGO_1588) has
195 previously been characterized as an activator of arginine catabolism genes (Zeng et al., 2006).
196 The closest match to *E. coli* ArgR was encoded by SGO_2057, and is termed here *S. gordonii*
197 ArgR. Searching with *Bacillus subtilis* AhrC identified an *S. gordonii* AhrC orthologue,
198 encoded by gene SGO_0697. Each of the predicted *S. gordonii* polypeptides include
199 conserved amino acids that have been shown to be important for arginine-dependent
200 transcriptional regulation (Fig. S2).

201 To investigate the function of *S. gordonii* ArgR/AhrC family regulators, each of the
202 three genes (*arcR*, *argR* and *ahrC*) was disrupted by allelic exchange mutagenesis using a
203 non-polar antibiotic insertion cassette, and double and triple mutants were produced as
204 described in the Materials and Methods. To ensure that the observed effects of gene
205 disruptions were not due to the introduction of antibiotic resistance cassettes, *argR*, *ahrC* and
206 *arcR* were each disrupted with two different antibiotics, and patterns of regulation were
207 shown to be similar in each mutant. Predicted Rho-independent terminators were identified
208 downstream of *arcR* and *argR*. However, the *ahrC* gene is predicted to be in an operon with
209 the DNA repair protein gene *recN* and SGO_0699 (Table S1). To ensure that the knockouts
210 of *argR*, *ahrC* and *arcR* did not have polar effects on downstream genes, *S. gordonii* DL1
211 (wild-type), *argR* and *ahrC* mutants were cultured in THB medium supplemented with
212 5 g L^{-1} yeast extract to mid-exponential phase (OD_{600}=0.5-0.7), RNA was extracted and
213 expression of *recN* and *mutS* (the gene downstream of *argR*) were assessed by qRT-PCR,
214 normalised to levels of 16S rDNA expression. There were no significant differences in
215 expression of *recN* or *mutS* in any of the strains (<1.5 fold change between all strains). For *S.*
216 *gordonii arcR*, the expression of the downstream gene *arcT* was assessed in high (5 mM) and

no arginine as part of an ongoing microarray analysis, and in each case there was no difference in expression between the wild-type and mutant (data not shown). Therefore, disruptions of *argR*, *ahrC* and *arcR* did not have polar effects on downstream genes. In addition, the expression of *argR*, *ahrC* or *arcR* was not significantly altered in mutants lacking one or more of the ArgR/AhrC family regulators (data not shown).

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

In *S. gordonii* DL1 (wild-type), arginine biosynthesis genes *argC*, *argG* and $pyrA_b$ were strongly up-regulated in response to a shift to no arginine (400-fold, 210-fold and 11-fold, respectively; $P<0.001$ in each case) (Fig. 4A-C). Disruption of *argR* or *ahrC* resulted in strong expression of *argC* or $pyrA_b$ 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 expression of *argG* under high arginine compared with *S. gordonii* DL1 under the same conditions ($P<0.001$). The effects of *arcR* disruption were independent of the presence or absence of ArgR or AhrC. Thus, the expression of *argG* in mutants disrupted in either *arcR* or *argR*/*ahrC* was partially reduced in 5 mM arginine compared with no arginine, whereas disruption of *arcR* in addition to *argR* and/or *ahrC* resulted in strong expression of *argG* independent of the arginine concentration.

On the *S. gordonii* genome, the *arcD* gene encoding an arginine-ornithine antiporter is immediately downstream and in the same direction as the *arcABC* genes, and it has been suggested that *arcD* may be part of the same operon (Dong et al., 2002). However, in contrast to *arcA* or *arcB*, the expression of *arcD* was up-regulated in low-arginine compared with 5 mM arginine (Fig. 4D; $P<0.001$). In all mutants lacking *argR* or *ahrC*, expression of *arcD* was high, independent of arginine levels. Expression of *arcD* in the *arcR* single mutant was not significantly different from the wild-type under 5 mM arginine or no arginine, indicating that ArcR does not regulate *arcD*. Using the promoter finding algorithm within PePPER (http://genome2d.molgenrug.nl/), a putative promoter was identified immediately upstream of *arcD*. It is possible that *arcD* is also co-transcribed to some extent from the P_{arcA} promoter and that differences in mRNA stability across the transcript may also affect mRNA levels detected by qRT-PCR. Nevertheless, the above data strongly indicate that *arcD* expression is subject to different regulatory controls compared with *arcA* or *arcB*.

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

transporter genes are down-regulated in high arginine, and catabolism genes are up-regulated is similar to that identified in *E. coli* (Cho et al., 2012).

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

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

PCR or microarray was used and independent of whether cells were shifted to no arginine from 5 mM arginine or from 0.5 mM arginine.

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

Several COGFun groups contained more members that were down-regulated than up-regulated when cells were exposed to CDM without arginine. In general, these pathways cover a diverse range of metabolic and biosynthetic pathways that are involved in cell maintenance and growth. Apart from genes with function unknown, only COGFun groups energy production and conversion (C), nucleotide metabolism and transport (F) and transcription (K) contained more members that were up-regulated in no arginine than down-regulated. Many of the genes involved in transcription encoded predicted transcription

316 regulators, and it is possible that these were involved in co-ordinating the wider gene
317 regulation response to arginine depletion. Overall, the effects of arginine depletion were
318 consistent with an active reduction in cell growth.

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

339 ***Gene regulation responses to arginine compared with other stimuli.*** To determine whether
340 the observed regulatory responses were specific to arginine, or whether they were indicative

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

We have previously identified 23 genes in *S. gordonii* that were regulated in response to coaggregation with *A. oris*, including nine gene involved in arginine biosynthesis (Jakubovics et al., 2008a). Since arginine biosynthesis genes are regulated in response to changes in arginine concentration, we hypothesized that arginine may be a key signal for 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.

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

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

optimal range for enhanced biofilm development was observed when the 25% saliva was supplemented with between 0.5 μM and 500 μM arginine (Fig. 8).

A pre-treatment of the glass surfaces in the Bioflux microfluidic device with L-arginine at concentration up to 500 mM did not appear to affect initial adhesion of *S. gordonii* cells to the substratum (Fig. S4). Three dimensional rendering showed that *S. gordonii* biofilms grown for 22 h in 25% saliva formed thin, patchy biofilms (Fig. 8A) with an average biovolume of 0.89 $\mu m^3/\mu m^2$. 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 3-fold. This was coincident with an increase in average biofilm thickness, although only biofilms developed in 500 μM arginine were significantly thicker than biofilms grown without added arginine ($P<0.05$). Biofilms were structured in heterogeneous stack-like micro-colonies and there was a great deal of variation in thickness within individual samples, resulting in high error bars for this parameter (Fig. 8B-8E). Based upon biofilm biovolume, average biofilm thickness, and biofilm roughness, *S. gordonii* biofilms developed in 25% saliva supplemented with 5 mM arginine were not statistically different ($P>0.05$) from those developed in non-supplemented 25% saliva. However, biofilms developed in 50 mM-500 mM arginine were drastically altered in biofilm architecture and biomass. Architecturally, the biofilms were increasingly patchy as the arginine concentration increased and the likelihood of detecting the presence of aggregated micro-colonies was reduced. When developed in saliva containing 500 mM arginine, the biomass was significantly ($P<0.05$) reduced by 15-fold, as compared with no added arginine, and possessed significantly reduced average thickness (35-fold decrease, $P<0.05$). Roughness, which is a description of the variation in biofilm thickness, was also significantly different ($P<0.05$). High concentrations of L-arginine, up to 500 mM, did not affect the growth of *S. gordonii* in planktonic cultures in CDM (data not shown). In addition, viable counts of *S. gordonii* in unamended saliva or in saliva adjusted to 500 mM L-

arginine remained stable over 24 h, indicating that high concentrations of L-arginine were not toxic to *S. gordonii* in saliva (data not shown). The addition of L-arginine (for all experiments, as HCl salt) had little effect on the pH in the growth medium. The pH of saliva without arginine or with different L-arginine concentrations varied between 7.1-7.9. In general, the pH of the effluent was slightly higher, and ranged between 7.9-8.3. The above data indicate that arginine stimulates *S. gordonii* biofilm development at lower concentrations (0.5 μM-500 μM) and retards biofilm development at higher concentrations (50 mM-500 mM).

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

Discussion

The work presented here demonstrates that arginine has a concentration-dependent effect on *S. gordonii* gene expression and can alter the ability of this oral bacterium to form

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

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

direction, or that its anabolic OTCase activity was very weak (Haas *et al.*, 1977). Our data indicate that *S. gordonii* ArcB can function for arginine biosynthesis in *S. gordonii*, but biosynthesis is only sufficient to sustain rapid growth under certain conditions, such as the gradual depletion of arginine during exponential growth in CDM (Jakubovics et al., 2008a). *S. gordonii* DL1 does not grow aerobically after a rapid shift to no arginine, possibly due to a lack of time to accumulate a pool of carbamoyl phosphate as a substrate for ArcB or due to increased demand for arginine in the presence of oxygen. Other strains of *S. gordonii*, appear to have similar phenotypes since *S. gordonii* Blackburn, Channon, FSS2 FSS3, M5 and PK488, also failed to grow in CDM without arginine (data not shown). Growth of all strains except *S. gordonii* Channon, was restored in CDM containing high (8 mM) arginine.

The ability of ArcB to function in an anabolic direction may also be limited by poor gene expression following arginine depletion. The *arcB* gene is part of a six gene *arcABCDTR* cluster, in which *arcR* is present in reverse orientation compared with the other genes (Dong et al., 2002). The promoter upstream of *arcA* (P_{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, Dong et al., 2004, Liu et al., 2008, Liu & Burne, 2009). Further, in glucose-grown cells, arginine sensing by ArcR results in approximately four-fold induction of expression from P_{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 up-regulated following arginine restriction significantly improved growth in low arginine, suggesting that poor expression of

arcB is a major restriction on arginine biosynthesis in *S. gordonii in vitro*. However, even though relocation of *arcB* increased the levels of *arcB* transcripts 150-fold in low arginine, it did not enable aerobic growth in the absence of arginine.

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

To the best of our knowledge, our data represent the first holistic analysis of the roles of three ArgR/AhrC family regulators in any organism. As in other bacteria, *S. gordonii*

514 ArgR and AhrC act cooperatively to repress the expression of arginine biosynthesis and
515 transport genes in high arginine. ArcR has already been shown to induce arginine catabolism
516 genes under high arginine (Zeng et al., 2006). Here, we have shown that ArcR also strongly
517 represses *argGH* under high arginine. This presumably reduces the conversion of citrulline to
518 arginine, and channels citrulline to the arginine catabolism pathway under high arginine (see
519 Fig. S1). Under low arginine, ArcR had a minor stimulatory effect on the expression of
520 *argCJBD* and $pyrA_apyrA_b$. Therefore, all three ArgR/AhrC family regulators are required for
521 the co-ordinated control of arginine metabolism gene expression in *S. gordonii.* A model for
522 the functions of ArgR, AhrC and ArcR in the regulation of arginine metabolism genes is
523 presented in Fig. 9. It is important to note that we have not investigated direct binding of
524 ArgR/AhrC regulators to promoter regions and it is possible that some regulatory effects may
525 occur through other transcriptional regulators or by differential mRNA degradation.

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

535 Global gene expression in response to arginine limitation or to disruption in
536 ArgR/AhrC family regulators has been investigated in a number of bacteria. In *L. lactis*,
537 disruption of *argR* and/or *ahrC* led to de-repression of arginine biosynthesis genes in high
538 arginine (Larsen et al., 2008). Disruption of *ahrC* also led to down-regulation of arginine

539 catabolism genes, while *argR* knockout resulted in slight increases in pyrimidine biosynthesis
540 genes. In *S. pneumoniae*, growth in low arginine resulted in the up-regulation of 13 genes
541 including genes involved in amino acid or oligopeptide transport and arginine biosynthesis,
542 and down-regulation of five genes including *pyrD* which is required for pyrimidine
543 biosynthesis (Kloosterman & Kuipers, 2011). In *E. coli*, the ArgR regulon is extensive, and
544 includes 423 genes (Cho et al., 2012). Many of these are controlled indirectly through the
545 action of ArgR on other transcriptional regulators. Genes that are controlled directly by ArgR
546 include those involved in arginine biosynthesis and transport, histidine biosynthesis and the
547 biosynthesis of glutamate, aromatic amino acids and lysine. Our microarray analysis
548 demonstrates that *S. gordonii* also mounts a major restructuring of gene expression in
549 response to arginine restriction involving changes in expression of >450 genes. As with other
550 organisms, amino acid metabolism and transport are among the functions most strongly
551 regulated by arginine. In addition, arginine modulates expression of genes in the pyrimidine
552 metabolism pathway, which is closely linked to arginine metabolism. However, in *S. gordonii*
553 the overall impact of arginine restriction appears to be a reduction in processes associated
554 with growth and metabolic activity such as protein synthesis, biosynthetic pathways and cell
555 envelope biogenesis. In addition, among the most strongly regulated genes were those
556 associated with adhesion and biofilm formation.

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

We have previously shown that *S. gordonii* responds to coaggregation with *A. oris* by up-regulating genes involved in arginine biosynthesis and biofilm formation (Jakubovics et al., 2008a). Here, we have demonstrated that arginine restriction influences the expression of 21 of the 23 genes that were shown to be responsive to coaggregation. The effects of coaggregation on gene expression were not seen in co-cultures of *S. gordonii* and *A. oris* in which cells were dispersed, indicating that physical contact with a surface is required for gene regulation (Jakubovics et al., 2008a). A number of previous studies have identified connections between arginine and biofilm formation in different bacteria. For example, at physiological concentrations found in cystic fibrosis sputum, arginine promotes *P. aeruginosa* biofilm formation and prevents swarming motility (Bernier *et al.*, 2011a). In model *P. aeruginosa* biofilms, arginine catabolism genes are up-regulated compared with planktonic cells, leading to anaerobic metabolism and increased susceptibility to ciprofloxacin and tobramycin (Sauer *et al.*, 2002, Borriello *et al.*, 2004, Xu *et al.*, 2013). Similarly, arginine deiminase activity is up-regulated in *S. aureus* and *S. pneumoniae*

biofilms compared with planktonic cells (Zhu *et al.*, 2007, Allan *et al.*, 2014). In *E. faecalis*, the arginine-dependent regulators ArgR and AhrC are critical for biofilm formation *in vitro* (Kristich *et al.*, 2008), and AhrC is also required *in vivo* in a mouse model of catheter-associated urinary tract infection (Frank *et al.*, 2013). Interestingly, under static conditions in nutrient-rich media we have observed that *S. gordonii* strains disrupted in the *arcR* gene form approximately 50% reduced biofilms compared with the isogenic wild-type progenitor (data not shown). This does not appear to be directly associated with the role of ArcR in regulating arginine biosynthesis, transport or catabolism genes since mutants in *argH*, *arcA*, *arcB* or *arcD* are not impaired in biofilm formation. Therefore it is possible that a different target of ArcR gene regulation plays a key role in biofilm formation, and we are currently investigating this hypothesis.

Using a microfluidic system that facilitates the growth of biofilms in flowing pooled human cell-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 since high concentrations of arginine are currently being incorporated into oral healthcare products (Sullivan et al., 2014). The catabolism of arginine produces ammonia, which is alkaline, and trials are currently underway to assess the potential for arginine to be used as an anti-caries agent on the basis

614 that the ammonia released by bacterial metabolism of arginine neutralises dental plaque acid
615 (Nascimento *et al.*, 2009, Nascimento *et al.*, 2013a, Nascimento *et al.*, 2013b). It is possible
616 that excessive alkaline production in biofilm cells of *S. gordonii* may trigger the release of
617 cells from surfaces, Ammonia itself has been shown to be a signalling molecule that
618 modulates biofilm formation and resistance to antibiotics (Nijland & Burgess, 2010, Bernier
619 *et al.*, 2011b). However, in the microfluidics biofilm system, used in this study, high
620 concentrations of arginine did not result in significantly greater increases in pH of the effluent
621 during biofilm growth than low arginine concentrations. Thus, arginine may inhibit cell-cell
622 interactions directly. It is already known that arginine inhibits or retards coaggregation
623 (Kamaguchi *et al.*, 1994, Levesque *et al.*, 2003) and autoaggregation (Merritt *et al.*, 2009).
624 Changes in cell-cell interactions will likely be most apparent in biofilm models that
625 incorporate fluid flow such as the microfluidic system employed in this study. Whether these
626 effects extend to multi-species oral biofilms is currently being examined (manuscript in
627 press).

628 In summary, our data strongly support the concept that arginine plays a major role in
629 modulating key processes including growth and biofilm formation in *S. gordonii*. The
630 regulatory response network for arginine is set up to allow arginine biosynthesis and growth
631 when changes in external arginine are gradual, but to shut down cell growth in response to
632 rapid depletion of arginine. We hypothesise that this regulatory architecture prevents *S.*
633 *gordonii* from over-committing resources to cell growth when arginine transiently reaches
634 high concentrations, such as during a meal. High concentrations of arginine trigger the
635 dispersal of *S. gordonii* from biofilms, which could potentially enable *S. gordonii* to relocate
636 to distant sites in the mouth. We are now undertaking investigations into the mechanisms
637 underlying this process. Early colonisers such as *S. gordonii* are critical for the initiation of
638 dental plaque development and for recruitment of potentially pathogenic microorganisms.

Ultimately, therefore, it may be possible to develop new strategies for oral biofilm control based on interfering with arginine sensing by oral bacteria.

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Materials and Methods

Bacterial media and growth conditions. *S. gordonii* was routinely cultured in Todd Hewitt Broth (THB) medium (Difco, Detroit, MI) without shaking at 37°C or on THB solidified by the addition of 15g/L Bacto-agar at 37°C in a candle jar. For some experiments, *S. gordonii* was cultured in chemically defined medium (CDM), prepared as described previously (Jakubovics et al., 2008a) and incubated either aerobically or in an anaerobic environment under 90% 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 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 & 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

688 replacing. Plasmid inserts were amplified with arcRF1/R2, argRF1/R2 or ahrCF1/R2 as
689 appropriate, and products were used to transform *S. gordonii* DL1 (Challis) as previously
690 described (Jakubovics *et al.*, 2005). All mutants were checked by PCR amplification and
691 sequencing.

692 For disruption of *arcB* or *pyrB*, or replacement of *arcR* with the *aad9* spectinomycin
693 resistance cassette amplified from plasmid pDL278 (LeBlanc *et al.*, 1992), mutagenesis was
694 employed without a cloning step. Primers were designed to amplify approximately 500 bp
695 upstream or downstream of the gene of interest. Extensions were added to the 5' end of the
696 'R1' and 'F2' primers to overlap primers for amplification of the antibiotic resistance
697 cassette. Following PCR amplification of the regions upstream and downstream of the gene
698 of interest and the antibiotic resistance cassette, the three fragments were combined in
699 equimolar quantities and used as template for a second round of PCR. For replacement of
700 *arcB* with *aphA3*, the upstream and downstream regions of *arcB* were amplified with
701 arcBF1/R1 and arcBF2/R2, respectively, and the *aphA3* cassette was amplified from plasmid
702 pSF151 with primers aphA3F2/R2. To replace *arcB* with *ermAM*, *arcB* was amplified from *S.*
703 *gordonii* chromosomal DNA using primers arcBF1/R3 and arcBF3/R2, in which 5' overlap
704 extensions were included in the 'R3' and 'F3' primers. The *ermAM* cassette was amplified
705 from pCM18 with ermF1/R1. Similarly, for *pyrB* mutagenesis, regions around the *pyrB* gene
706 were amplified with pyrBF1/R1 and pyrBF2/R2 for disruption with *aphA3*, or with
707 pyrBF1/R3 and pyrBF3/R2 for disruption with *ermAM*. Overall, these reactions generated
708 products *arcB::ermAM*, *arcB::aphA3*, *pyrB::ermAM*, *pyrB::aphA3* or *arcB::aad9*. Fragments
709 were cleaned and used for transformation of *S. gordonii* DL1 (Challis). A similar approach
710 was employed to generate the *argD-arcB* complementation strain. Approximately 500 bp
711 regions surrounding a predicted Rho-independent terminator (5'-
712 AAAAGGATTCAGTTTGAGCTGGATTCTTTTT-3') downstream of *argD* were amplified

with primers argDF1/R1 and argDF2/R2 (Table 3). The *arcB* gene was amplified with primers arcBF4/R4. Following PCR amplification, the three products were mixed and used as a template for a second round of PCR with primers argDF1/R2. The *argD-arcB* fragment generated was cleaned and used for transformation of *S. gordonii* DL1. Transformants were selected on solidified CDM-arg medium. All transformants were checked by PCR amplification and DNA sequencing. For complementation of *arcB* mutants with *arcB* gene immediately downstream of the promoter P_{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 25-30 Klett Units (KU), measured using a Klett-Summerson colorimeter with a 660 nm filter (Klett Manufacturing Co., New York). Cultures were incubated at 37°C anaerobically (90% 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 (+++)].

738 In most cases at least two of the three cultures had the same growth yield on the four point
739 scale, and this value was reported. Occasionally, three different values were obtained for the
740 same strain at one arginine concentration, in which case the median value was given.

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

750 ***RNA extraction and RT-PCR/qRT-PCR.*** Intracellular RNA was stabilised by the addition of
751 2 volumes of RNAProtect (Qiagen, Valencia, CA) and vortex mixing for 5 s, and RNA was
752 extracted as previously described (Jakubovics et al., 2008a). Briefly, cells were pelleted by
753 centrifugation, the supernatant was removed and cells were stored at -70°C for up to 72 h.
754 Cells were re-suspended in 1 ml Trizol reagent (Invitrogen, Carlsbad, CA), mixed with lysing
755 matrix B (Qbiogene, Morgan, Irvine, CA) and disrupted in a FastPrep bead beater
756 (Qbiogene). Subsequently, RNA was extracted using the Trizol manufacturer's protocol.
757 Extracted RNA was treated for 1 h at 37°C with RQ1 DNase I (Promega, Madison, WI) and
758 purified using RNeasy MinElute columns (Qiagen). A sample of RNA was analysed on a
759 0.8% (wt/vol) agarose gel containing 3% (vol/vol) formaldehyde to check for degradation.
760 The concentration of RNA in each sample was estimated with a NanoDrop ND-1000
761 spectrophotometer (Labtech, Uckfield, East Sussex).

For RT-PCR and qRT-PCR analysis, samples were reverse transcribed with Superscript III reverse transcriptase (Invitrogen) and cleaned using MinElute columns (Qiagen). Primers 1446F/R and 1447F/R for RT-PCR analysis of *arcA*/*arcB* are described in Table S2. Reactions were carried out using REDTaq polymerase (Sigma-Aldrich, St Louis, MO) with the following thermocycle protocol: denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 90 s, and a final elongation at 72°C for 5 min. Primers 1446F/R and 1447F/R were also used for qRT-PCR analysis of *arcA* and *arcB*. Other qRT-PCR primers are shown in Table S2, except the following which have previously been reported: 16SSgF1/R1 (16S rDNA), 0175F/R (*argG*), 1590F/R (*arcD*), 1569F/R (*argC*), 1104F/R ($pyrA_b$) and 1075F/R (*amyB*) (Jakubovics et al., 2008a). Reactions (25 µL total volume) contained 0 to 10 ng cDNA template, 12.5 µL Power SyBr Green PCR mix (Applied Biosystems, Foster City, CA) and forward/reverse primers each at 300 nM, with the exception of 16SSgF1/R1 reactions, which contained primers at 100 nM. An MX3005P thermocycler (Stratagene, La Jolla, CA) was employed for qRT-PCR using the protocol: 95°C for 10 min, 40 cycles of 95°C for 30 s, 56°C for 1 min and 72°C for 30 s, and a dissociation curve consisting of 95°C for 1 min, 56°C for 30 s, and incremental increases in temperature up to 95°C. Fluorescence readings were collected following the 56°C primer annealing step and throughout the dissociation curve. Specific amplification of the desired fragments was assessed by the presence of a single sharp fluorescence decrease during the dissociation phase, and by analysis of representative samples on agarose gels. Reaction efficiencies were estimated by performing three independent reactions for each set of primers using dilutions of one *S. gordonii* cDNA sample as template over a 6-log range of concentrations. All primers gave reaction efficiencies >80%. Relative quantities of transcripts were calculated from three independent experiments by normalising against the 16S rDNA

786 gene as described previously (Jakubovics et al., 2008a). The heatmap was drawn in R (R
787 Core Team, 2014)

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

808 ***Saliva and inoculum preparation for biofilm experiments.*** Human saliva was collected from
809 volunteers in accordance with the University of Michigan Institutional Review Board
810 evaluated protocol (HUM00042954) described by Nance et al. (2013). Cell-free saliva (CFS)

811 was used as the lone nutrient source. This was prepared using a similar protocol to that
812 described by Rao *et al*. (2011). Briefly, saliva was gathered from five healthy adults who had
813 not consumed anything but water for at least two hours prior to collection. All donors had not
814 taken any antibiotics for at least 3 months and did not smoke. The saliva from each donor was
815 pooled and 2.5 mM DTT was added before standing on ice for 10 min. The pooled saliva was
816 then centrifuged at 20,000 *g* for 30 min. The supernatant was collected and diluted with
817 distilled water to a final concentration of 25%. The 25% saliva was then filter sterilized using
818 a 0.22 μm pore-size filter (Nalgene) to yield CFS. For long-term storage, CFS was separated
819 into 30 ml aliquots and stored at -80°C. Prior to use, arginine was added to CFS to final
820 concentrations of 0.5 μM, 5 μM, 50 μM, 500 μM, 5 mM, 50 mM, or 500 mM. A control with
821 CFS containing no arginine was used for all experiments. Inocula were prepared by growing
822 *S. gordonii* DL1 in 5 ml of Schaedler's broth that had been pre-reduced in and atmosphere
823 containing 5% CO_2. The culture was grown under an atmosphere containing 5% CO_2 at 37°C
824 until an OD_{600} of 0.4 was reached, whereupon it was used as an inoculum for microfluidics
825 experiments.

826 ***Microfluidics system.*** Biofilms were developed using a Bioflux 200 microfluidics system
827 (Fluxion, San Francisco, CA) with attached 48-well Bioflux microfluidics plates. Each
828 Bioflux plate was conditioned with CFS prior to use. One hundred μl of each concentration
829 of arginine-supplemented CFS were added in triplicate to each inlet well and flowed at 1.0
830 dyne/cm^2 for 2-3 min at 20°C, followed by 20 min at 20°C with no flow in order to condition
831 the channels for cell attachment. Exponentially growing *S. gordonii* DL1 cell suspensions in
832 Schaedler's broth at an OD_{600} of 0.4 were flowed into the Bioflux system for 6 s from the
833 outlet port at a speed of 1.0 dyne/cm^2, to facilitate inoculation of the viewing area and not
834 further upstream (ie preventing contamination of the inlet reservoir). The plate was then
835 incubated at 37°C for 45 min with no flow. The outlet wells containing the inoculum were

aspirated and 900 µl of each of the respective arginine concentrations of CFS were added to each inlet well to bring the total inlet volume to approximately 1 ml. The CFS then flowed from the inlet to outlet wells for 20 h at 0.2 dyne/cm^2 at 37°C.

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

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

After image collection, IMARIS software (Bitplane, Zurich, Switzerland) was used to visualize the biofilms in 2D and 3D. Additionally, IMARIS was used to prepare the images for analysis using COMSTAT software (Heydorn *et al.*, 2000) and IMAGEJ (Schneider *et al.*, 2012). COMSTAT was used to quantify the biomass of the biofilm, as well as its average thickness and roughness. Thickness was measured from where one surface of the biofilm

contacts the glass on the bottom of the plate to the top of the opposite surface in the channel in the field of view, while roughness was a measurement of thickness variability in a single field of view. For the LIVE/DEAD quantification, IMAGEJ was used. Green represented viable cells, while red represented dead or damaged cells and the pixel intensity of red and green was measured in throughout the image stacks using the *Histogram* function for each channel. The percentage of green and red, and therefore the percentage of viable and non-viable cells, was subsequently determined for each image stack. This was performed using Excel (Microsoft, Redmond, WA) to multiply the total pixels by the intensity (8 bit images, 0-255 in intensity levels). All software programs were run on a computer containing an Intel i5 processor (Intel, Santa Clara, CA) and a Radeon 5850 graphics card with 1 GB of RAM (AMD, Sunnyvale, CA).

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

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Tables

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

	Anaerobic	Aerobic							
Strain	0	0	8	16	32	64	128	256	512 μM arginine
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 (+++).

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).

Figure legends

Figure 1. Analysis of *arcA-arcB* gene transcript by RT-PCR. Combinations of *arcA*F1, *arcA*R1, *arcB*F1 and *arcB*R1 primers were used to amplify fragments of the *arcB* gene (111 bp; lanes 1, 4 and 7), *arcA* gene (173 bp; lanes 3, 6 and 9), or a region spanning *arcA-arcB* (1,438 bp; lanes 2, 5 and 8). Positive control reactions (lanes 1-3) employed chromosomal DNA as a template for PCR. Alternatively, DNase I-treated RNA preparations were used as PCR templates either without reverse transcriptase (RT) (lanes 4-6) or with RT (lanes 7-9). The presence of a band at 1,438 bp from cDNA template (lane 8) indicates that *arcA* and *arcB* are co-transcribed.

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

Figure 3. Constructs for mutagenesis and complementation of *arcB*. A. The native position of *arcB* in the *arcABC* gene cluster is shown, along with the site of insertion of the *aphA3* cassette in the *arcB*::*aphA3* gene knockout construct. An additional strain was constructed in which *arcB* was replaced with the *ermAM* cassette at the same location (not

shown). In the complementation strain, *arcB*$_{Comp}$, the *arcB* gene was inserted immediately downstream of *argD* and upstream of the predicted Rho-independent transcription terminator. An additional construct containing two copies of *arcB*, one downstream of *arcA* and one downstream of *argD* was also produced (*arcB*++). Predicted gene promoters are indicated by bent lines with arrows. Under arginine restriction, *arcA* promoter activity is reduced whereas *argC* promoter activity is increased. Expression of *arcB* in the different strains after a shift from 5 mM arginine to either 5 mM arginine (black bars) or no arginine (grey bars) was determined by qRT-PCR (B). Values are means and SDs of $\log_2$ fold change compared with *S. gordonii* DL1 in 0.5 mM arginine (marked as 'C' for comparator).

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

Figure 5. Comparison between microarray data and qRT-PCR. Total RNA was extracted from *S. gordonii* following 30 min exposure to CDM either without or with arginine. The

relative levels of gene expression in the absence of arginine compared with arginine-containing medium determined by microarray were plotted against levels assessed by qRT-PCR. The relative levels of expression of six arginine metabolism/transport genes (*argC*, *argG*, *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.

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

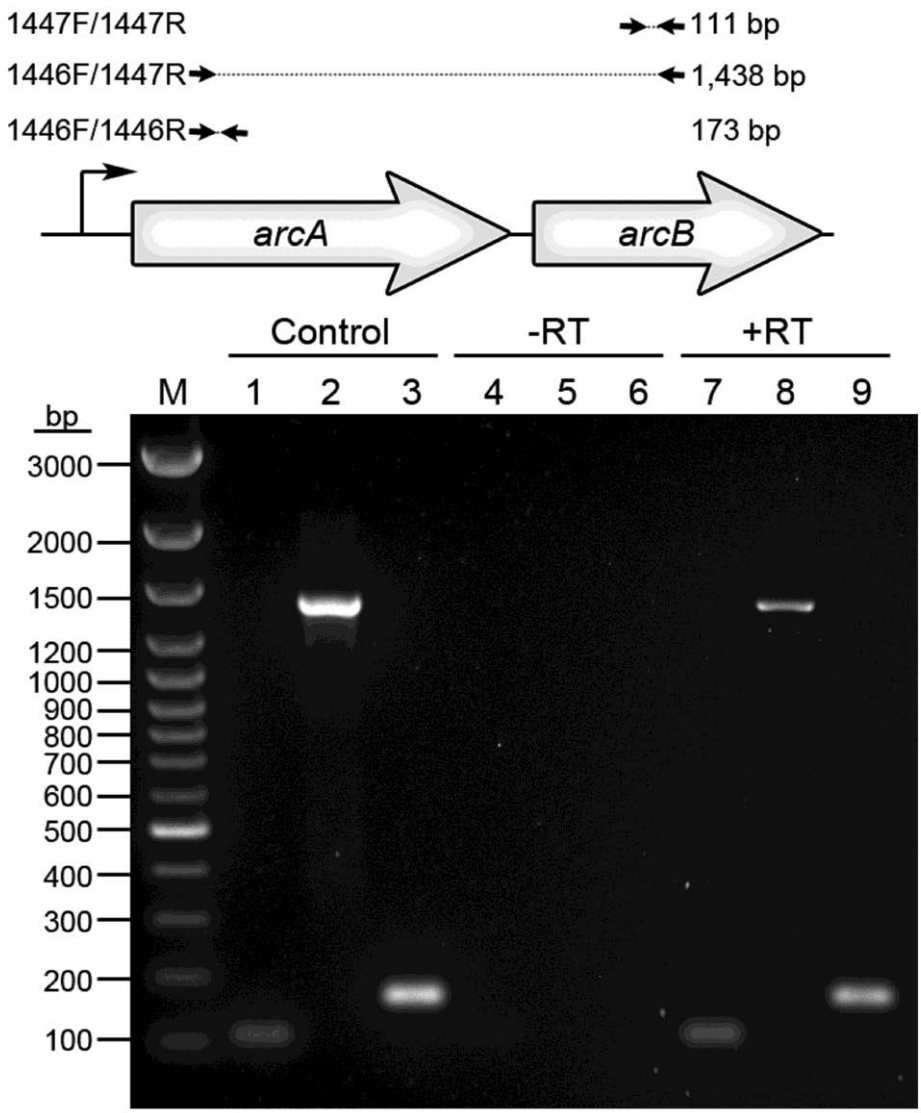
Figure 7. Comparison between regulation of *S. gordonii* genes by coaggregation and by arginine depletion. The arginine-dependent expression of genes that had previously been identified as being regulated by coaggregation with *A. oris* was assessed using DNA

microarrays. All genes that were significantly up-regulated in monocultures compared with coaggregates were also up-regulated in low arginine compared with high arginine. Most genes that were down-regulated in monocultures were also down-regulated in low arginine, with the exception of *spxB* (pyruvate oxidase) and SGO_1308 (hypothetical protein), which were not regulated by arginine (circled).

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

Figure 9. A model of regulation of arginine metabolism genes by ArcR, ArgR and AhrC. ArgR and AhrC are dependent on each other for activity and here they are represented as a functional protein complex. A. In the presence of arginine, ArcR, ArgR and AhrC are activated. This is shown as direct binding by 6 arginine residues (Arg_6). Activated ArgR/AhrC represses transcription of genes involved in arginine biosynthesis (shaded arrows) or accessory arginine-related functions (black arrows), indicated by lines with capped ends. In the presence of arginine, ArcR positively regulates expression of arginine catabolism genes (white arrows), shown by a line with an arrowhead, and negatively regulates *argGH* expression. Elements upstream of these genes that have consensus ARG box signatures are indicated by shaded boxes. Predicted promoters are indicated by thin right-facing arrows, and

terminators are shown as loops and vertical lines. B. In very low or no arginine, ArcR weakly up-regulates (dashed lines) promoters upstream of *argC* and *pyrR*.



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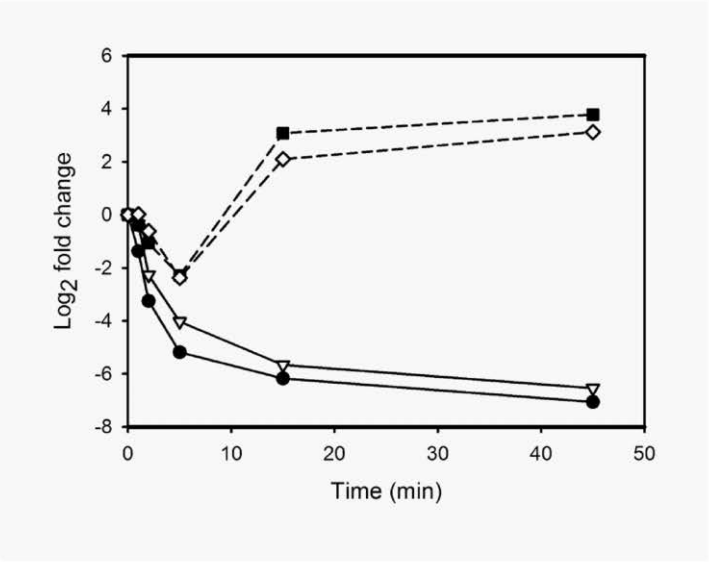

Figure 2

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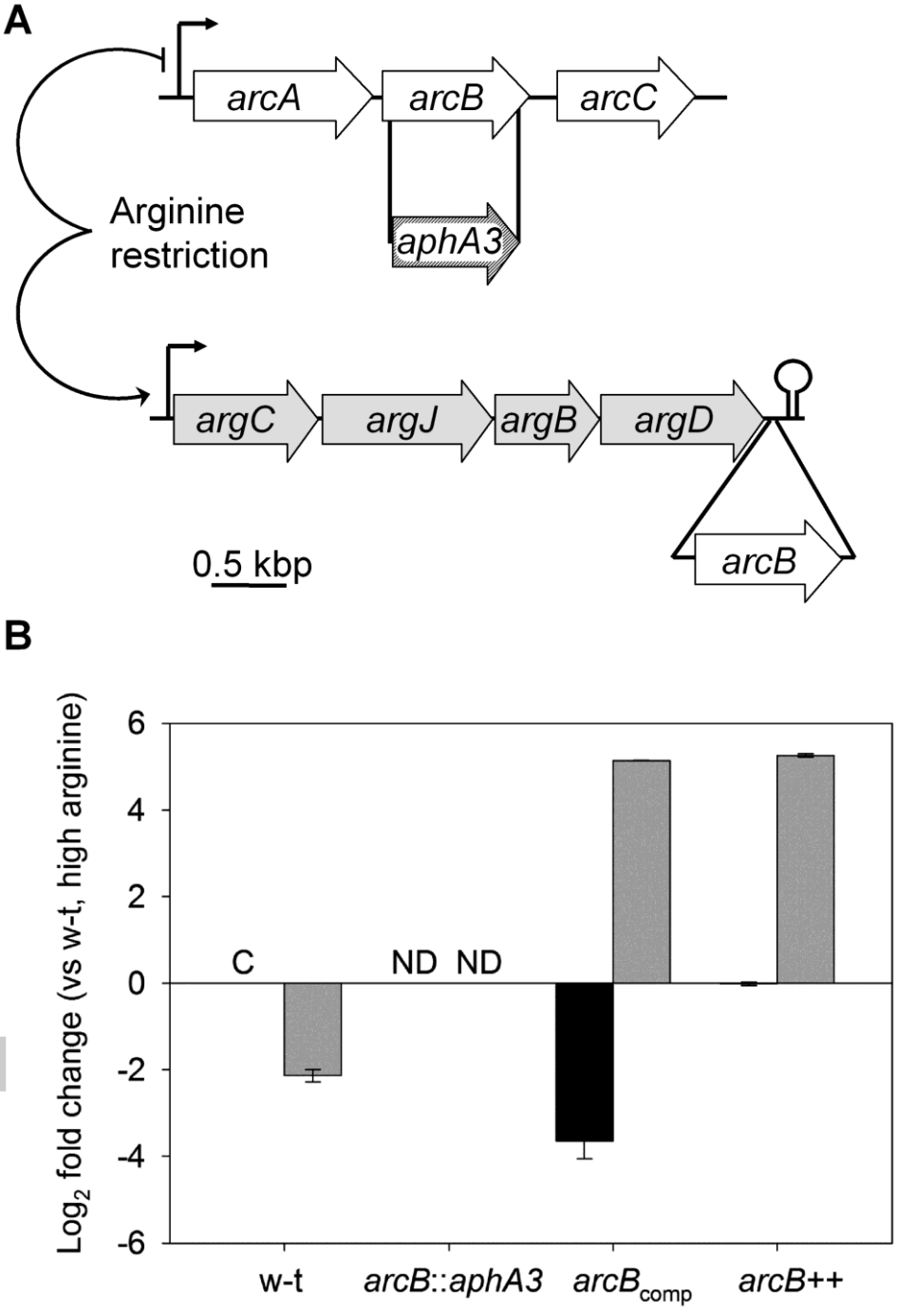

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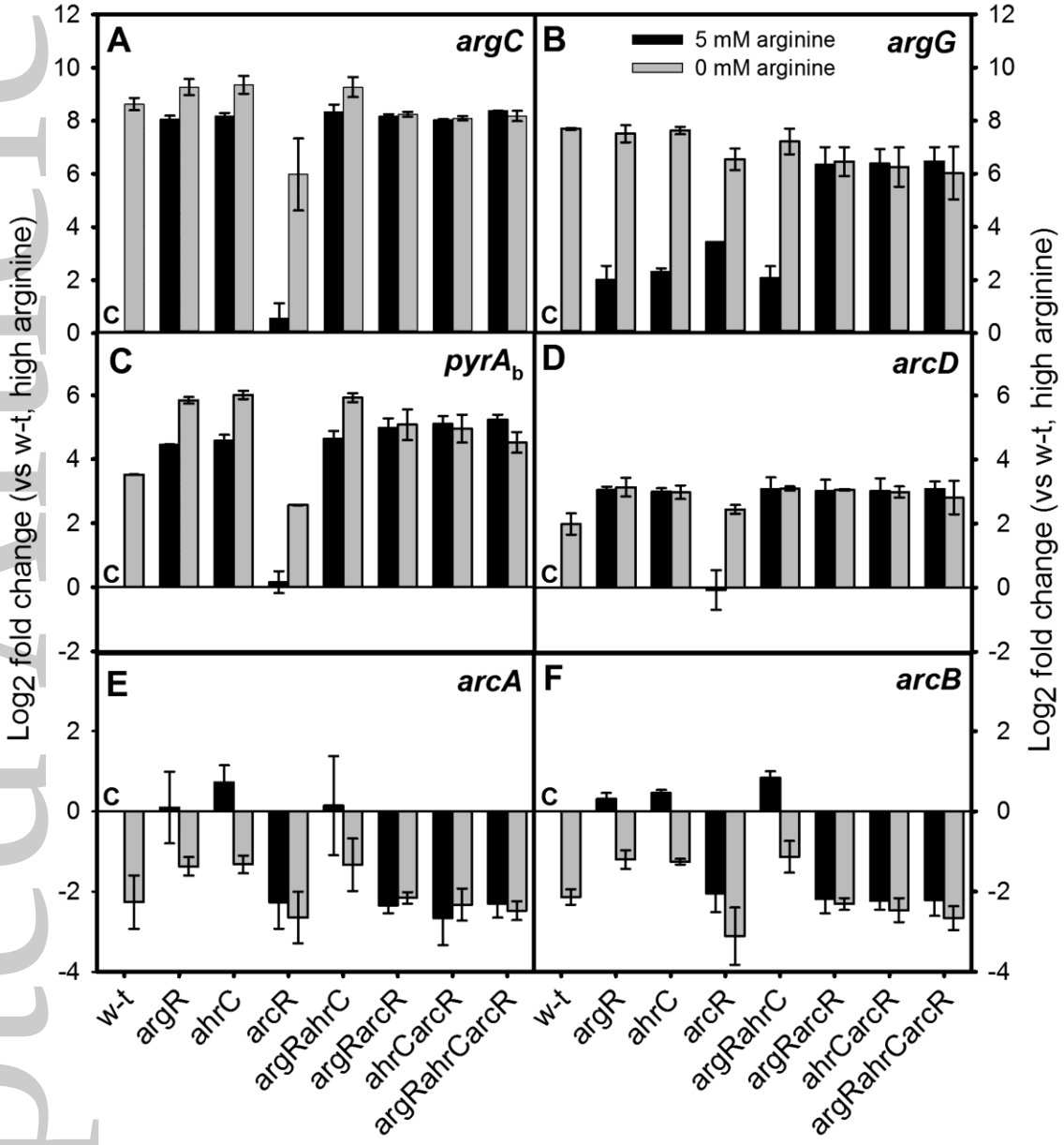

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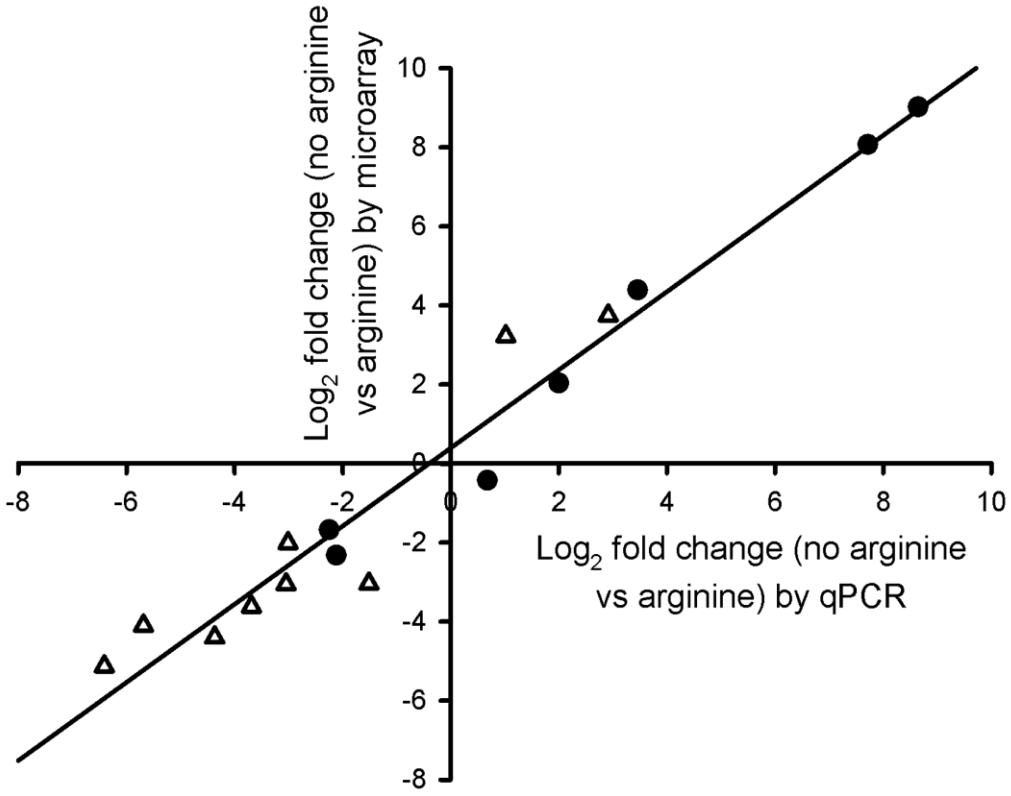

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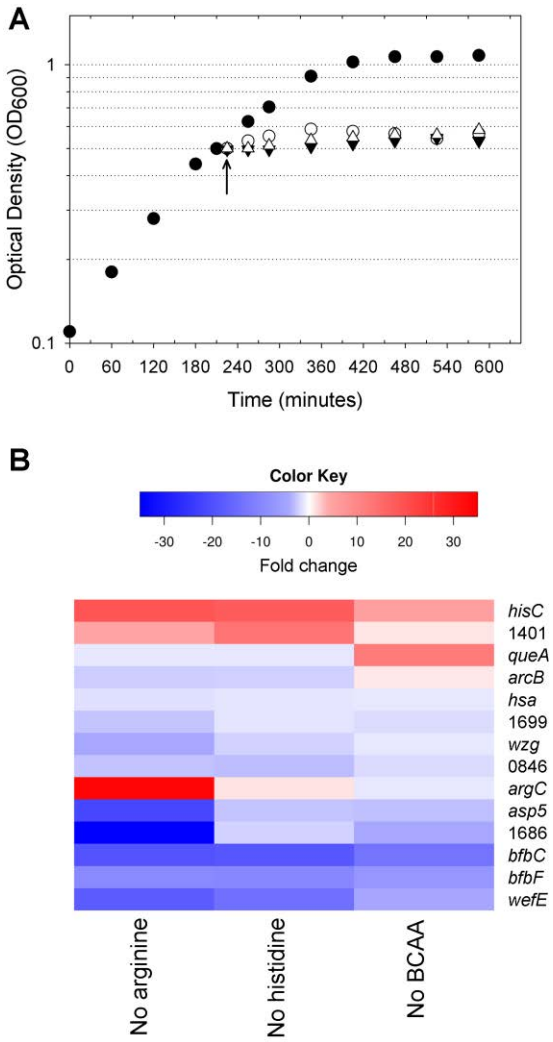

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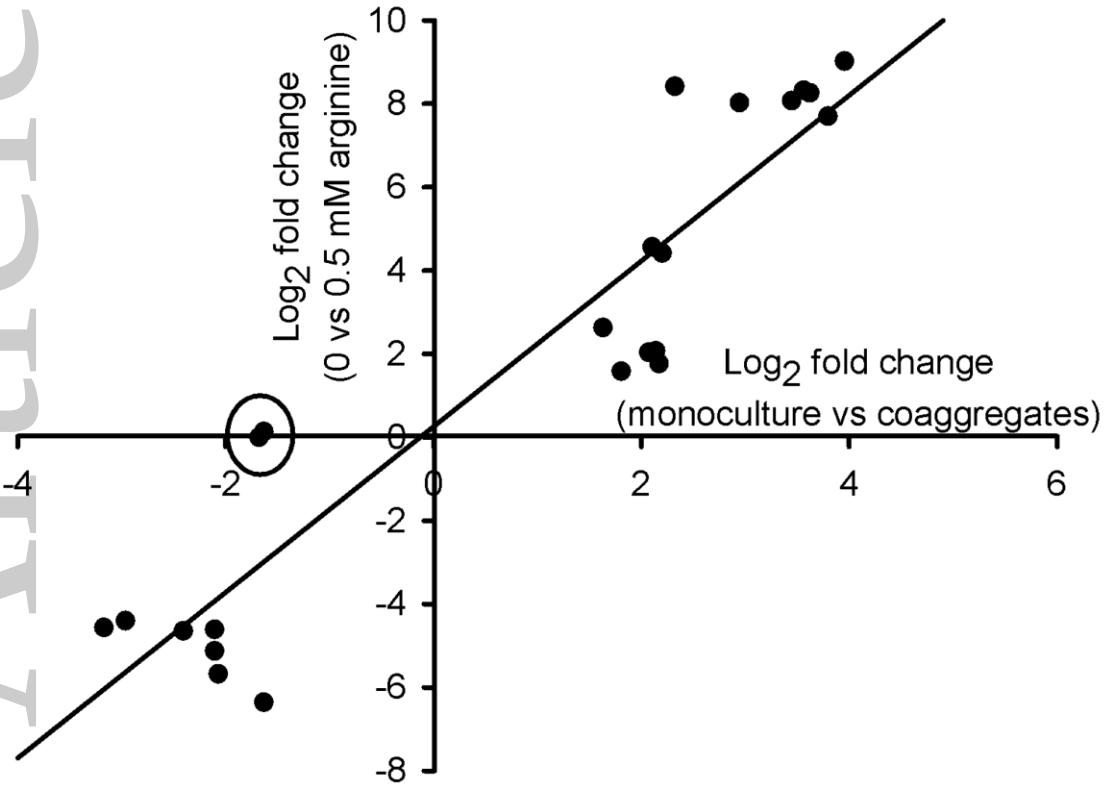

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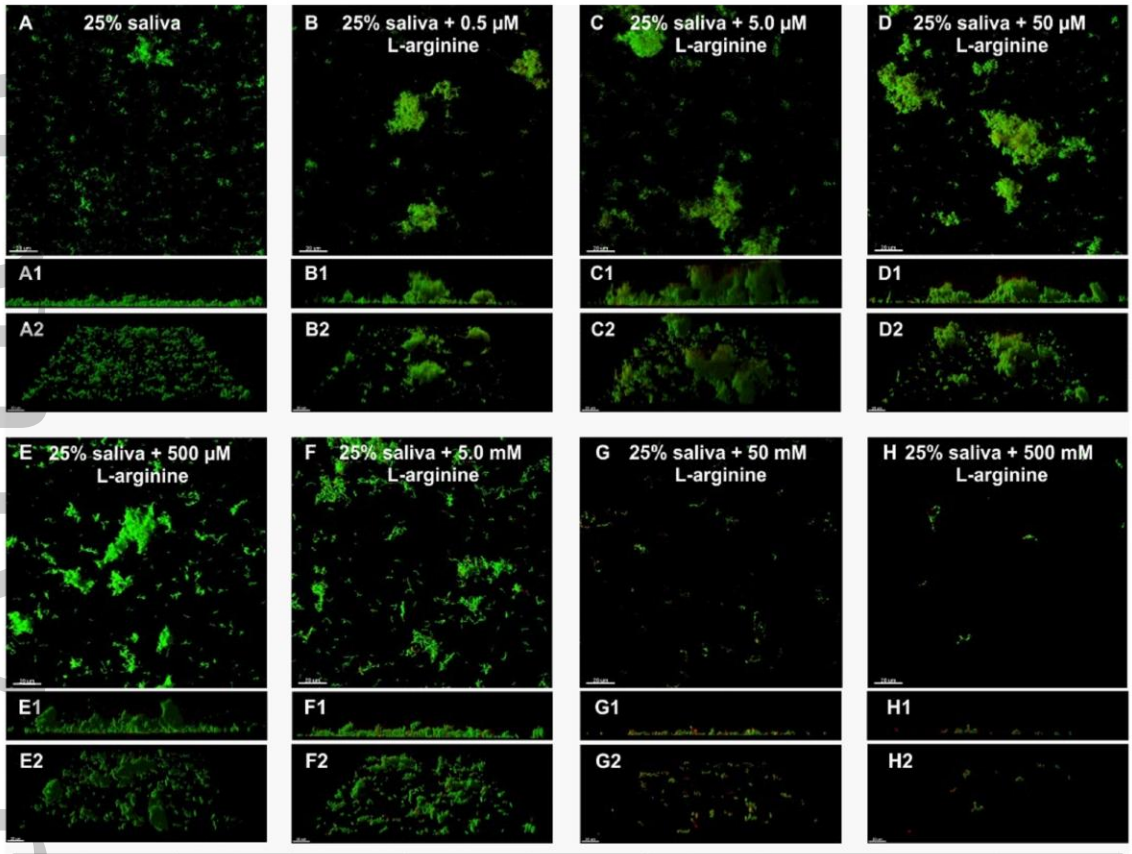
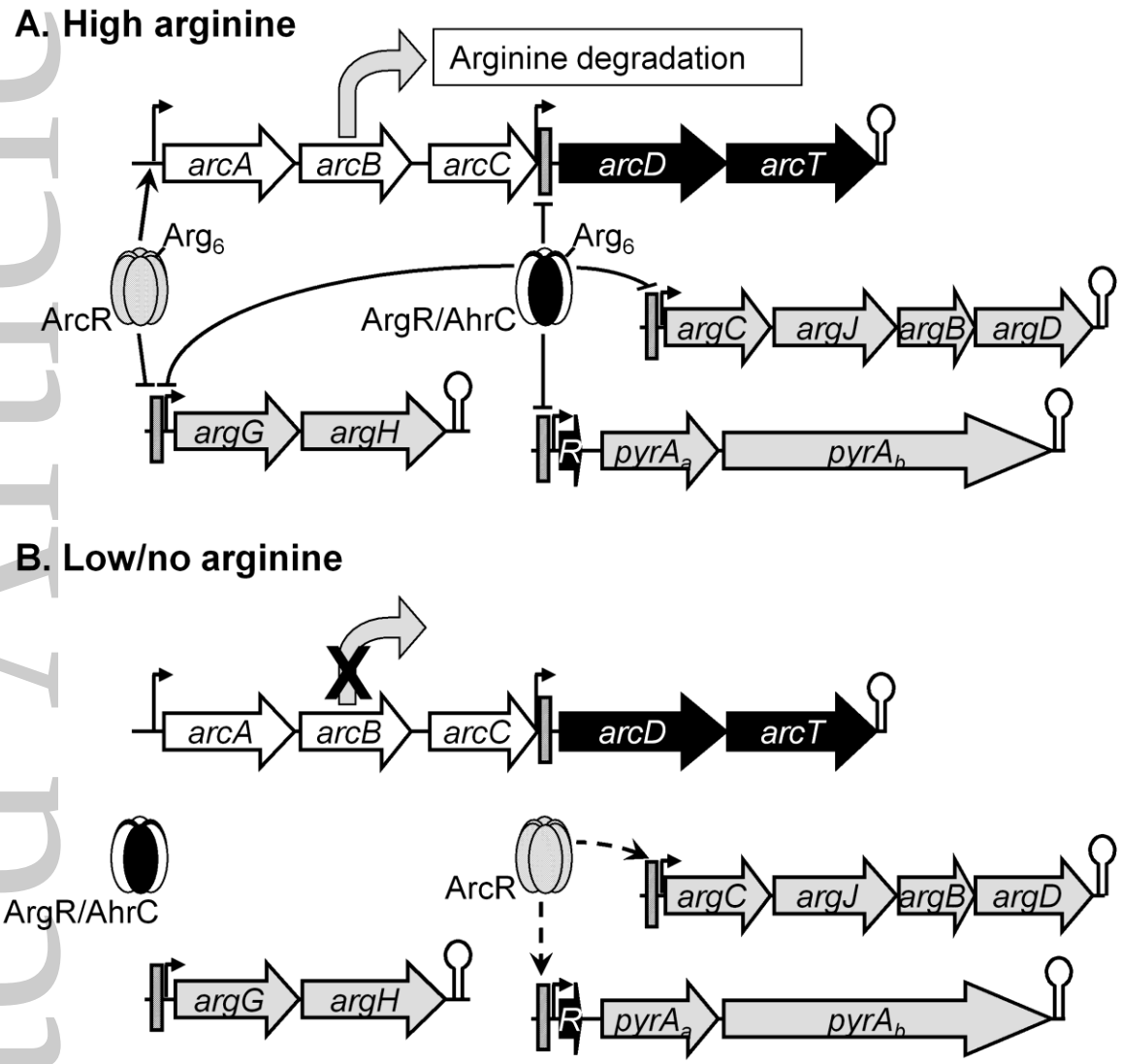


Biofilm Parameter	Control	0.5 µM	5 µM	50 µM	500 µM	5 mM	50 mM	500 mM
Viability (%)	84.00 (4.56)	69.99 (8.69)**	76.78 (5.37)**	82.34 (4.42)	81.24 (4.05)	86.30 (3.34)	81.65 (4.99)	75.37 (7.09)**
Biomass ($\mu m^3/\mu m^2$)	0.89 (0.89)	1.12 (1.11)*	2.53 (1.68)*	1.78 (0.64)*	2.30 (1.32)*	0.90 (1.11)	0.21 (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

MMI_13023_F8



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