Streptococcus gordonii Challisin protease is required for sensing cell-cell contact with *Actinomyces oris*

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Running head: Protease-mediated coaggregation sensing by *S. gordonii*

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ABSTRACT

The ability of microorganisms to regulate gene expression is thought to be critical for survival and growth during the development of polymicrobial biofilms such as dental plaque. The commensal dental plaque colonizer, *Streptococcus gordonii*, responds to cell-cell contact (coaggregation) with *Actinomyces oris* by regulating >20 genes, including those involved in arginine biosynthesis. We hypothesized that an *S. gordonii* extracellular protease is critical for sensing by providing amino acids that modulate gene expression. *S. gordonii* coaggregated strongly with *A. oris* in buffer, saliva or chemically defined medium (CDM). In wild-type *S. gordonii*, expression of arginine biosynthesis genes *argC* and *argG* increased within two hours' growth in CDM in monocultures, but not following coaggregation with *A. oris.* By contrast, coaggregation of *A. oris* with an *S. gordonii* mutant lacking *sgc*, encoding the extracellular protease Challisin, resulted in increases in *argC* and *argG* gene expression that were similar to monocultures. Genetic complementation of *sgc* restored the ability of *S. gordonii* to sense coaggregation with *A. oris*. Coaggregation enabled growth of *S. gordonii* in low/no arginine and disruption of *sgc* did not affect this ability. We propose that extracellular bacterial proteases may be key mediators of cell-cell contact sensing by diverse microbial species.

INTRODUCTION

Human dental plaque is a microbial biofilm on tooth surfaces that typically contains 100 or more microbial species encased within a polymeric extracellular matrix (Wang *et al.* 2017). The accumulation of dental plaque begins with the adherence of early microbial colonizers such as *Streptococcus* spp., *Gemella haemolysans*, *Actinomyces* spp., *Haemophilus* spp., *Rothia* spp., *Neisseria* spp., *Kingella oralis*, *Slackia exigua* and *Veillonella* spp. to the acquired enamel pellicle that coats the tooth surface (Heller *et al.* 2016).

Cell-cell interactions, known as coaggregation, are thought to be important for initial colonization and for the subsequent recruitment of later colonizers to the biofilm (Jakubovics 2015, Katharios-Lanwermeyer *et al.* 2014). Oral streptococci may form hubs for coaggregation-mediated interaction networks since many of them possess multiple adhesin proteins on the cell surface that mediate interactions with a wide variety of host or bacterial receptors (Nobbs *et al.* 2011). For example, *Streptococcus gordonii* DL1 (Challis) employs cell surface proteins CshA, SspA, SspB and Hsa to coaggregate with other oral microorganisms including *Candida albicans*, *Veillonella parvula*, *Porphyromonas gingivalis* and *Actinomyces oris* (Back *et al.* 2015, Bamford *et al.* 2015, Patil *et al.* 2016, Zhou *et al.* 2015). *Actinomyces* spp., and *A. oris* in particular, are among the most common bacteria in early dental plaque (Teles *et al.* 2012). The genus *Actinomyces* has undergone extensive reclassification over the last few decades. For example, *A. oris* MG1 was originally isolated from gingival dental plaque of a patient with mild gingivitis and was described as *A. viscosus* (Delisle et al. 1978). Subsequently *A. viscosus* strains isolated from humans were shown to be very similar to *A. naeslundii* and were reclassified as *A. naeslundii* genospecies 2 (Johnson *et al.* 1990). In 2009, a molecular analysis of *A. naeslundii*

strains split the group into three separate species and strain MG1 was designated *A. oris* (Henssge et al. 2009).

Coaggregation between *S. gordonii* DL1 and *A. oris* MG1 has been characterized in detail and shown to involve adhesion of *S. gordonii* SspB to a glucose, mannose and galactose-containing polysaccharide of *A. oris* (Back *et al.* 2015). The impact of coaggregation on gene regulation in *S. gordonii* has been investigated using DNA microarrays (Jakubovics *et al.* 2008). Twenty-three genes were identified in *S. gordonii* that were differentially regulated in coaggregates compared with monocultures, including the biofilm-associated locus *bfbCDARBGF* (up-regulated 3- to 9-fold) and nine genes involved in arginine biosynthesis or transport (down-regulated 4- to 14-fold). Of these, 21 genes were also regulated in response to changes in arginine concentration (Jakubovics *et al.* 2015), indicating that arginine itself may be involved in the coaggregation-sensing pathway. *S. gordonii* is a functional arginine auxotroph since it has the capacity to biosynthesize arginine, but does not grow when transferred directly into chemically defined medium lacking arginine (Jakubovics *et al.* 2008). Interestingly, coaggregation with *A. oris* overcomes this limitation and enables *S. gordonii* growth in no arginine (Jakubovics *et al.* 2008). Arginine sensing in *S. gordonii* is mediated by three ArgR-family transcriptional regulators: ArgR and AhrC, which act in concert to suppress arginine biosynthesis gene expression in arginine-replete conditions, and ArcR, which is required for activation of arginine catabolism gene expression under high arginine (Jakubovics *et al.* 2015, Robinson *et al.* 2018). On the basis of these observations, we propose a model whereby *S. gordonii* scavenges arginine from the *A. oris* cell surface through the action of an extracellular protease. This leads to arginine-containing peptides or free arginine that is internalized and sensed by one or more ArgR-family regulator, leading to repression of arginine biosynthesis

gene expression and an ability to initiate growth in the absence of arginine. However, until now the identity of the protease involved in this putative cell-cell sensing pathway has not been investigated.

At least three different protease activities have been detected in culture supernatants of *S. gordonii* DL1 (Juarez and Stinson 1999). It is possible that these correspond to three different putative secreted serine proteases that are annotated in the *S. gordonii* genome and encoded by SGO_RS01555 (166 kDa), SGO_RS01560 (165 kDa) and SGO_RS02790 (165 kDa). Of these, SGO_RS02790 has been studied in detail and is now known as 'Challisin'. Challisin is thought to play a role in infective endocarditis as a pro-coagulant since it has activity homologous to angiotensin converting enzyme as well as the ability to degrade fibrinogen (Harty *et al.* 2012, Harty and Hunter 2011). Challisin also protects *S. gordonii* in polymicrobial biofilms by degrading bacteriocins produced by other species such as *Streptococcus mutans* (Wang and Kuramitsu 2005). In addition to the secreted serine proteases, *S. gordonii* FSS2 produces an extracellular arginine aminopeptidase (RAP) that cleaves N-terminal arginine residues from peptides (Goldstein *et al.* 2002). The N-terminus of RAP has been sequenced and is 100% identical to that of SGO_RS03550 of *S. gordonii* DL1, which encodes a 65 kDa protein. This paper aimed to investigate the possible involvement of an *S. gordonii* extracellular protease in the sensing of coaggregation with *A. oris*. Understanding the mechanisms underpinning coaggregation sensing by bacteria could potentially lead to new approaches for modulating interspecies interactions and for controlling the accumulation of biofilms such as dental plaque.

MATERIALS & METHODS

Culture media and growth conditions

Strains used in this study are listed in Table 1. To examine coaggregation, *S. gordonii* DL1 and *A. oris* MG1 were routinely cultured in 20 ml Todd Hewitt Yeast Extract (THYE) medium, containing 30 g L^{-1} Todd Hewitt Broth (Difco, Detroit, MI) and 5 g L^{-1} Yeast Extract. Cells were cultured anaerobically in a Bugbox Plus anaerobic workstation (Baker Ruskinn, Bridgend, UK) at 37°C and 90% N_2/5% H_2/5% CO_2 atmosphere for 18 h without shaking. *E. coli* was cultured in Luria Bertani (LB) broth (Melford Laboratories Ltd.) aerobically at 37°C for 8-12 h with shaking at 180 rpm. Where necessary, the appropriate antibiotics (all Sigma Aldrich, Irvine, UK) were added to *E. coli* cultures: ampicillin (100 μg/mL), kanamycin (25 μg/mL) and erythromycin (400 μg/mL) or to *S. gordonii* cultures: kanamycin (250 μg/mL), spectinomycin (100 μg/mL) or erythromycin (2 μg/mL).

Coaggregation assays

To induce coaggregation, bacterial cells were grown as monocultures in THYE medium at 37°C for 18 h. Cells were harvested at 3,800 *g*, 4°C for 10 min in a swing-out rotor, washed three times with one volume of phosphate buffer saline (PBS, pH 7.3) and resuspended in chemically defined medium (CDM), prepared as described by Jakubovics *et al.* (2008). This medium is a minor modification of the original Fortified M1 medium plus citrate (FMC) developed by Terleckyj *et al.* (1975) and contains a mixture of vitamins, amino acids, nucleotides, metal salts, glucose and phosphate buffer. Cells were adjusted with CDM to OD_{600} = 1.1 ± 0.1 (approximately $5x10^9$ CFU/ml). Equal volumes (300 μl) of *S. gordonii* and *A. oris* cells were added to a glass test tube, mixed by vortex for 10 sec and gently rocked until coaggregation was apparent. Semi-quantitative scoring of coaggregation between *S. gordonii* DL1 and *A. oris*

MG1 cells was conducted using the visual scoring system developed by Cisar *et al.* (1979). *S. gordonii* UB1360 Δ*sspA*Δ*sspB* (Heddle *et al.* 2003) was employed as a negative control since this strain does not coaggregate with *A. oris* MG1 (Jakubovics *et al.* 2005).

Coaggregation was visualized by 3D imaging using confocal laser scanning microscopy (CLSM) or by transmission electron microscopy (TEM). For CLSM labeling, rubber O rings of 0.2 mm diameter (W&H Ltd, Bürmoos, Austria) were fixed to glass microscope slides using sticky wax or superglue. To keep the coaggregation stable during imaging, approximately one quarter of the total volume within the O rings was filled with agarose (1.25% w/v). *S. gordonii* cells were stained with 5 μM Picogreen (Thermo Fisher, Waltham, MA, USA) and *A. oris* cells were stained with 30 μM propidium iodide (Thermo Fisher) at 37°C for 30 min. Prior to mixing for coaggregation, cells were washed three times with coaggregation buffer (1mM Tris-HCl, pH 8.0, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 150 mM NaCl, and 0.02% NaN_3). Coaggregated cells or monoculture controls were placed in the middle of the rubber O ring and a coverslip was added. Coaggregation samples were visualized using a Leica SP2 CLSM microscope (Leica, Microsystems, Heidelberg, Germany) using excitation (Ex) at 530 nm and emission (Em) at 630 nm for propidium iodide and Ex/Em = 485 nm/530 nm for Picogreen. For high resolution imaging of coaggregation by TEM, samples were placed into 2% glutaraldehyde in 100 mM phosphate buffer, pH 7.0, immediately following coaggregation, and stored at 4°C. Samples were dehydrated through a series of ethanol washes, embedded in in epoxy resin and sectioned at the Electron Microscopy Research Services, Newcastle University. Sections were analyzed in a Philips CM100 transmission electron microscope.

Mutagenesis and complementation of protease-encoding genes in *S. gordonii*

S. gordonii genomic DNA was purified using the Masterpure Gram Positive DNA purification kit (Epicentre® Biotechnologies, Madison, WI, USA), as instructed by the manufacturer. Overlap extension PCR was employed to knock out either the *rapA* gene, encoding arginine aminopeptidase, or the *sgc* gene, encoding Challisin (Jakubovics *et al.* 2015). All primers are listed in Table 2. For *rapA* gene deletion, a 285 bp region immediately upstream of *rapA* and a 574 bp region containing a 3' region of the gene with downstream sequence, were amplified by PCR using primers *rapA*F1/R1 and *rapA*F2/R2, respectively. In a separate PCR reaction a 738 bp region of the non-polar *ermAM* antibiotic resistance cassette was amplified from plasmid pVA838 (Macrina *et al.* 1982) using primers *erm*F1/R1. The three products were mixed in equimolar ratios and stitched together in a second round of PCR, which generated a 1,597 bp fragment. This was subsequently used for transformation of *S. gordonii* DL1. The *sgc* gene was disrupted using a similar approach. A 521 bp fragment upstream of *sgc* was amplified using *adhP* HF1/HR1, and a 516 bp fragment downstream of *sgc* was amplified with *glyQ* HF2/HR2. The non-polar *aphA3* kanamycin resistance cassette (795 bp) was amplified from plasmid pSF151 (Tao 1992) using primers *aphA3*F1/R1. A 1,795 bp fragment containing the upstream and downstream regions of *sgc* stitched to *aphA3* was generated in a second round of PCR. This product was used for transformation of *S. gordonii* DL1. Disruption of *rapA* in *S. gordonii* Δ*rapA* (NU04) and *sgc* in *S. gordonii* Δ*sgc* (NU12) was confirmed by PCR and sequencing. To produce a genetic complementation strain, *S. gordonii* Sgc_{Comp}, the In-Fusion ligation-independent cloning strategy was used (Takara Bio Europe SAS, Saint-Germain-en-Laye, France). Primers pPE1010F and pPE1010R were used to amplify a 5,811 bp fragment of vector pPE1010 (Egland *et al.* 2004). Primers *sgc*CompF and *sgc*CompR, containing 20 bp regions of overlap with vector pPE1010, were employed to amplify a 4,562 bp fragment

containing the *sgc* gene from *S. gordonii* genomic DNA. The In-Fusion HD PCR ligation cloning kit was utilized to fuse the *sgc* gene into the pPE1010 vector to generate plasmid pSgc$_{Comp}$. The integrity of the inserted *sgc* gene was confirmed by sequencing, and pSgc$_{Comp}$ was used for transformation of *S. gordonii* Δ*sgc::kan* to generate *S. gordonii* Sgc$_{Comp}$.

Challisin activity assay

To measure Challisin activity in cell fractions or culture supernatants, *S. gordonii* DL1, Δ*sgc* and Sgc$_{Comp}$ were cultured for 18 h in 20 mL THYE medium. Bacterial cells were harvested by centrifugation at 3,800 *g* for 10 min at 4°C in a swing-out rotor and supernatants were collected. For cellular and cell surface protein fractions, cells were disrupted using mutanolysin as described by Jakubovics *et al.* (2005) with minor modifications. Cells were washed twice with distilled water and resuspended in 100 µl of spheroplasting buffer containing 26% (w/v) raffinose, 10 mM $MgCl_2$, 20 mM Tris-HCl, pH 6.8. Mutanolysin was added to a concentration of 500 U ml^{-1} and the solution was incubated at 37°C for 15 min. Following centrifugation at 10,000 *g*, 4°C for 10 min, surface proteins that had been released into the supernatant were collected. Cells were resuspended in 200 µl spheroplasting buffer, 50 µl of 0.1 mm glass beads were added and cells were lysed by vigorous vortex mixing for 5 min. Cell debris was removed by centrifugation at 10,000 *g*, 4°C for 10 min and proteins in the supernatant were collected. Protein concentrations in supernatants and cell extracts were determined with the Pierce Coomassie (Bradford) Protein Assay Kit (Thermo Fisher) using bovine serum albumin as standard. Protease activity was measured by the azocasein method essentially as described by Sarath *et al.* (1989). The reaction was carried out at 37°C for 30 min in 500 µl buffer containing 100 µl cell supernatant, cellular protein or cell surface protein fraction, 0.8% (w/v) azocasein, 0.6

177 mM $NaHCO_3$, pH 8.3. The reaction was stopped by adding 500 µl of 10% (w/v) trichloroacetic
178 acid and immediately mixed by vortex. Samples were incubated at 20°C for 5 min, and the
179 precipitate was removed by centrifugation at 10,000 *g* for 5 min at 4°C. One hundred µl of the
180 supernatant were added to 200 µl of 1M NaOH and the $A_{440\ nm}$ was read in a microplate reader.
181 All experiments were performed three times independently.
182
183 **RNA extraction**
184 To study the impact of coaggregation on gene regulation in *S. gordonii*, monocultures,
185 cocultures or coaggregate cultures were prepared in CDM as previously described (Jakubovics *et*
186 *al.* 2008). Cocultures consisted of *S. gordonii* and *A. oris* cells that were mixed in equal ratios
187 but not forced into coaggregates by vortexing. By this method, cells remain separated for several
188 hours before coaggregates slowly start to form (Jakubovics *et al.* 2008). By contrast,
189 coaggregates consisted of *S. gordonii* and *A. oris* cells that were vortex-mixed at relatively high
190 concentrations to induce strong coaggregation before being diluted gently. For monocultures,
191 300 µl of *S. gordonii* and 300 µl of *A. oris* cell suspensions at a final concentration of
192 approximately 5 x 10^9 CFU/ml were separately added to 14.7 ml CDM (pre-warmed to 37°C) in
193 a capped 15 ml glass tube, giving a final concentration of ~1 x 10^8 CFU/ml. Cocultures, in which
194 *S. gordonii* and *A. oris* were cultured together in the same tube without first inducing
195 coaggregation, were established by adding 300 µl of *S. gordonii* and 300 µl of *A. oris* cells, each
196 at a concentration of 5 x 10^9 CFU/ml, to 14.4 ml CDM and mixed gently by inversion. To induce
197 coaggregation, 300 µl of *S. gordonii* and 300 µl of *A. oris* cells were each added to an Eppendorf
198 tube and vortex mixed for 10 sec. Large aggregates were separated from single cells or small
199 aggregates by centrifugation at 1,000 *g* for 3 min. The supernatant was removed, and pellets

were adjusted to 15 ml with FMC, resulting in a final concentration of ~1 x 10^8 CFU/ml of each species. All tubes were incubated at 37°C in a water bath for up to 4 h. When required, RNA was stabilized by the addition of 5 ml RNA Later (Thermo Fisher) to 5 ml sample, vortex mixing for 5 sec and incubation at 20°C for 5 min. Cells were harvested at 3,000 *g* for 15 min at 20°C. The supernatant was discarded and pellets were frozen at -80°C for RNA extraction within 72 h.

To extract RNA, samples were thawed at 20°C and resuspended in 100 µl spheroplasting buffer containing 0.1 mg/mL spectinomycin (Jakubovics *et al.* 2015). Mutanolysin was added to 500 U ml^{-1} and cells were incubated at 37°C for 5 min. Total RNA was extracted using the Ambion RiboPure Bacteria RNA Purification kit (Life Technologies) according to the manufacturer's instructions. RNA concentrations were determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). To ensure that RNA had not degraded during extraction, an aliquot of each sample was analyzed by agarose gel electrophoresis.

Reverse transcription and Quantitative real-time PCR (RT-qPCR)

One µg of total RNA from bacterial cells was reverse transcribed using the QuantiTect Reverse Transcription kit (Qiagen, Germany, Hilden) in accordance with the manufacturer's instructions. Briefly, RNA samples were incubated with Wipeout Buffer and RNase-free water at 42°C for 2 min to remove contaminating genomic DNA. Reverse transcription reactions contained Quantiscript RT buffer (1x), long random primers (10 µM) and oligo-dT RNA sample (1.0 µM). Reactions were incubated at 42°C for 15 min, then at 95°C for 3 min to inactivate the Quantiscript reverse transcriptase. For RT-qPCR, reaction mixtures were prepared containing 0.25 µl of cDNA samples and QuantiTect SYBR Green mix (Qiagen, Germany), 1 µM forward and reverse primer (Table 2) in a total volume of 20 µl. Reactions were performed in triplicate

using an Opticon 2 DNA Engine (Bio-Rad Laboratories Ltd., Hertfordshire, UK) with thermocycling conditions as follows: initial denaturation at 95°C for 2 min and 40 cycles of amplification at 95°C for 15 s, 55°C for 10 s and 72°C for 30 s. The Comparative CT Method (ΔΔCT) was used to analyze RT-qPCR data, and data were normalized to the 16S rRNA gene as a reference. All RT-qPCR reactions were validated using melt curve analysis, and selected samples were analyzed by agarose gel electrophoresis. Three independent repeats were performed for all culture conditions.

Batch growth in CDM

To assess the growth of monocultures and coaggregate cultures in CDM, samples were set up using the same approach as described for RNA extraction. Cultures were incubated at 37°C and the turbidity was checked hourly for up to 10 h. Representative samples were checked by phase contrast microscopy to ensure that there was no contamination and to qualitatively assess the relative growth of *S. gordonii* and *A. oris*. All experiments were performed two to three times independently, and growth was very similar each time (SD <0.15 relative light units for all samples).

RESULTS

Visualization of coaggregation

To confirm that *S. gordonii* DL1 and *A. oris* MG1 physically interact in coaggregation buffer, coaggregation assays were performed by mixing concentrated suspensions of each cell type and vigorously mixing by vortex (see Materials and Methods). Strong coaggregation was observed in the test tube, which was scored '4+' using the visual scoring system developed by

Cisar *et al.* (1979). By CSLM, the two species were intermixed throughout the three dimensional structure of the aggregates (Fig. 1). Using TEM to visualise interactions at higher resolution, cell walls of *S. gordonii* were shown to be in close contact with *A. oris* cells (Fig. 2).

Disruption of genes encoding extracellular proteases

Initially, we hypothesized that the *S. gordonii* arginine aminopeptidase (Rap) may be involved in cell-cell sensing with *A. oris*. Partial sequence of the gene encoding Rap has been obtained from *S. gordonii* FSS2 (Goldstein *et al.* 2002). We identified a region of the *S. gordonii* genome that was 100% identical to this sequence and encoded a putative 65 kDa aminopeptidase protein at locus SGO_RS03550. We termed this gene '*rapA*'. In preliminary studies, *rapA* was knocked out by allelic replacement with the *ermAM* erythromycin resistance cassette. However, following coaggregation with *A. oris*, there was no apparent difference in the expression of the arginine biosynthesis genes *argC* or *argG* in *S. gordonii* Δ*rapA* compared with the wild-type (data not shown). Therefore, our focus turned to the previously characterized serine protease Challisin.

To study the role of Challisin in cell-cell sensing, the *sgc* gene encoding Challisin was knocked out by allelic replacement with the *aphA3* kanamycin resistance cassette. Successful gene disruption was confirmed by PCR and DNA sequencing. To confirm that disruption of *sgc* reduced extracellular protease activity, *S. gordonii* DL1 and Δ*sgc* were assayed for the production of challisin using an azocasein-based colourimetric assay (Fig. 3). Supernatant fluids from *S. gordonii* DL1 had detectable proteolytic activity that was significantly reduced in *S. gordonii* Δ*sgc* ($P<0.05$). To ensure that the reduction in activity was caused directly by the disruption of *sgc*, a complemented strain (*S. gordonii* Sgc_{Comp}) was generated in which the *sgc*

gene was restored on a plasmid (see Materials and Methods). Protease activity of *S. gordonii* Sgc_{Comp} was significantly higher than that of *S. gordonii* Δ*sgc* and was not significantly different from that of the wild-type, confirming that the Challisin (Sgc) was responsible for the observed protease activity. Supernatants were used for these studies since Challisin is predicted to be secreted from cells and has previously been demonstrated to be present in supernatant fluids (Wang and Kuramitsu 2005). To ensure that there was not significant Challisin protease activity in cell-associated fractions, cellular proteins and cell surface associated proteins were extracted and protease activity was determined by the azocasein assay. In all strains, there was no detectable protease activity in cell surface protein fractions, and there was no activity in cellular protein fraction of *S. gordonii* Δ*sgc*. Very low levels of protease activity were detected in cellular protein extracts of *S. gordonii* wild type and Sgc_{Comp} that were $>10^3$-fold lower than the activity in an equivalent mass of protein from culture supernatants. Therefore, the vast majority of Challisin activity was localized to culture supernatants.

Coaggregation sensing over time

To assess the effect of the Challisin mutant on gene regulation in mixed-species cultures containing *A. oris*, *S. gordonii* cells were cultured in BHY medium, harvested and resuspended in CDM containing 0.5 mM arginine. Growth of the cells was followed for up to 4 h in monoculture, co-culture (*S. gordonii* and *A. oris* mixed gently without inducing coaggregation) and coaggregation. The impact of coaggregation on gene regulation was assessed by monitoring expression of two arginine biosynthesis genes, *argC* and *argG* that are in different loci on the *S. gordonii* chromosome and have previously been shown to be regulated by coaggregation (Jakubovics *et al.* 2008). In addition, a control gene, *amyB*, that is not regulated by coaggregation

293 with *A. oris*, was monitored. As expected, *amyB* expression did not change significantly over the
294 course of the experiment in *S. gordonii* DL1 or *S. gordonii* Δ*sgc* (Fig. 4). By contrast, *argC* and
295 *argG* were strongly up-regulated in *S. gordonii* DL1 monocultures or co-cultures with *A. oris*
296 after 2 h and remained elevated until the final measurement at 4 h. In cultures of *S. gordonii*
297 where coaggregation with *A. oris* was induced by vortex mixing, *argC* and *argG* expression
298 remained essentially unchanged over 4 h. Like the wild-type, the expression of *argC* in *S.*
299 *gordonii* Δ*sgc* monocultures or co-cultures was strongly up-regulated (100- to 200-fold) between
300 0 and 2 h, and then remained relatively stable up to 4 h after inoculation. The increase in *argC*
301 expression was detected earlier in *S. gordonii* Δ*sgc* than in the wild-type. After 1 h, there was
302 approximately 18- and 36-fold increased levels of *argC* in monocultures and co-cultures of *S.*
303 *gordonii* Δ*sgc*, respectively, compared with the equivalent wild-type cultures. In these *S.*
304 *gordonii* Δ*sgc* cultures, *argG* expression followed a similar pattern to the wild-type: it was
305 strongly up-regulated after 2 h and remained high up to 4 h. In coaggregates, however, there was
306 a marked difference in the expression of *argC* and *argG* in *S. gordonii* Δ*sgc* compared with *S.*
307 *gordonii* DL1. Specifically, *argC* and *argG* expression in *S. gordonii* Δ*sgc* in coaggregates was
308 similar to that in monocultures or cocultures at all time points, indicating that coaggregation no
309 longer suppressed the up-regulation of these genes in the absence of Challisin (Fig. 4).
310
311 **Effect of *sgc* complementation on coaggregation-mediated gene regulation**
312 To verify that the observed gene regulation effects were due to the disruption of Challisin
313 and not an unidentified second site mutation, coaggregation experiments were set up with the
314 complemented strain *S. gordonii* Sgc_{Comp}. We focused on 2 h after the initiation of coaggregation
315 for these experiments, since this was the first time point where the expression of *argC* and *argG*

was strongly up-regulated in wild-type monocultures (Fig. 4). Expression of *argC* and *argG* was strongly up-regulated in monocultures of *S. gordonii* DL1, Δ*sgc* and Sgc_{Comp} at this point (Fig. 5A). In coaggregate cultures, the up-regulation of these genes was suppressed in the wild-type but not in *S. gordonii* Δ*sgc* (Fig. 5B). Levels of *argC* and *argG* expression were low in *S. gordonii* Sgc_{Comp} indicating that genetic complementation with the *sgc* gene restored the suppression of arginine biosynthesis genes in response to coaggregation. As a control, *amyB* expression was shown to be stable in all cultures (Fig. 5).

Impact of Challisin knockout on coaggregation-mediated growth in low arginine.

It was previously demonstrated that coaggregation with *A. oris* enables the growth of *S. gordonii* in the absence of arginine under conditions where *S. gordonii* does not grow in monoculture (Jakubovics *et al.* 2008). To determine whether Challisin is required for coaggregation-mediated growth promotion, *S. gordonii* Δ*sgc* and *S. gordonii* DL1 were inoculated in CDM containing either no arginine or 0.5 mM arginine, in monoculture or after inducing coaggregation with *A. oris* (Fig. 6). Neither *S. gordonii* Δ*sgc* nor *S. gordonii* DL1 grew without arginine in monoculture, and these strains grew slowly in CDM containing 0.5 mM arginine. In both media, growth of each strain was clearly enhanced by coaggregation with *A. oris*. *A. oris* does not grow in the CDM used here (Fig. 6 and Jakubovics *et al.* (2008)), and therefore the increase in absorbance reflected growth of *S. gordonii* only. This was confirmed by examination of cultures under phase contrast microscopy following growth (data not shown). In all cases, there was very little difference in growth rates or yields of *S. gordonii* Δ*sgc* and *S. gordonii* DL1 when cultured under similar conditions, indicating that Challisin is not involved in the enhancement of arginine-limited growth by coaggregation.

To further explore the link between arginine sensing and growth in no arginine, the role of ArgR-family regulators in growth was examined using isogenic mutants. Since ArgR and AhrC appear to function as a single unit (Robinson *et al.* 2018), a single mutant disrupted in both genes was employed for this work, together with a strain disrupted in the gene encoding the third regulator, ArcR. *S. gordonii* Δ*argRahrC* and *S. gordonii* Δ*arcR* were cultured in monoculture or following coaggregation with *A. oris* (Fig. 7). In the absence of arginine, *S. gordonii* Δ*arcR* showed no detectable growth in monoculture, and grew slowly following inoculation in coaggregates. Growth was very similar to the wild-type in both monoculture and coaggregates. By contrast, *S. gordonii* Δ*argRahrC* grew relatively well in this medium. This strain reached stationary phase by 6 h in monoculture at a relatively low yield ($OD_{600\ nm}$ <0.5). In coaggregate cultures, growth continued beyond this point and reached ~$OD_{600\ nm}$ = 1.0 by 10 h. In 0.5 mM arginine, *S. gordonii* Δ*argRahrC* grew strongly and to a high yield ($OD_{600\ nm}$ > 1.2 after 9 h) both in monoculture and from inoculation as coaggregates. By contrast, *S. gordonii* Δ*arcR* grew slowly in this medium in monoculture and had not reached $OD_{600\ nm}$ = 0.5 after 10 h. In contrast to the wild-type, growth was only very slightly enhanced in cultures inoculated with coaggregates. Overall, these data demonstrate that coaggregation enhances growth of *S. gordonii* lacking either ArgR/AhrC or ArcR in the absence of arginine, but that coaggregation has very little impact on cultures grown in 0.5 mM arginine. *S. gordonii* Δ*argRahrC* generally grew more strongly than the wild-type under low or no arginine, whereas *S. gordonii* Δ*arcR* was specifically impaired in growth from coaggregate cultures in 0.5 mM arginine.

DISCUSSION

Here, we have identified a key role of the *S. gordonii* serine protease Challisin in modulating gene regulation in response to coaggregation with *A. oris*. Challisin has previously been shown to be involved in interactions with *S. mutans* GS5, since it degrades *S. mutans* competence stimulating peptide (CSP) and inhibits bacteriocin production and biofilm colonization (Wang *et al.* 2011, Wang and Kuramitsu 2005). It is possible that Challisin may degrade a secreted peptide of *A. oris*. However, the impact of Challisin on *S. gordonii* sensing and gene regulation is restricted to coaggregate cultures rather than cocultures where cells are physically separated. Therefore the target of Challisin must be concentrated in the vicinity of aggregates, and it is possible that Challisin cleaves a protein, or proteins, that are exposed on the cell surface of *A. oris*.

In addition to targeting bacterial proteins, Challisin cleaves host substrates such as fibrinogen and angiotensin (Harty *et al.* 2012, Harty and Hunter 2011). Studies on fibrinogen degradation have revealed that the Challisin requires a proline residue two amino acids upstream of the cleavage site and a neutral or basic unbranched residue at the point of cleavage (Harty *et al.* 2012). In a preliminary investigation, we have demonstrated that treatment of *A. oris* cells with Challisin-containing supernatant from *S. gordonii* cultures leads to the loss of at least one *A. oris* cell surface protein, a band migrating at ~65 kDa on a polyacrylamide gel (Figure S1). Lower molecular weight bands were apparent following treatment with *S. gordonii* supernatant that were not present in untreated samples, including a protein of ~34 kDa that appeared after 1 h and a protein of ~58 kDa that appeared after 24 h. The absence of a ~65 kDa protein following incubation with *S. gordonii* supernatants may have resulted from proteolytic cleavage by Challisin and it is possible that the appearance of bands at ~34 kDa and ~58 kDa may represent

the generation of cleavage products from higher molecular weight proteins. However, it is also possible that the changes in protein profile were the result of altered production of proteins, or from a component of the supernatant other than Challisin. Further studies will aim to identify the specific targets of Challisin that are involved in sensing *A. oris* by treating cells with purified Challisin and assessing changes in cell surface proteins using proteomic approaches.

S. gordonii produces several extracellular proteases, including three serine proteases and an arginine aminopeptidase (Goldstein *et al.* 2002, Juarez and Stinson 1999). Disruption of *rapA*, encoding the arginine aminopeptidase, did not appear to affect coaggregation-mediated gene regulation. Nevertheless, it is possible that this enzyme plays a role in digesting substrates and liberating arginine once the initial cleavage of proteins has been performed by Challisin. Similarly, it is possible that other serine proteases, encoded by tandem genes (SGO_RS01555 and SGO_RS01560) in the *S. gordonii* chromosome, may play a role in sensing coaggregation with *A. oris*. Nevertheless, Challisin appears to be the major protease for coaggregation sensing under the conditions employed here since disruption of the *sgc* gene encoding Challisin abrogated the coaggregation sensing phenotype. To assess the evolutionary relationship between Challisin and the predicted products of SGO_RS01555 and SGO_RS01560, sequences were aligned using the Clustal W algorithm in MEGA7 software (Kumar *et al.* 2016). Distances were calculated using with the Poisson correction model, with gaps and missing data eliminated (Zuckerkandl and Pauling 1965). From this, SGO_RS01555 and SGO_RS01560 were found to be more closely related to one another than either is to Challisin (pairwise distance of 0.74 amino acid substitutions between SGO_RS01555 and SGO_RS01560, and 1.13-1.16 between either one and Challisin). Overall, it is likely that SGO_RS01555 and SGO_RS01560 have different activities and functional roles in the biology of *S. gordonii* compared with Challisin.

406 It is noteworthy that the expression of *argC*, but not *argG* was strongly elevated in *S.*
407 *gordonii* Δ*sgc* monocultures or cocultures after just one hour of culture in CDM, whereas up-
408 regulation was not observed in the wild-type until 2 h. Expression of *argC* is exquisitely
409 sensitive to the extracellular concentration of arginine and *argC* is strongly regulated as arginine
410 levels change (Jakubovics *et al.* 2015, Robinson *et al.* 2018). It is unlikely that the proteolytic
411 activity of Challisin is involved in scavenging arginine from CDM since this medium does not
412 contain protein. However, prior to culture in CDM, cells were cultured in THYE. It is possible
413 that Challisin is required for scavenging arginine from proteins in THYE, and that this leads to
414 increased loading of wild-type *S. gordonii* cells with arginine. This would then lead to
415 suppression of expression of arginine biosynthesis genes, which would be observed most
416 strongly with the *argCJBD* operon.
417 Interestingly, abrogation of coaggregation sensing by disruption of the *sgc* gene did not
418 affect the ability of coaggregation with *A. oris* to promote the growth of *S. gordonii*, particularly
419 under arginine-limiting conditions. We have previously shown that *S. gordonii* is a functional
420 arginine auxotroph, and does not grow aerobically in monoculture following transfer to growth
421 media without arginine (Jakubovics *et al.* 2008). Coaggregation with *A. oris* is sufficient to
422 enable full growth of wild-type *S. gordonii*, but not an *argH* mutant that is unable to
423 biosynthesise arginine. Therefore, coaggregation provides a signal for growth rather than simply
424 supplying enough arginine to enable full growth of *S. gordonii* (Jakubovics *et al*. 2008). Even
425 though Challisin is critical for coaggregation sensing, it is apparently not needed for growth
426 promotion since disruption of *sgc* did not affect growth following coaggregation with *A. oris*.
427 Coaggregation sensing triggers a set of genes that are regulated by ArgR family regulators ArgR
428 and AhrC (Jakubovics *et al.* 2015). Here, disruption of genes encoding ArgR and AhrC led to

enhanced growth in moderate (0.5 mM) and no arginine, which is consistent with their roles as repressors of arginine biosynthesis gene expression. Conversely, disruption of the gene encoding a third ArgR family regulator, ArcR, prevented the beneficial effects of coaggregation on growth specifically under moderate arginine. In no arginine, growth of this strain was similar to wild-type and was enhanced by coaggregation. Therefore, it appears that ArcR plays a role in linking coaggregation sensing to growth under moderate arginine, but that there is another pathway for this in the absence of exogenous arginine. It is not clear how ArcR stimulates growth. One possibility is that growth may be triggered by the catabolism of arginine, since ArcR is a key activator of transcription of arginine catabolism genes when arginine is available (Liu *et al.* 2008). It is also possible that ArcR links coaggregation sensing to biofilm formation. It has recently been shown that this regulator is critical for biofilm formation by *S. gordonii* (Robinson *et al.* 2018).

In the absence of arginine, it is possible that active growth is initiated by the high cell density in coaggregates rather than by sensing a specific feature of *A. oris*. Previous studies have shown that a number of phenotypes of oral bacteria are affected by cell density. For example, a density-dependent signal termed 'START' has previously been observed to trigger the rapid growth of dental plaque after an initial colonization stage (Liljemark *et al.* 1997). Cell density has been shown to modulate phenotypes in the oral streptococcus *S. mutans* including acid adaptation and bacteriocin expression (Kreth *et al.* 2006, Li *et al.* 2001). Both of these phenotypes are mediated by the ComCDE sensing system. In future it will be of interest to determine whether ComCDE is involved in coaggregation-mediated growth of *S. gordonii*.

In summary, we have identified Challisin as a key component in the pathway of cell-cell sensing of *A. oris* by *S. gordonii*. We propose a model whereby *S. gordonii* sense *A. oris* through

the uptake of arginine or arginine-containing peptides that are released from *A. oris* by the activity of Challisin protease. This cell-cell sensing is likely a key driver from oral biofilm formation, and thus represents a potential target for strategies to control the accumulation of dental plaque. Further work is required to identify the polypeptide targets of Challisin in this interaction and to characterize the signal for coaggregation-mediated growth enhancement, which appears to be independent of Challisin. Extracellular proteases are produced by a wide variety of bacteria (Rawlings *et al.* 2016), and it is likely that protease-mediated amino acid scavenging may be a conserved mechanism for sensing of cell-cell interactions in many different species.

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560
561

Table 1. Bacterial strains used in this study.

Strain	Description[a]	Source or Reference
A. oris MG1	Wild type	(Delisle *et al*., 1977)
S. gordonii DL1	Wild type	Howard Jenkinson, Bristol
S. gordonii UB1360	*sspAB*::*aad9* ($Spec^R$)	(Heddle *et al.*, 2003)
S. gordonii NU04 (Δ*rapA*)	*rapA*::*ermAM* (Erm^R) in *S. gordonii* DL1 background	This study
S. gordonii NU12 (Δ*sgc*)	*sgc*::*aphA3* (Kan^R) in *S. gordonii* DL1 background	This study
S. gordonii NU38 (Sgc_{Comp})	NU12 strain with plasmid $pSgc_{Comp}$ (Erm^R)	This study
S. gordonii PK3349	*argR*::*aphA3* *ahrC*::*ermAM* (Kan^R Erm^R)	(Jakubovics *et al.*, 2015)
S. gordonii PK3351	*arcR*::*ermAM* (Erm^R)	(Jakubovics *et al.*, 2015)

[a]Abbreviations: $Spec^R$, spectinomycin resistant; Erm^R, erythromycin resistant; Kan^R, kanamycin resistant.

Table 2. PCR primers used in this study.

Target gene	Forward /reverse	Primer Sequence	Product Length (bp)	Source or Reference
16S rRNA	F	AGACACGGCCCAGACTCCTAC	138	(Jakubovics *et al.*, 2008)
	R	TCACACCCGTTCTTCTCTTACAA		
argG	F	AAACGATCAGGTCCGTTTTG	123	(Jakubovics *et al*., 2008)
	R	GATTTCTTCCTCCCGAGACC		
argC	F	AAAGAGCCTGCTGAAGACCA	118	(Jakubovics *et al*., 2008)
	R	AGGGAATCAAGGCCAACTCT		
amyB	F	GACAGCGAAAACGGAAACTATGAC	109	(Jakubovics *et al*., 2008)
	R	CCAATCGGAAGCCCTGTAT		
aphA3	F	AAAATATTGAGGAGTTTTGTatgattgaacaagatggattgcacgc	795	This Study
	R	AGTAAGCTTGAAACTTtcagaagaactcgtcaagaaggcg		
adhP	F	GACCAAGGATACGGCCTGGG	521	This Study
	R	caatccatcttgttcaatcatACAAAACTCCTCAATATTTTTTAAATCATATTATAATAAAAATTGTCT		
glyQ	F	tgacgagttcttctgaAAGTTTCAAGCTTACTTTAGAATTAGAATCCCTTAGG	516	This Study

	R	AATAGCTCGCAGGAAGGTATAAGGACT A		
rapA1	F	GCTTTGCCCAAATCAAGAAC	1,731	This Study
	R	GTTCATGTAATCACTCCTTTTCTTCATAGT CTATCTC		
rapA2	F	ACGGGAGGAAATAATTCAAGAACAAACCG AACGCGAC	2,590	This Study
	R	TTCCCCCAAGTGCCTACCTT		
ermAM	F	CTTAGGAATTCAAGTTAAATTAAATGCTA	738	This Study
	R	TTCCGAATTCACAAAAGCGACTCATAG		

FIGURE LEGENDS

Figure 1. Visualization of coaggregation between *S. gordonii* and *A. oris*. Cells were pre-labelled with Pico-green (*S. gordonii*, green) or propidium iodide (*A. oris*, red) prior to coaggregation, and visualised by confocal laser scanning microscopy.

Figure 2. High resolution transmission electron microscopy analysis of coaggregation between *S. gordonii* and *A. oris*. The enlargement (right panel) shows streptococcal cells (cocci) in close contact with *A. oris* (rod-shaped).

Figure 3. Extracellular protease activity in *S. gordonii* culture supernatants, measured using the azocasein assay. Bars represent means from three independent experiments and standard errors are shown. Protease activity was significantly lower in *S. gordonii* Δ*sgc* than either the wild-type or the genetically complemented strain (Sgc_{Comp}), $P < 0.001$, double asterisk.

Figure 4. Changes in *S. gordonii* gene expression during growth in CDM (0.5 mM arginine). Cells were grown in monoculture (closed circles), coculture (open triangles) or culture as coaggregates with *A. oris* (closed squares), and the expression of *amyB* (control) and arginine biosynthesis genes *argC* and *argG* was measured by RT-qPCR. Fold change was normalised to 16S rRNA gene expression, and relative fold changes compared with *S. gordonii* wild-type at 1 h are shown as mean ± SD of three independent experiments.

Figure 5. Effects of complementing the *sgc* mutation on coaggregation-mediated gene regulation. Expression of *amyB, argC* and *argG* was monitored by RT-qPCR after 2 h growth in CDM as monocultures, cocultures or coaggregates with *A. oris*. Bars represent the relative change in gene expression compared with *S. gordonii* wild-type at 1 h, shown as mean and standard error from three independent experiments.

Figure 6. Impact of coaggregation with *A. oris* on the growth of *S. gordonii* wild-type (circles) or Δ*sgc* (squares) in CDM adjusted to no arginine or 0.5 mM arginine. Growth in monoculture (open symbols) or following inoculation as coaggregates (closed symbols) is shown. *A. oris* monocultures are shown as open triangles. All values represent means from two to three independent repeats, which all gave similar patterns of growth.

Figure 7. Effects of disrupting ArgR family regulators on *S. gordonii* growth in monoculture (open symbols) or following coaggregation with *A. oris* (closed symbols). Growth of *S. gordonii* wild-type (circles), Δ*arcR* (diamonds) or Δ*argRahrC* (triangles) is plotted as means from three independent repeats.

Figure 1

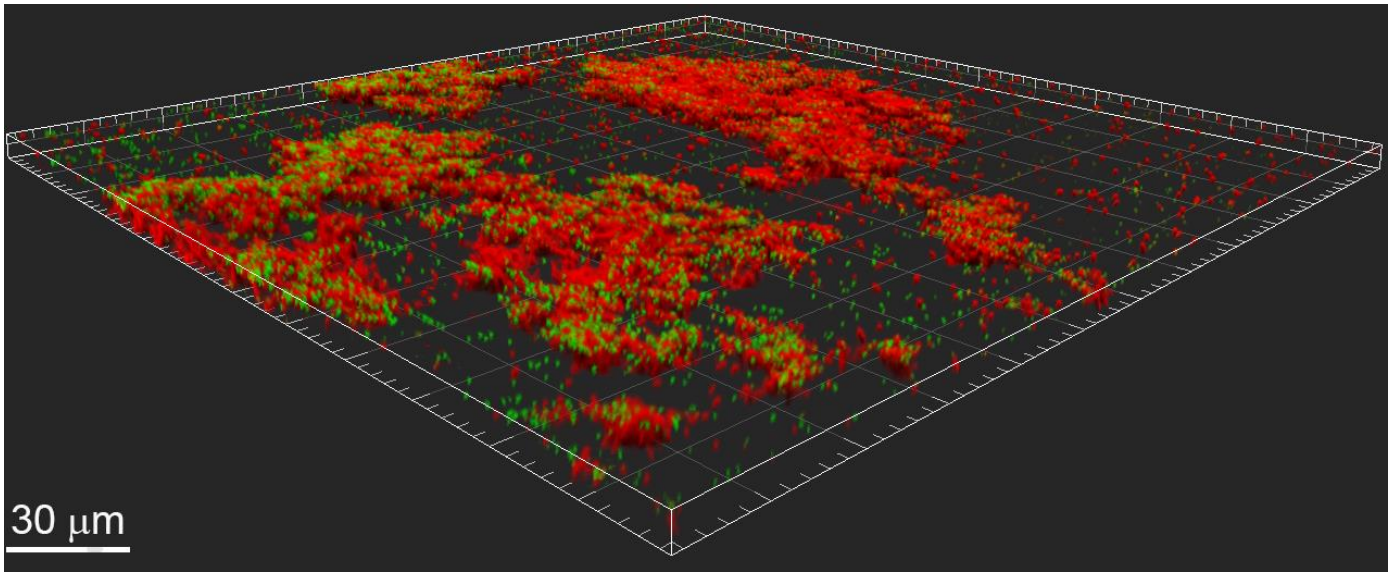


Figure 2

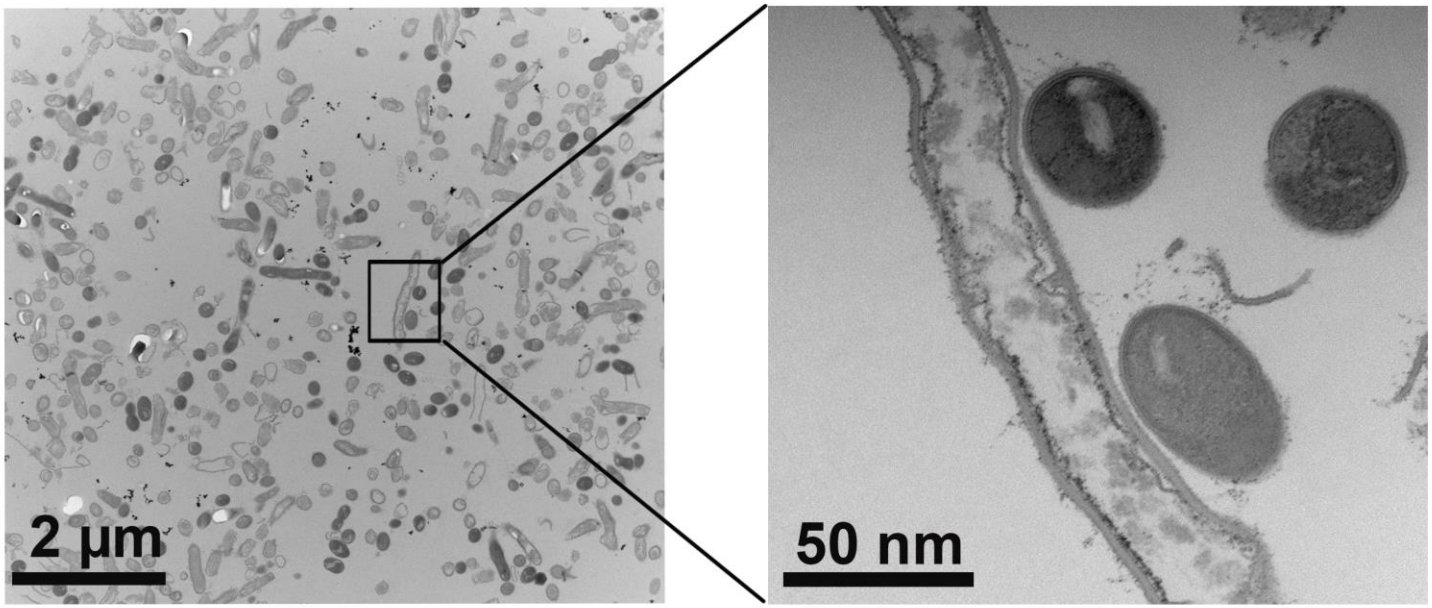


Figure 3

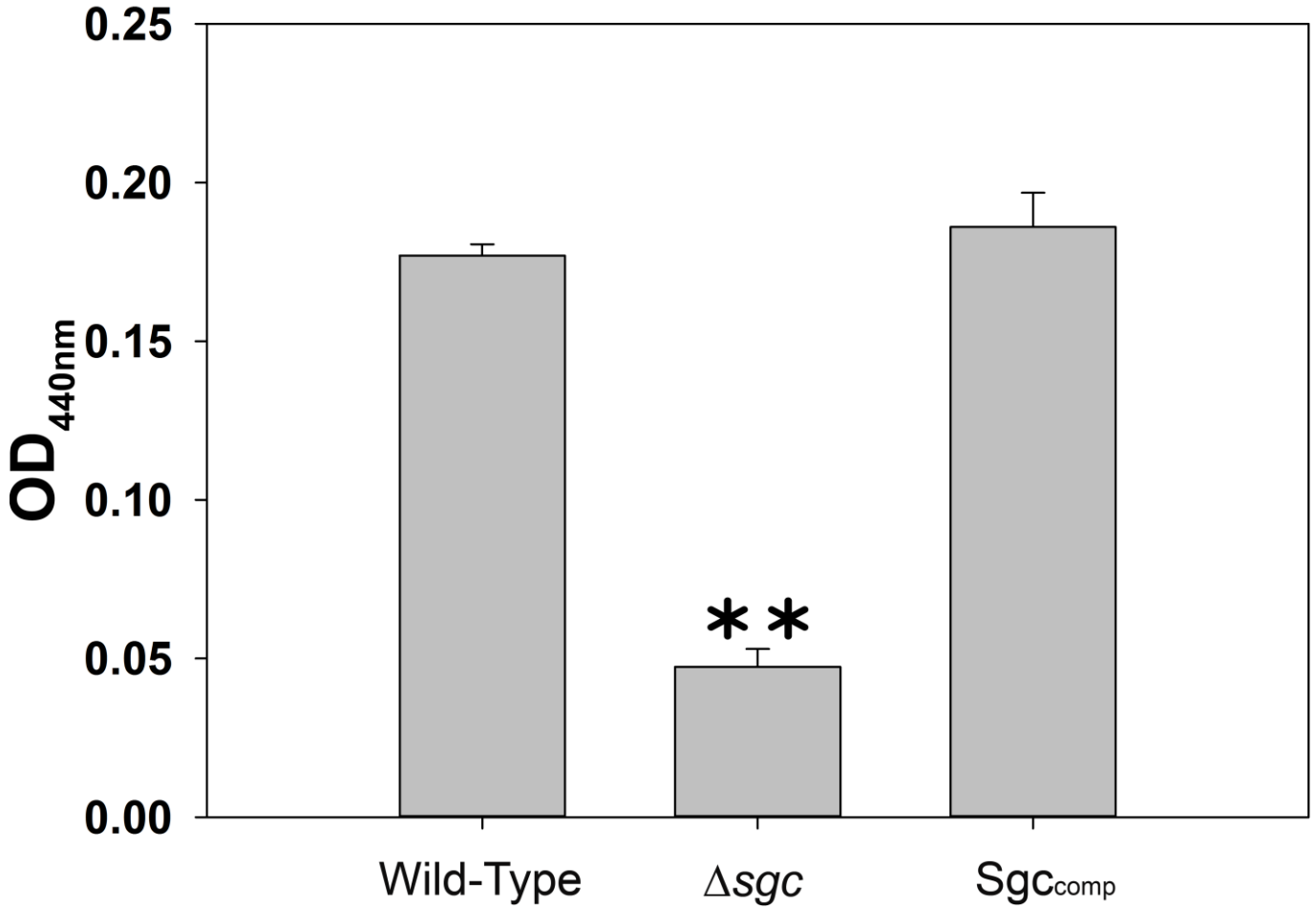


Figure 4

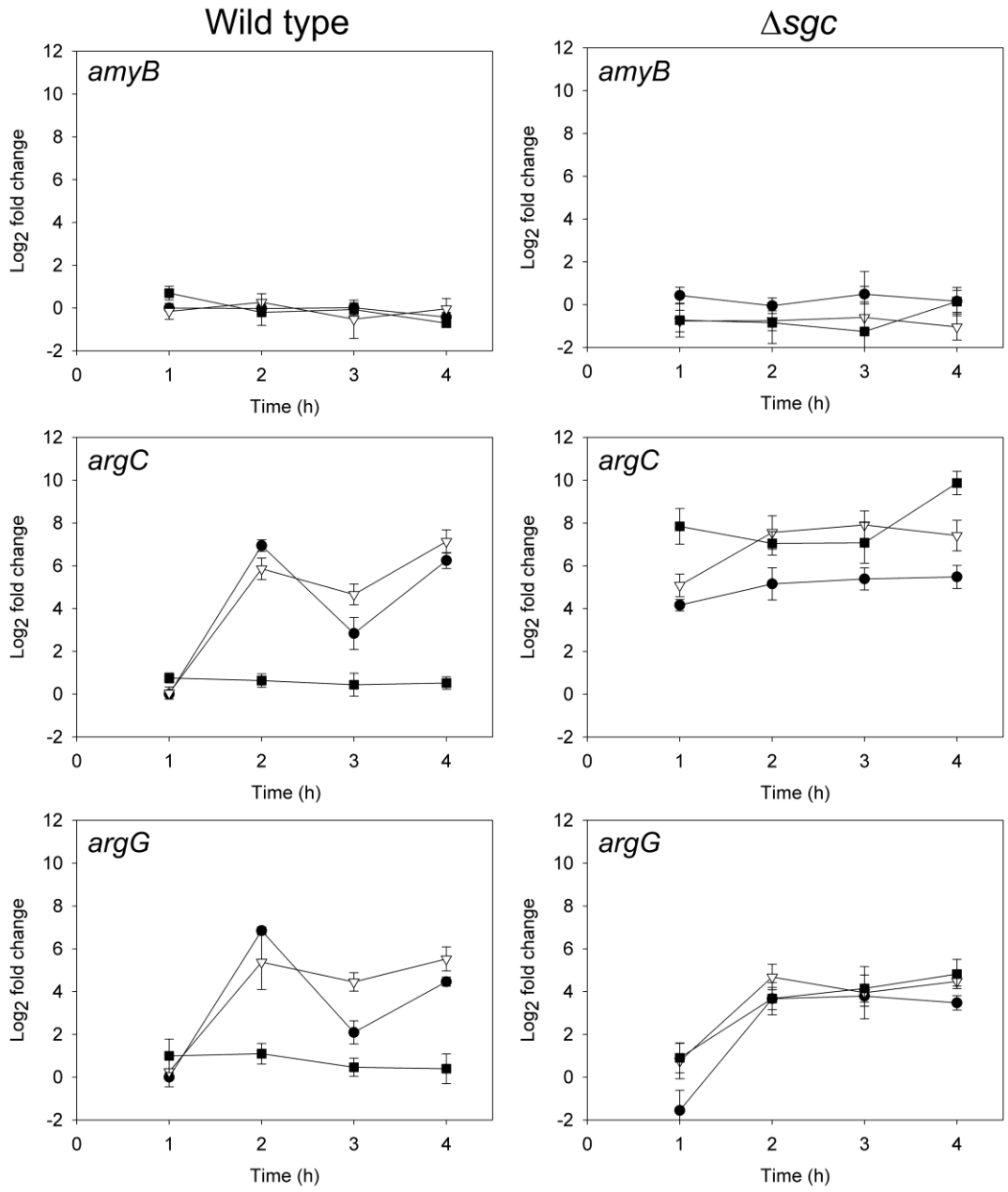


Figure 5

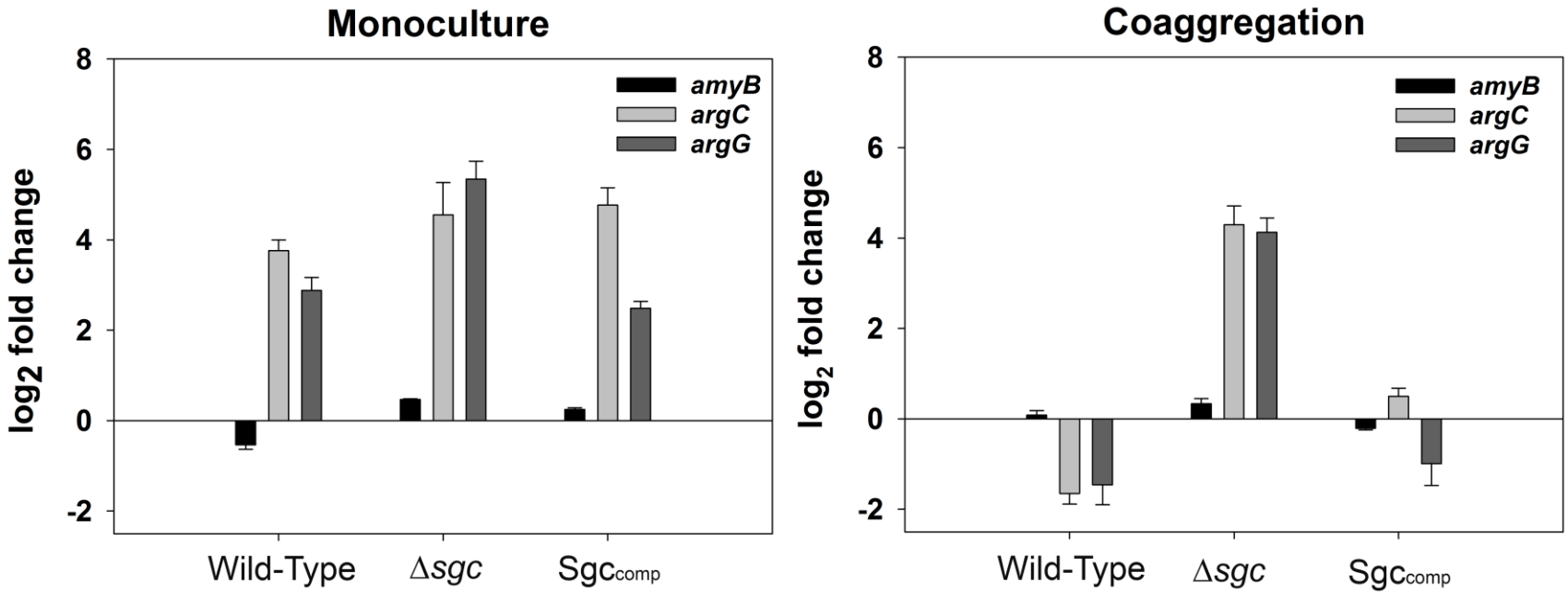

Monoculture
Coaggregation
log2 fold change
log2 fold change
amyB
argC
argG
8
6
4
2
0
-2
Wild-Type
Δsgc
Sgccomp

Figure 6

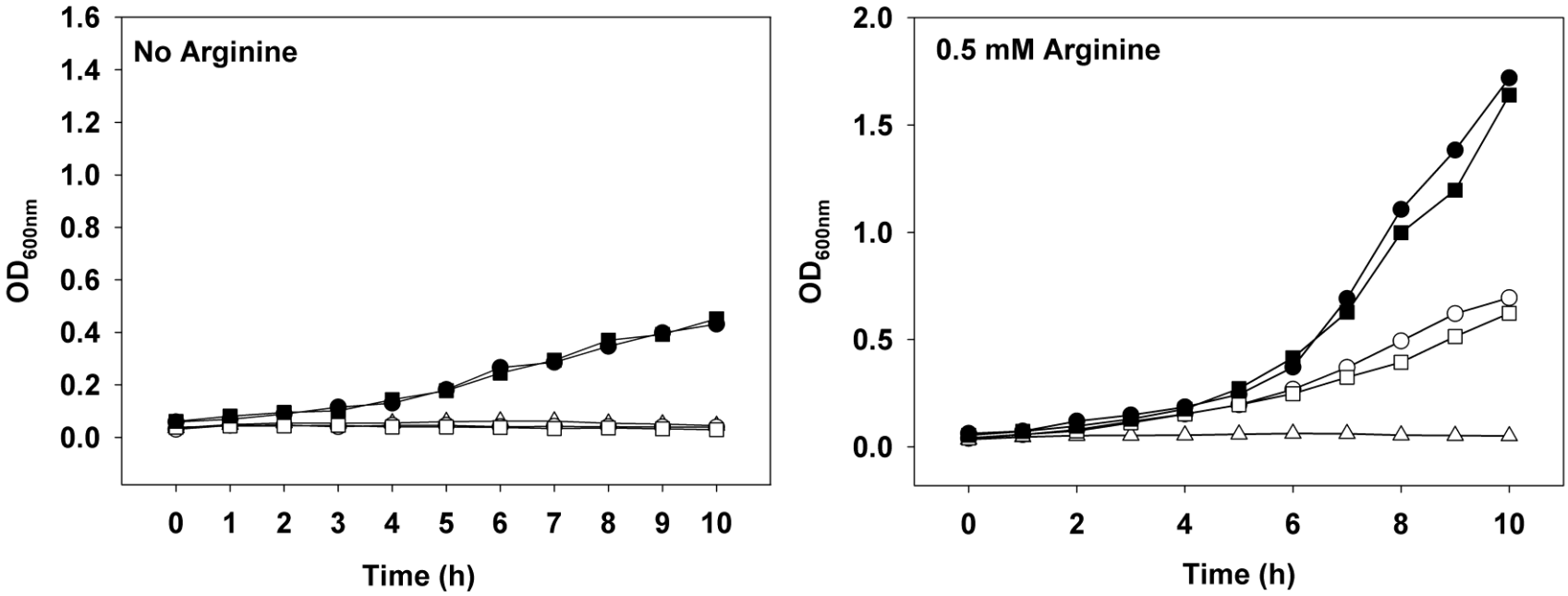
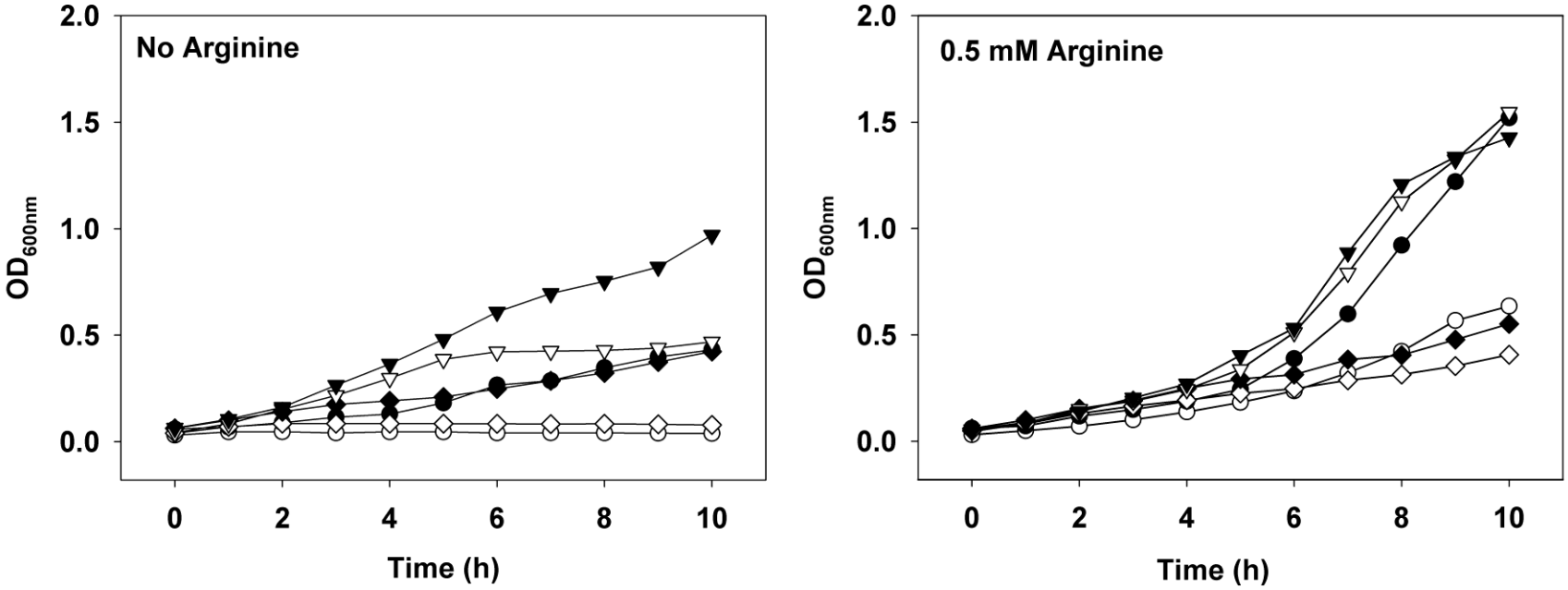

No Arginine
0.5 mM Arginine
OD600nm
Time (h)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
2.0
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8 9 10
0 2 4 6 8 10

Figure 7


No Arginine
0.5 mM Arginine
OD600nm
Time (h)
2.0
1.5
1.0
0.5
0.0
0
2
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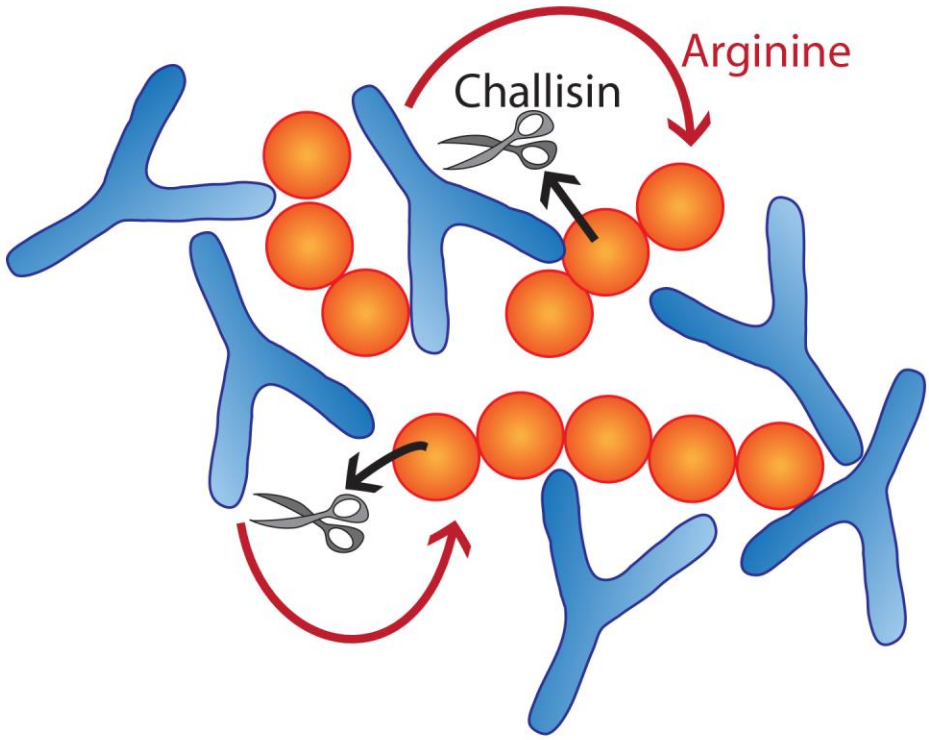
Graphical Abstract



Supplementary Material: *Streptococcus gordonii* Challisin protease is required for sensing cell-cell contact with *Actinomyces oris*

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SUPPLEMENTAL METHODS

Analysis of *A. oris* cell surface proteins following treatment with *S. gordonii* culture supernatants

S. gordonii DL1 was cultured for 18 h at 37°C in 20 ml THYE medium. Cells were harvested and supernatants were collected. Challisin activity in the supernatants was determined by the azocasein assay (see Materials & Methods). To assess the effects of Challisin-containing supernatants on *A. oris* cell surface proteins, *A. oris* MG1 was cultured for 18 h in 20 ml THYE at 37°C. Cells were harvested, resuspended in 10 ml of *S. gordonii* culture supernatants and incubated at 37°C. After 1 h, 6 h or 24 h, cells were harvested by centrifugation and cell surface proteins were extracted as described in Materials & Methods. Protein concentrations were determined using the Pierce Coomassie (Bradford) Protein Assay Kit (Thermo Fisher) with bovine serum albumin as standard. Proteins (10 µg per lane) were separated on a 12% polyacrylamide gel (Laemmli 1970) with an Unstained Protein Standard, Broad Range 10-200 kDa (New England Biolabs, Ipswich, MA, USA).

REFERENCE

Laemmli UK. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* 1970;**227**: 680.

FIGURE S1

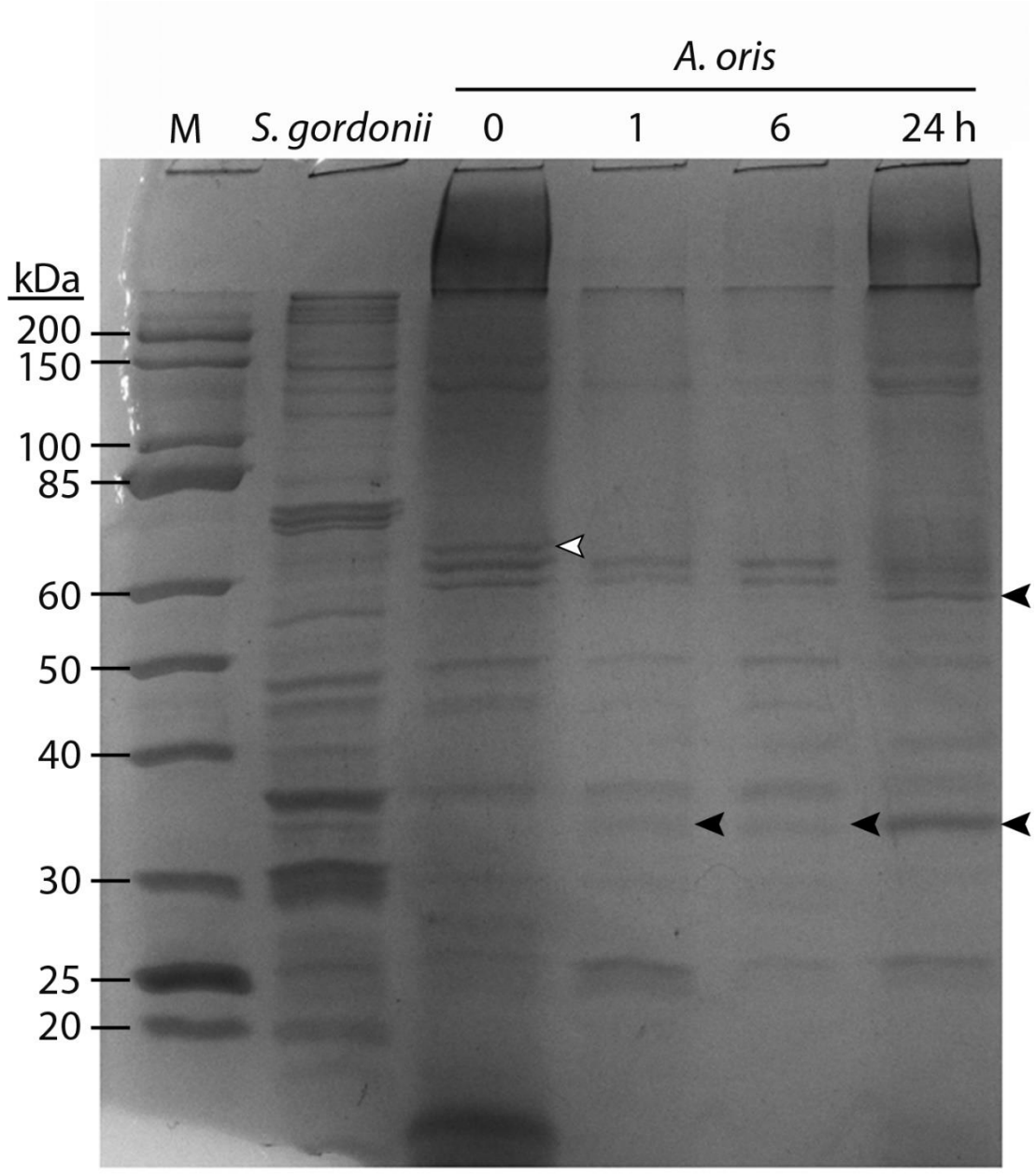

Figure S1. Effects of *S. gordonii* extracellular supernatant on *A. oris* cell surface proteins. *A. oris* MG1 cells were treated with Challisin-containing supernatant from *S. gordonii* DL1 for up to 24 h. Cell surface proteins were extracted from *A. oris* and separated on a 12% polyacrylamide gel. A band at approximately 65 kDa was seen before treatment (white arrowhead) that was not visible following treatment. Lower molecular weight bands at ~58 kDa and ~34 kDa were visible in samples treated with *S. gordonii* supernatants, and not in lanes without treatment. M = Molecular weight markers.