The Major Autolysin of *Streptococcus gordonii* is Subject to Complex Regulation and Modulates Stress Tolerance, Biofilm Formation and eDNA Release

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ABSTRACT

A gene, designated atlS, encoding a major autolysin from Streptococcus gordonii, was identified and characterized. The predicted AtlS protein is 1160 amino acids, 127 kDa and has a conserved β1, 4-N-acetylmuramidase domain. Zymographic analysis of wild-type S. gordonii revealed peptidoglycan hydrolase activities with molecular masses of 130 and 90 kDa that were absent in an atlS deletion mutant. Western blotting revealed that the 90-kDa band was derived from the 130-kDa protein. Inactivation of atlS resulted in formation of long chains by the cells, markedly decreased autolytic capacity, poor biofilm formation, diminished tolerance of acid and oxidative stress, and decreased production of extracellular DNA (eDNA). The biofilm-forming capacity of the atlS mutant could be almost completely restored to that of the wild-type strain by adding purified recombinant AtlA autolysin of S. mutans, but was only partially restored by addition of eDNA. Autolysis, eDNA release and atlS expression increased sharply when cells entered stationary phase and were greatly enhanced in cells growing with aeration. The LytST and VicRK two-component systems were both required for the induction of atlS by aeration, and purified LytT was able to bind to the promoter region of atlS in vitro. Thus, AtlS and its associated regulatory cascade dominantly control phenotypes of S. gordonii that are critical to colonization, persistence, and competition with other commensal and pathogenic oral bacteria in response to the redox environment and growth domain.
INTRODUCTION

Autolysis, which is triggered by a self-digestion of the cell wall by peptidoglycan hydrolases, can be a mechanism for programmed cell death in bacteria, but also plays critical roles in cell wall turnover, cell separation, antibiotic resistance, adherence, genetic competence and protein secretion (10, 23, 25, 41, 61). In recent years, autolysis has been shown to play important roles in biofilm development and dispersal (4, 8, 16, 54), although the underlying mechanisms are not fully understood. One of the benefits of autolysis to biofilm formation and persistence is believed to be the removal of old or damaged cells in a way that can promote the survival of the population during stresses (53, 68). In addition, extracellular DNA (eDNA) released by autolysis could promote intercellular adherence and thus stabilize biofilms (6, 17, 49, 51, 62, 70, 72).

The importance of autolysins of oral streptococci has been highlighted in recent years. Initial studies using functional genomic analyses demonstrated that the AtlA protein of S. mutans was crucial for biofilm development (3, 4, 11, 59). A role for this protein in a variety of virulence-related phenotypes, including surface protein biogenesis, was also demonstrated (3, 4, 11, 59). Subsequently, characterization of apparent atlA homologues in Streptococcus sobrinus (73) and Streptococcus downei (67) provided further evidence of the importance of these proteins to cellular physiology, homeostasis, and properties related to colonization and persistence. More recently, Ahn and co-workers showed that a complex post-transcriptional network affected by oxygen and glucose concentration modulates autolysin gene expression, as well as localization, maturation and activity of AtlA of S. mutans (5).
Streptococcus gordonii, an early colonizer of the oral cavity of infants and of the teeth, can comprise a substantial proportion of the biofilms on healthy dental surfaces (1, 9, 47, 65). Colonization by *S. gordonii* is believed to be beneficial to the host because of its contribution to pH homeostasis in oral biofilms through the hydrolysis of arginine in saliva and the diet (13, 44). *S. gordonii* also promotes biofilm development and diversity, and is believed to enhance the colonization and growth of some oral commensals through metabolic interdependence, surface protein interactions and quorum sensing (21, 29, 45, 46, 66). Another important function of *S. gordonii* in oral biofilms is associated with its demonstrated abilities to antagonize the growth of the caries pathogen *Streptococcus mutans* (26, 31, 71).

The relatively recent discovery of the importance of eDNA release by bacteria in biofilm formation and stability (6, 17, 49, 51, 62, 70, 72) has stimulated interest in the mechanisms regulating externalization of DNA. The release of eDNA is typically a consequence of cell lysis, as reported for *Streptococcus pneumoniae*, in which a subpopulation of cells undergoes lysis during competence development (43, 63). In contrast, eDNA release from *S. gordonii* was reported to be inducible by exogenous H$_2$O$_2$, but independent of cell lysis (30). By computer analysis of the genome of *S. gordonii* strain Challis at [http://www.oralgen.lanl.gov/](http://www.oralgen.lanl.gov/), we identified a gene, SGO.2013, that encodes a protein with 32% identity to the AtlA autolysin of *S. mutans*. In this report, we present data on the characterization and regulation of SGO.2013 and the involvement of its gene product in eDNA release, biofilm formation and stress tolerance.
MATERIALS AND METHODS

**Bacterial strains, growth conditions and reagents.** *Escherichia coli* DH10B was grown in Luria broth and *S. gordonii* DL1 and its derivatives were cultured in brain heart infusion (BHI) broth (Difco). For selection of antibiotic-resistant colonies after genetic transformation, ampicillin (100 µg ml\(^{-1}\) for *E. coli*), erythromycin (300 µg ml\(^{-1}\) for *E. coli* or 10 µg ml\(^{-1}\) for *S. gordonii*), or kanamycin (50 µg ml\(^{-1}\)) for *E. coli* or 1 mg ml\(^{-1}\) for *S. gordonii*) were added to the media. For biofilm formation assays, *S. gordonii* strains were grown in the 1/4 strength BHI medium (BHI medium diluted 1:4 with dH\(_2\)O) supplemented with 10 mM sucrose. Chemical reagents and antibiotics were obtained from Sigma (St. Louis, MO).

**Construction of mutant strains.** Strains used in this study are listed in Table 1 and primers used for deletion mutagenesis are listed in Table 2. To construct a reporter gene fusion for measuring transcription from the *atlS* promoter (SGO.2013), a 624-bp fragment immediately 5' to the start codon of *atlS* was amplified by PCR with primers P\(_{SG2013-5'}\) and P\(_{SG2013-3'}\) (3). To construct a reporter gene fusion for measuring transcription from the *lytT* gene promoter, a 300-bp fragment immediately 5' to the start codon of *lytT* was amplified by PCR with primers P\(_{lytT-5'}\) and P\(_{lytT-3'}\) (3) that included *Bam*HI recognition sequences. The products harboring P\(_{SG2013}\) and P\(_{lytT}\) were fused with the promoterless *cat* gene derived from pC194 (14, 27). After confirming the correct sequence of the promoter fusion, the P\(_{SG2013-cat}\) and P\(_{lytT-cat}\) constructs were cloned onto plasmid pYQ1 (20), which allows for stable integration of the gene fusion into the *gtfG* gene of *S. gordonii*. The constructs were transformed into competent *S. gordonii* DL1
to construct strains SgWT and SgWT2, which carry the $P_{SG2013}$-cat and $P_{lytT}$-cat gene fusions, respectively (3). All strains were then verified by PCR followed by DNA sequence analysis.

To make deletions of the genes of interest, 5′- and 3′-flanking regions of each gene were amplified from chromosomal DNA from *S. gordonii* DL1, ligated together using *Bam*HI sites designed into each primer set, and cloned into the pGEM-T Easy vector (Promega, Madison, WI). These plasmids were digested with *Bam*HI and a non-polar kanamycin (NPKm) cassette from pALH124 (3), which lacks its own promoter, was inserted (3). The desired mutagenic plasmids were identified by PCR amplification using vector-originated M13 primers. The plasmids were isolated and used to transform competent SgWT or SgWT2, selecting on BHI agar containing Km. In all cases, double-crossover mutants of each gene were confirmed by PCR and DNA sequencing, including sequencing the flanking regions to insure that no undesired mutations were inadvertently introduced. To construct *lyt* and *vic* complemented strains, DNA fragments containing *lytST* and *vicRK* along with their promoter regions were amplified with primers (Table 1) and inserted into the *E. coli-Streptococcus* shuttle vector pDL278 (32), to yield plasmids pDL-lytST and pDL-vicRK, respectively. These recombinant plasmids were introduced into strains carrying $P_{atlS}$-cat gene fusions, including the wild-type background and strains lacking LytST or VicRK. All strains were then verified by PCR followed by DNA sequence analysis.

**Growth kinetics.** Growth of strains of *S. gordonii* in BHI (pH 7.0) or BHI that was acidified to pH 5.5 with HCl (BHI/HCl), under aerobic or anaerobic conditions (4) was
monitored using a Bioscreen C (Growth Curves USA, NJ) with multi-well disposable microtiter plates. An aliquot (3 µl) from an overnight culture was inoculated in at least triplicate into wells containing 300 µl of BHI or BHI/HCl. All inocula were adjusted to the same OD<sub>600</sub> before dilution. To assess the ability of cells to grow in the presence of oxidative stressors, cells from overnight cultures were transferred to pre-warmed BHI and grown at 37°C in a 5% CO<sub>2</sub> aerobic atmosphere to OD<sub>600</sub> = 0.5. The cells were then diluted into fresh BHI containing 0.003% H<sub>2</sub>O<sub>2</sub> (Fisher Scientific) or 25 mM paraquat (methyl viologen; Sigma) and the impact of the agents on bacterial growth was monitored in a Bioscreen C at 37°C under aerobic conditions or with an overlay of two drops of sterile mineral oil to create relatively anaerobic conditions.

**Autolysis, biofilm, zymographic, CAT and eDNA assays.** Autolysis and the crystal violet/microtiter biofilm assays were performed as previously described (4). A zymographic analysis of cell wall-associated murein hydrolases was conducted as described by Qoronfleh and Wilkinson (52). To collect extracellular murein hydrolases of mutants of *S. gordonii*, cells were cultured in 50 ml BHI medium and collected at OD<sub>600</sub> = 0.9. Cell pellets were resuspended in 500 µl of 4% SDS and incubated for 60 min at room temperature with agitation, followed by centrifugation at 13,000 × g for 5 min. The supernatant fluids were collected and combined with an equal volume of 50 mM Tris (pH 6.5), 10% Glycerol. To prepare substrates for the zymogram, an overnight culture (800 ml) of *S. gordonii* DL1 grown in BHI medium was collected and the pellet was washed for 4 times with distilled H<sub>2</sub>O. The pellet was suspended in 60 ml of 4% SDS and boiled for 30 min. The heat-killed cells were washed with five times in dH<sub>2</sub>O.
and the pellet was saved. The pellet was resuspended in dH₂O and the suspension was added to a polyacrylamide gel at a final concentration of 1% (v/v) (48), prior to casting and polymerization. CAT activity was measured by the spectrophotometric method of Shaw (58) as previously described (37). eDNA was collected and quantified as described by Kreth et al. (30).

Cloning, expression and purification of recombinant proteins. Recombinant plasmids expressing LytT lacking its predicted signal sequence was constructed by PCR cloning of the relevant DNA fragments into the vector pMAL-p2X (New England Biolabs) using primers listed in Table 1. The coding sequence for LytT was cloned in-frame behind the malE gene to create a maltose binding protein (MBP) fusion protein. The proteins were overproduced in E. coli DH10B by induction with isopropyl-β-D-thiogalactopyranoside (IPTG) and purified as soluble proteins on affinity columns as recommended by the supplier (Fisher Scientific).

Western blot assays. Exponentially-growing cells (OD₆₀₀ = 0.5 - 0.6) were centrifuged and washed twice with Tris-buffered saline (10 mM Tris, 0.9% NaCl, pH 7.4). Whole-cell lysates were obtained by homogenization in SDS boiling buffer (60 mM Tris-HCl, pH 6.8, 10% glycerol, 5% SDS) in the presence of glass beads, followed by centrifugation at 2000 × g for 10 min (15). Protein samples were separated by SDS-PAGE, blotted onto PVDF membranes, and incubated with affinity-purified (33) AtlA-antibody (3) followed by peroxidase-conjugated goat anti-rabbit IgG (KPL, Gaithersburg, MD). Signals were disclosed using the SuperSignal West Pico chemiluminescent kit (Thermo, Waltham, MA).
Electrophoretic Mobility Shift Assay (EMSA). EMSA was carried out following a previously published protocol (69). Briefly, a DNA fragment containing the predicted promoter region of atlS was amplified by PCR with biotin-labeled primers.

Approximately 10 fmol of biotin-labeled probe was used in combination with different concentrations of purified recombinant-LytT protein in a 10 µl reaction mixture containing 10 mM HEPES (pH 7.9), 50 mM KCl, 10 mM EDTA, 5 mM MgCl₂, and 2 µg poly (dI-dC). After incubation at room temperature for 1 h, the DNA-protein samples were resolved in a non-denaturing, low-ionic-strength polyacrylamide gel and blotted onto hybridization transfer membranes (Perkin-Elmer). Signals were developed using the Chemilluminescent Nucleic Acid Detection Module (Thermo Scientific).

RESULTS

Characterization of SGO2013 (AtlS) activity. To determine whether SGO.2013 encoded an autolysin, the autolytic activity of a SGO.2013-deficient mutant was compared with that of the wild-type strain. The mutant strain exhibited a markedly lower autolytic rate and extent of autolysis than the parental strain (Fig. 1). The autolysin profiles of the mutant and wild-type strains were also confirmed by zymographic analysis (Fig. 2A). Two major cell wall hydrolase bands with apparent masses of 130 and 90 kDa were present in whole-cell extracts from the wild-type strain (Fig. 2A), but neither band was detected in the SGO.2013 mutant (Fig. 2A). Western blotting using antibodies that were raised against a purified, recombinant S. mutans AtlA autolysin (3), which were affinity purified using a 6-His-tagged AtlA protein (3), also disclosed proteins of 130 and...
90 kDa (Fig. 2B). Thus, similar to atlA of S. mutans (Fig. 2B), SGO.2013 encodes a

gene product, heretofore designated AtlS, which is present as a higher molecular mass

species and as a lower molecular weight protein that likely arises from proteolysis of full

length AtlS.

AtlS affects stress tolerance by S. gordonii. The atlS mutant strain formed longer

chains of cells compared to the wild-type strain and clumped extensively in broth culture

(data not shown). To examine whether AtlS of S. gordonii affected acid tolerance,

SgWT and the atlS mutant were cultured in BHI (pH 7.0) or BHI/HCl broth (pH 5.5). In

pH 7.0 medium, the growth curve of the atlS mutant was similar to that of SgWT (Fig.

3A) and the atlS mutant achieved modestly higher final optical densities, probably due to

decreased autolysis of the mutant. In medium that had been adjusted to pH 5.5, the wild-
type strain grew more slowly and achieved substantially lower final optical densities than

were achieved at pH 7.0. In contrast to the parental strain, the atlS mutant was not able

to grow in BHI/HCl broth (Fig. 3A).

To examine whether AtlS of S. gordonii was required for oxidative stress tolerance,

the SgWT and atlS mutant strains were cultured in BHI medium with 0.003% hydrogen

peroxide or 25 mM paraquat under aerobic conditions, or with a mineral oil overlay to

create a more anaerobic environment (4). In the presence of hydrogen peroxide under

aerobic conditions, the SgWT strain displayed a slightly extended lag phase, modestly

slower growth rate and attained a maximum optical density similar to when cultured in

anaerobic conditions (Fig. 3B). However, lysis of the cells was much more rapid and

extensive in cells grown in aerobic conditions than those growing under anaerobic
conditions (Fig. 3B). The *atlS* mutant consistently displayed a longer lag phase, slower growth and achieved lower final optical densities in the presence of hydrogen peroxide under anaerobic and aerobic conditions (Fig. 3B) than SgWT, but no obvious lysis was detected in the *atlS* mutant. Similarly, in the presence of 25 mM paraquat, the *atlS* mutant grew more slowly and achieved lower final optical densities than the SgWT strain (Fig. 3C). Collectively, the results indicate that a compromised ability to cope with oxidative stress is associated with *atlS* deficiency and provide more evidence that the cells lacking *AtlS* are hyper-resistant to autolysis.

**AtlS-dependent eDNA release.** DNA release is typically a consequence of cell lysis. To examine the characteristics of extracellular DNA (eDNA) release by *S. gordonii*, the wild-type strain was cultured in BHI medium under aerobic and anaerobic conditions, and the cells were collected at different growth stages. The eDNA produced by a 1 ml culture was extracted and analyzed in a 0.8% agarose gel or quantified by real-time PCR with 16S rRNA primers (Table 2). When cells were cultured under aerobic conditions, eDNA released by the wild-type strain increased gradually from the early-exponential phase (OD$_{600}$=0.2–0.3) to stationary phase (OD$_{600}$= 0.9–1.0), with DNA bands characteristic of chromosomal DNA clearly visible (Fig. 4A). Notably, no eDNA production from anaerobically-cultured cells was detectable by gel electrophoresis (Fig. 4). Consistent with these observations, quantitative real-time PCR revealed that aerobic cultures that had reached stationary phase produced 100-fold more eDNA than anaerobically-grown cells in the same growth phase (Fig. 4B), indicating that oxygen or one of its metabolites plays an important role in triggering DNA release. In contrast to
the wild-type strain, very low levels of eDNA were detected in the \textit{atlS} mutant in all growth phases under aerobic or anaerobic conditions, providing strong evidence that AtlS and cell lysis contribute in major ways to eDNA production by \textit{S. gordonii}.

\textbf{Regulation of \textit{atlS} expression.} The SgWT strain, which carries a \textit{cat} gene fused to the \textit{atlS} promoter, was grown in TY medium containing 25 mM galactose under aerobic or anaerobic conditions. CAT activity arising from P_{\textit{atlS}} expression was examined at different growth phases. From the early-exponential phase (OD_{600}=0.2\sim0.3) to mid-exponential phase (OD_{600}=0.5\sim0.6), \textit{atlS} expression of cells was at a basal level, under both aerobic and anaerobic conditions (Fig. 6A). However, P_{\textit{atlS}} expression was increased more than 10-fold after cells entered stationary phase (OD_{600}= 0.9\sim1.0), with aerobically-grown cells showing 2.5-fold higher \textit{atlS} expression than anaerobically-grown cells in stationary phase (Fig. 6A). Thus, \textit{atlS} expression is both growth phase- and oxygen-dependent in \textit{S. gordonii}.

Previous studies demonstrated that the LytST and VicRK two-component systems (TCS) were involved in regulating autolysis in some Gram-positive bacteria, including \textit{S. pneumoniae}, \textit{S. aureus}, and \textit{S. mutans} (4, 24, 50, 57). To determine whether these signal transduction systems also affected autolysis in \textit{S. gordonii}, the entire \textit{lytST} or \textit{vicRK} operon were replaced by a non-polar kanamycin cassette (10). To evaluate the function of individual components of the TCS, the \textit{lytS}, \textit{lytT}, \textit{vicR} and \textit{vicK} genes were disrupted individually by non-polar insertions (Table 1). All \textit{vic} and \textit{lyt} mutants formed long chains and clumped when growing in BHI (data not shown). With the exception of the strain carrying only the \textit{vicK} mutation, SgvicK, all \textit{vic} and \textit{lyt} mutants were substantially
more resistant to autolysis than the SgWT strain (Fig. 5). When the vicRK and lytST mutants were complemented with plasmid-borne copies of these genes, growth curves similar to the wild-type genetic background were observed (Fig. 5).

To assess whether LytST and VicRK influenced autolysis of S. gordonii by regulating the expression of atlS, cells were grown to stationary phase in TY medium containing 25 mM galactose under aerobic or anaerobic conditions and CAT activities were measured. In the SgWT background, cells expressed 2.5-fold higher CAT activity from P_atlS under aerobic conditions than anaerobic conditions (Fig. 6B), whereas the lyt mutants displayed a basal level of P_atlS expression and no induction in aerobic conditions was evident (Fig. 6). A phenotype similar to the lyt mutants was observed in the SgvicR and SgvicRK mutants, whereas about a two-fold induction of P_atlS by oxygen still could be detected in the SgvicK mutant (Fig. 6B). The histidine kinase (LytS) and response regulators (LytT and VicR) were required for activation of the atlS gene expression by oxygen. Also of note, the complemented strains, SgvicRK-cvicRK and SglytST-clytST, and lytST or vicRK over-expressing strains (SgWT-cvicRK and SgWT-clytST) had higher expression from the atlS promoter than the wild-type or mutant strains carrying the empty vector pDL278 (Fig. 6B). Collectively, these data demonstrate that the Lyt and Vic systems influence autolysis of S. gordonii by regulating the expression of atlS in response to oxygen and possibly other factors.

Purified LytT binds to the promoter region of atlS in vitro. To explore whether the LytT response regulator could interact with the atlS promoter region, the entire lytT coding sequence was cloned onto plasmid pMAL-p2x to create a maltose binding fusion
protein to the N-terminus of LytT. Soluble LytT-MBP protein was overproduced in *E. coli* and purified by affinity chromatography. The purified protein displayed the expected apparent molecular mass of 68 kDa following SDS-PAGE (data not shown). A DNA probe was constructed by PCR amplification of 300-bp of DNA immediately 5’ to the coding sequence of *atlS* with biotin-labeled primers. In the absence of LytT-MBP, the probe migrated to the bottom of the gel (Fig. 7). When recombinant LytT was added, the migration of P$_{atlS}$ was retarded and increasing concentrations of LytT-MBP caused more P$_{atlS}$ to shift (Fig. 7). Unlabelled P$_{atlS}$ DNA could effectively compete the interaction with LytT-MBP (Fig. 7). An irrelevant MBP-tagged protein, ComE-MBP, was used in mobility shift assays in place of LytT-MBP. ComE-MBP, which was constructed by cloning the entire *comE* coding sequence from *S. mutans* onto plasmid pMAL-p2x to create a translational fusion that added maltose binding protein to the N-terminus of ComE. ComE-MBP was purified using the same protocol as for LytT-MBP. Previous experiments conducted in our lab confirmed that the ComE-MBP fusion protein was active in an EMSA with a known ComE-activated promoter (data not shown). No shift of the biotinylated P$_{atlS}$ was detected when ComE-MBP was used at same concentrations as LytT-MBP (Fig. 7), confirming that MBP was not responsible for the binding of recombinant LytT-MBP to its target DNA.

**Expression of lytST is affected by VicR.** Given the contrast in the behavior of the *vicK* mutant compared to that of the *vicR* or *vicRK* mutant strains in terms of *atlS* responsiveness to oxygen, we investigated the possibility that expression of the *vicRK* or *lytST* TCSs might be influenced in response to oxygen. Thus, the SgWT2 and the *vic*
mutant carrying the lytT promoter (P_{lytT}) fused to cat were cultured in BHI under aerobic
or anaerobic conditions. Cells were grown to OD_{600} = 0.5 ~ 0.6 and CAT activities were
measured. Around 2-fold higher expression of P_{lytT} was detected under aerobic
conditions compared to anaerobic conditions in the SgWT2 and the SgvicK2 mutant (Fig.
6C). However, both the SgvicR2 and SgvicRK2 mutants displayed a similar high level
of expression of lytT under aerobic and anaerobic conditions (Fig. 6C), suggesting a
possible role of the VicRK system in repression of lytT in response to redox.

**AtlS-dependent biofilm formation in response to oxygen.** AtlA of *S. mutans* was
originally identified by virtue of its profound effect on biofilm formation (3), so we
assessed the capacity of the SgWT and atlS mutant strains to form biofilms in 96-well
microtiter plates (Fig. 8). The strains were cultured in 200 µl of 1/4-strength BHI broth
with 10 mM sucrose for 48 h, with or without an overlay of three drops of sterile mineral
oil to create relatively anaerobic or aerobic culture conditions, respectively. SgWT
formed biofilms very efficiently, but the atlS mutant formed nearly 80% less biofilm (Fig.
8). Notably, the vic and lyt mutants were also defective in biofilm formation at a level
comparable to the atlS mutant, whereas the vic and lyt complemented strains formed
biofilms as efficiently as the wild-type strain (Fig. 8). To further confirm the
requirement for AtlS for efficient *in vitro* biofilm formation by *S. gordonii*, purified 6-
His-tagged, full-length AtlA protein from *S. mutans* (4) was added into the biofilm
culture medium at a final concentration of 2.5 µM. Similar to what was reported for
AtlA-deficient *S. mutans* (4), purified AtlA could restore biofilm formation by the atlS
mutant to near wild-type levels (Fig. 8).
The release of bacterial DNA is believed to stabilize cell-to-cell adherence and biofilm architecture (6, 17, 49, 51, 62, 70, 72). To test if eDNA release from AtlS-dependent lysis played a role in biofilm formation by *S. gordonii*, *atlS* mutants were cultured in biofilm medium with 50 ng/µl of eDNA extracted from aerobically-grown wild-type *S. gordonii* (Fig. 4). Under these conditions, the *atlS* mutant was able to form about 70% of the biofilm mass of the SgWT strain (Fig. 8), but doubling of the concentration of eDNA in the culture medium did not restore biofilm biomass to wild-type levels (data not shown). Thus, a compromised ability of the *atlS* mutant to form biofilms may be only partially attributable to decreased release of eDNA. Plasmid DNA (pDL278, 50 ng/µl) (32) was also used as the eDNA source with the *atlS* mutant and a similar level of biofilm biomass was formed as with added chromosomal DNA (Fig. 8), indicating that there is no specificity for eDNA to promote biofilm formation and that restoration of the biofilm phenotype did not require the presence of wild-type chromosomal DNA.

**DISCUSSION**

Dental caries remains a major health problem and this dieto-infectious disease has a strong ecological basis. Specifically, development of the disease is associated with increases in the proportions of acid-tolerant species, including *S. mutans* and lactobacilli, usually at the expense of beneficial commensal organisms (7, 18, 22, 60). Evidence continues to emerge that there is intense interspecies competition between oral commensals and caries pathogens. In addition to having similar nutritional requirements and thus competing for common nutrients, these organisms can antagonize the growth of
one another through metabolic pathways and production of antimicrobial peptides. The AtlA autolysin of *S. mutans* is tremendously important to cellular homeostasis and is highly regulated at the transcriptional and post-transcriptional levels by environmental factors that are relevant to oral biofilm ecology and composition, e.g. oxygen and carbohydrate availability (3, 4, 11, 59). Thus, the autolytic pathway represents a potential target for anti-caries therapies (3, 4, 11, 59). Importantly, though, an effective anti-caries therapy should not have a detrimental influence on beneficial commensal microorganisms. This report is the first description of an autolysin of the abundant oral commensal *S. gordonii* and its role in biofilm formation, stress tolerance, autolysis and eDNA production. Collectively, the data highlight similarities and some notable differences in the regulation and the roles in cellular processes of the apparent autolysin homologues of a health-associated commensal and a known caries pathogen (Summarized in part in Table 3).

Studies with various Gram-positive bacteria, including *S. pneumoniae*, *S. aureus* and *S. mutans*, demonstrate that induction of autolysis may be a response by cells to environmental stresses, often involving quorum sensing, that could enhance the survival of the population as a whole (53, 68). For example, LytA-mediated autolysis of *S. pneumoniae* can be triggered by competence development (24), acid stress (50) or antibiotics (34). In *S. aureus*, metabolism of carbohydrates regulates *cid-* and *lrg-* dependent autolysis, with both glucose and acetate affecting autolytic behavior (56). Regulation of the gene for, and the activity of, the AtlA autolysin of *S. mutans* (59), which dominantly controls cell separation, biofilm formation and autolysis (11, 3, 59), is very complex. For example, the *atlA* gene is in an operon with at least four additional
genes and transcription of\textit{atlA} is influenced by at least three promoters (11, 3, 59). Aeration is a major trigger for\textit{AtlA}-dependent autolysis in\textit{S. mutans} and the response to aerobiosis is controlled by the VicRK two-component system (4). While regulation of autolysis by\textit{S. gordonii} has some properties in common with these organisms, important differences exist.

The cross-reaction of anti-\textit{AtlA} antibody with the\textit{AtlS} protein by Western blot analysis (Fig. 2B) confirmed the prediction from computer algorithms that\textit{AtlS} and\textit{AtlA} share substantial amino acid sequence identity (32\%) and that these autolysins are produced as two active isoforms, presumably as a result of proteolytic cleavage of the full-length, secreted proteins (3). Also, as shown,\textit{AtlA} could function to restore biofilm formation by an\textit{atlS} mutant of\textit{S. gordonii}. In spite of these sequence and functional similarities, there are some important differences in the contribution of these enzymes to cellular physiology and homeostasis. For example, loss of\textit{AtlA} had a profoundly negative impact on the development of genetic competence in\textit{S. mutans} (4), whereas inactivation of\textit{atlS} did not adversely affect the transformation efficiency of\textit{S. gordonii} (data not shown). In addition, compromised tolerance of acid stress was strongly associated with a deficiency of\textit{AtlS} in\textit{S. gordonii} (Fig. 3A), but strains of\textit{S. mutans} lacking\textit{AtlA} did not show a similar phenotype (4).

Substantial variation in transcriptional and post-transcriptional control mechanisms for\textit{atlA} and\textit{atlS} are also apparent. Transcriptional studies revealed that\textit{atlA} is part of a multi-gene operon under the control of at least three promoters (3), but\textit{AtlS} appears to be encoded as a single cistron (Fig. 6A, 10) driven from a proximal promoter. Proper maturation of\textit{AtlA} (3, Fig. 2) of\textit{S. mutans} requires the ThmA and SMu0629 proteins.
encoded in the atlA operon (3, 4). In contrast, the genes encoding proteins with the
highest degree of similarity to ThmA and Smu0629 are not genetically linked to atlS or to
one another. Thus, basic aspects of the organization of transcription and of the
regulatory machinery for autolysin maturation differ in these two organisms.

In both S. mutans and S. gordonii, oxygen was a major trigger for AtlS- and AtlA-
dependent autolysis (4), but clear differences in the signal transduction pathways for this
activation exist. In S. mutans, the VicRK TCS affects AtlA levels and maturation by
regulating the transcription of the SMu0629 gene, which is required for efficient
maturation of AtlA (4). In contrast, the expression of atlS of S. gordonii is regulated by
oxygen primarily at the transcriptional level (Fig. 6A), and both the VicRK and LytST
TCSs were required for the induction of atlS transcription in response to oxygen (Fig.
6B). The affect of LytT on atlS transcription is apparently direct, as LytT could bind to
the promoter region of atlS in vitro (Fig. 7). In contrast, the effects of Vic appear to be
exerted through effects on the expression of the lytST operon. As evidenced by the
different behaviors of strains lacking VicK or VicR (Fig. 6C), it could be proposed that
the VicR response regulator maybe repress lytT expression in the absence of VicK kinase
activity. In this case, VicR phosphorylation by VicK in response to redox may alleviate
lytT repression, leading to induction of lytT by oxygen. However, cross-regulation of
VicR by the LytT histidine kinase or other kinases in response to oxygen or other signals
may contribute to differential expression of atlS. Collectively, then, it appears that S.
gordonii modulates atlS expression in response to the redox environment, and possibly
other signals, through a VicRK-LytST cascade, which would allow the organism to alter
envelope biogenesis, autolytic activity and eDNA release to adapt to microenvironments.
It is also notable that the Vic, Com and Cia TCS have different impacts on expression of the genes for the agmatine deiminase and arginine deiminase pathways, which are highly similar alkali-generating systems of *S. mutans* and *S. gordonii*, respectively (35, 36).

Thus, this study provides further support for the idea that ecological and physiologic pressures may have lead to evolutionary divergence in the roles of the Vic, Com and Cia TCS of these two organisms.

It is also of interest that apparent LytST homologues in *S. mutans* affected the regulation of autolysis of *S. mutans* not by influencing *atlA* expression, but by regulating the transcription of *lrgAB* and *cidAB*, which encode predicted holin:antiholin complexes (5). Similar to that of *S. pneumoniae* and *S. aureus*, the *lrgAB* operon of *S. mutans* is located adjacent to the *lytST* operon (12, 55, 57). In contrast, the *S. gordonii lrgAB* genes are distant from the *lytST* operon and we could find no evidence for transcriptional regulation of *lrg* by LytST in this organism (data not shown). In addition, we were unable to identify *cidAB* genes or other genes that encoded LrgAB-like proteins in the *S. gordonii* genome. In addition to availability of oxygen, glucose levels were revealed to affect autolysis of *S. mutans* through the CcpA protein (2), which was able to bind directly to the promoter region of *lrgA* (7). However, we have no evidence to support that autolysis of *S. gordonii* is regulated by glucose availability, nor have we been able to observe binding of a purified, recombinant CcpA protein (2, 18) to the promoter regions of the *atlS* gene of *S. gordonii* (data not shown). These findings highlight additional fundamental differences in control of autolysin expression, maturation and localization in *S. gordonii* compared to *S. mutans*.

*S. gordonii* and *S. pneumoniae* are both naturally competent for genetic...
transformation and have evolutionarily similar competence regulatory pathways, whereas

the CSP system of *S. mutans* appears to have evolved from a bacteriocin regulatory
cascade (26, 28) and *comX* expression is governed primarily by the ComRS system (40).

Notably, the expression of *cbpD* and *lytA*, which encode autolysins of *S. pneumoniae*,

increases sharply when cells enter stationary phase and can be induced by addition of

CSP (63), but cells can be protected from self-lysis by induction of an immunity protein,

*ComM* (63). *S. mutans* can also be induced to lyse by high concentrations (2 µM) of its
cognate CSP through pathways distinct from those in *S. pneumoniae* (24), although we

found that *atlA* expression can be stimulated by high concentrations of *S. mutans* CSP

(data not shown). Notably, pure CSP that was synthesized based on the *S. gordonii*

ComC sequence could activate *atlS* transcription and induce cell lysis (data not shown),

but *S. gordonii* lacks apparent *cbpD* and *comM* homologues. Therefore, there is

considerable evolutionary divergence in the interconnection of the competence and lytic

control pathways among the oral streptococci, as well as between the viridans group of

streptococci and *S. pneumoniae*.

In this report, we provide direct evidence that AtlS is required for efficient eDNA

release by *S. gordonii* (Fig. 4) and that aeration is a major environmental trigger for

eDNA release. Previously, it was reported that eDNA release of *S. gordonii* could be

stimulated by 1 mM H₂O₂ and occurred without detectable lysis of the cells (30). More

recently, Kreth and co-workers showed that H₂O₂ production by pyruvate oxidase is a

contributing factor to lysis of *S. sanguinis*, a close relative of *S. gordonii* (74). In this

study we observed that 0.003% H₂O₂ (4) was not sufficient to trigger cell lysis of *S.

*gordonii* (Fig. 3B) or to enhance *atlS* expression (data not shown) when cells were
cultured anaerobically, suggesting that oxygen itself or a metabolite of oxygen other than H$_2$O$_2$ serves as a stimulus for cell lysis. While it was noted that there was a basal level of eDNA released from cells that were cultured under relatively anaerobic conditions, the production of the overwhelming majority of eDNA by *S. gordonii* in aerobic conditions and in stationary phase under the conditions utilized in this study is dependent on the presence of the AtlS autolysin and associated with cell lysis.

In many species, eDNA contributes to biofilm formation as a component of the extracellular biofilm matrix, impacting cell-to-cell adherence and biofilm architecture (6, 17, 49, 51, 62, 70, 72). Supporting a role for eDNA in the development of *S. gordonii* biofilms is the observation that biofilm formation by strains lacking AtlA could be partially restored by the addition of either chromosomal or plasmid DNA (Fig. 8). The fact that complete restoration of biofilm formation by eDNA could not be achieved could simply indicate that the production of eDNA must be spatially or temporally regulated for optimal development of stable biofilms. As likely, the impact of loss of AtlS on the biogenesis of a normal cell surface, which was a striking phenotype of the *atlA* mutant of *S. mutans* (3), could account for a failure of the mutant strain to produce wild-type levels of biofilm. From a mechanistic perspective, altered peptidoglycan structure in the *atlS* mutant could impact osmo-adaptation or adaptation to other stresses associated with the mass transport limitations in three-dimensional biofilms (38). Likewise, long cell-chains and the associated decrease in available cell surface area for biofilm-promoting interactions may contribute to poor cell coaggregation and biofilm formation (64).

It was of significant interest that the biofilm forming capacity of the *atlS* mutant could be restored to a level comparable to the wild-type strain by addition of 2.5 µM
AtlA protein to the cultures (Fig. 8), which implies that AtlS is able to recognize S. mutans cells walls as a suitable substrate. Interestingly, addition of relatively high concentrations of S. mutans AtlA protein caused obvious cell lysis of S. gordonii (data not shown). We are presently examining whether a similar antagonistic impact of AtlS on S. mutans can be observed in vitro, but these preliminary results point to the possibility that autolysin release in dental biofilms could modulate the composition and activity of oral microbial populations. Such antagonistic potential would add to the growing body of evidence that there are multiple strategies used by oral streptococci to establish and persist in healthy and cariogenic biofilms.

S. gordonii is an earlier colonizer on dental surface, and is also thought to be important for the cosubsequent colonization of other oral commensals (21, 29, 47). It is known that the oxygen tension and overall redox potential in early biofilms is very high compared to that of immature biofilms (39, 42). Whereas biofilm formation by S. gordonii does not appear to be adversely affected by aerobic conditions, cultivation of S. mutans under similar conditions causes potent inhibition of biofilm development by this caries pathogen. Given the differences in the signal transduction pathways and regulation mechanisms controlling the expression of the genes for, and the maturation of, the AtlA and AtlS autolysins, development of therapeutic strategies that selectively disrupt the autolytic pathways of a prominent caries pathogen without disrupting normal functions of the commensal streptococci may be an effective means of modulating oral biofilm composition, and thus oral biofilm pathogenic potential. Efforts are underway to identify the signals regulating autolysin gene expression, processing and activity.
ACKNOWLEDGEMENTS

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33  
gordonii surface glycoproteins GspB and Hsa to specific carbohydrate structures 

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inhibition of Streptococcus mutans bacteriocin production by Streptococcus 


of an autolysin gene, atlg, from Streptococcus sobrinus. FEMS. Microbiol. Lett. 
291:17-23. 

protein A Controls hydrogen peroxide production and cell death in Streptococcus 
Table 1. Strains used in this study.

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<thead>
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<th>Strain</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<tbody>
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<td>SgWT</td>
<td><em>S. gordonii</em> DL1/ <em>P</em>&lt;sub&gt;atlS&lt;/sub&gt;-cat</td>
<td>This study</td>
</tr>
<tr>
<td>UA159</td>
<td>Wild type</td>
<td>University of Alabama, Birmingham</td>
</tr>
<tr>
<td>ΔatlS/S. gordonii / <em>P</em>&lt;sub&gt;atlS&lt;/sub&gt;-cat</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>ΔlytT/S. gordonii / <em>P</em>&lt;sub&gt;atlS&lt;/sub&gt;-cat</td>
<td></td>
<td>This study</td>
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<tr>
<td>ΔlytS/S. gordonii / <em>P</em>&lt;sub&gt;atlS&lt;/sub&gt;-cat</td>
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<td>This study</td>
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<tr>
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<td></td>
<td>This study</td>
</tr>
<tr>
<td>ΔvicK/S. gordonii / <em>P</em>&lt;sub&gt;atlS&lt;/sub&gt;-cat</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>ΔvicR/S. gordonii / <em>P</em>&lt;sub&gt;atlS&lt;/sub&gt;-cat</td>
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<td>This study</td>
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<tr>
<td>ΔvicRK/S. gordonii / <em>P</em>&lt;sub&gt;atlS&lt;/sub&gt;-cat</td>
<td></td>
<td>This study</td>
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<td>SgWT2</td>
<td><em>S. gordonii</em> DL1/ <em>P</em>&lt;sub&gt;lytT&lt;/sub&gt;-cat</td>
<td>This study</td>
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<td>This study</td>
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<tr>
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<td>ΔlytST/pDL-lytST</td>
<td>This study</td>
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<td>ΔvicRK-ΔvicRK</td>
<td>ΔvicRK/pDL-vicRK</td>
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Table 2. Primers used in this study.

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<th>Application</th>
<th>source</th>
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<td>Deletion of <em>atlS</em></td>
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<td><em>P</em>_atlS-5'-biotin</td>
<td>5'-/5Bios/CGATGGCAGTTAGGAC-3'</td>
<td>Amplification of <em>atlS</em> promoter probe with biotin for EMSA</td>
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<td>LytT-5'</td>
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<td>This study</td>
</tr>
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<td>LytT-BamHI-3'</td>
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<td>Deletion of <em>lytT</em></td>
<td>This study</td>
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<tr>
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<td>This study</td>
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<td>Deletion of <em>lytS</em></td>
<td>This study</td>
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<tr>
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<td>LytS-BamHI-5'</td>
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<td>ClytST-BamHI-5'</td>
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<td>Construction of <em>lytST</em> complementation</td>
<td>This study</td>
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<td>ClytST-EcoRI-3'</td>
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<td>Deletion of <em>vicK</em></td>
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<tr>
<td>VicR-BamHI-3'</td>
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<td>Deletion of <em>vicK</em></td>
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<td>VicK-BamHI-5'</td>
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<td>VicR-5'-2</td>
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<td>(35)</td>
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<td>VicR-BamHI-3'-2</td>
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<td>Deletion of <em>vicR</em></td>
<td>(35)</td>
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<td>VicR-BamHI-5'</td>
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<td>Deletion of <em>vicR</em></td>
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<td>VicR-3'</td>
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<td>(35)</td>
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<td>CvicRK-BamHI-5'</td>
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<td><em>P</em>_lytT-3'</td>
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<td>16S rRNA-S</td>
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<td>eDNA quantification</td>
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<td>eDNA quantification</td>
<td>This study</td>
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* Boldface indicates engineered restriction sites.
Table 3. Comparison of AtlA and AtlS.

<table>
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<tr>
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<th>AtlA</th>
<th>AtlS</th>
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<td><strong>Origins</strong></td>
<td><em>S. mutans</em></td>
<td><em>S. gordonii</em></td>
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<tr>
<td><strong>Encoding genes</strong></td>
<td>SMu0630</td>
<td>SGO02013</td>
</tr>
<tr>
<td><strong>Gene locus</strong></td>
<td>Multigene operon</td>
<td>Single-gene operon</td>
</tr>
<tr>
<td><strong>Protein characteristics</strong></td>
<td>977 amino acids, 107 kD and has a conserved $\beta_1$, $4-N$-acetylmuramidase domain located at C-terminal (3).</td>
<td>1160 amino acids, 127 kD and has a conserved $\beta_1$, $4-N$-acetylmuramidase domain located at N-terminal.</td>
</tr>
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<td><strong>Involvement in the cell physiology</strong></td>
<td>Normal cell wall biosynthesis, stress tolerance, autolysis, competence and biofilm formation (3, 4)</td>
<td>Normal cell wall biosynthesis, stress tolerance, autolysis and biofilm formation</td>
</tr>
<tr>
<td><strong>Contribution to eDNA release</strong></td>
<td>NR*</td>
<td>Major</td>
</tr>
<tr>
<td><strong>Processing for protein maturation</strong></td>
<td>Cleavage (3)</td>
<td>Cleavage</td>
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<td><strong>Inducible conditions</strong></td>
<td>Oxidative stress (4)</td>
<td>Oxidative stress and CSP</td>
</tr>
<tr>
<td><strong>Transcriptional regulation</strong></td>
<td>At least three promoters (3)</td>
<td>One proximal promoter</td>
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<td><strong>Direct regulator</strong></td>
<td>NR*</td>
<td>LytT</td>
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<tr>
<td><strong>Post-transcription regulation</strong></td>
<td>ThmA, Smu0629 and VicRK (4)</td>
<td>ND*</td>
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</table>

* NR - not reported.

# ND - not determined.
FIGURE LEGENDS

Figure 1. Comparison of autolysis by the SgWT and atlS mutant strains. An autolysis assay was carried out as described in Materials and Methods.

Figure 2. (A) Zymographic assay: autolysin profiles of the SgWT and AtlS-deficient strains determined by renaturing SDS-PAGE using a 7.5% polyacrylamide gel containing 1% (wet weight) of cell walls prepared from S. gordonii. (B) Western blot analysis of proteins extracted from SgWT and atlS mutant strains after incubation in 4% SDS extracts. Of note, in order to detect similar signals for the AtlA and AtlS proteins with the anti-AtlA antibody, it was necessary to apply 10-fold more surface protein extract from S. gordonii to the gel than required for S. mutans (Fig. 2B). Thus, duplicate lanes labeled as 1 x UA159 contain proteins extracted from S. mutans UA159. Duplicate lanes labeled as 10 x SgWT and lanes labeled as 10 x AtlS indicate that 10-fold more protein from the wild-type or atlS mutants of S. gordonii, respectively, were loaded on the gel than in the S. mutans lanes. Following SDS-PAGE, proteins were transferred onto a nitrocellulose membrane and subjected to Western blotting using an affinity-purified anti-AtlA polyclonal antiserum at the dilution of 1:500. See Materials and Methods for more details.

Figure 3. Growth curves of strains SgWT and atlS mutant strains under aerobic or anaerobic conditions (A) in BHI (pH 7.0) or BHI that was acidified to pH 5.5 with HCl; (B) in BHI broth with or without 0.003 % hydrogen peroxide; (C) in BHI broth with or without 25 mM paraquat. Optical density at 600 nm was determined every 30 min for
Figure 4. eDNA release by SgWT and AtlS-deficient S. gordonii as a function of growth domain. Cells were cultured in BHI broth under aerobic or anaerobic conditions. (A) Agarose gel electrophoresis (0.8%) of eDNA stained with ethidium bromide as described in the Materials and Methods section. The optical density (OD$_{600}$) of the culture when harvested is presented below each panel and the photographs are representative of three independent experiments that yielded similar results. (B) Quantitative real-time PCR of eDNA using the 16S rRNA gene as the amplification target. Data was normalized as detailed in the methods section and represent the means and standard deviations of two independent experiments done in duplicate on different days. *, statistically significant differences among the same strain collected at different growth phases ($P < 0.05$ [Student $t$ test]). †, statistically significant differences between the same strain cultured anaerobically and aerobically ($P < 0.05$ [Student $t$ test]).

Figure 5. Growth and lysis of strains SgWT and lyt mutants (A) and SgWT and vic mutants (B) in BHI broth cultured under aerobic conditions. Optical density at 600 nm was determined every 30 min after shaking using a Bioscreen C.

Figure 6. CAT activities of (A) SgWT carrying $P_{atls}$-cat cultured in TY medium containing 25 mM galactose at different growth phases, under aerobic or anaerobic conditions; (B) SgWT and its derivatives cultured in TY medium containing 25 mM galactose and collected at stationary phase, under aerobic or anaerobic conditions; (C)
SgWT2 carrying \( P_{\text{lytT-cat}} \) and its derivatives cultured in BHI broth, under aerobic or anaerobic conditions. Cells were collected at the mid-exponential phase of growth. The values of the columns are the average of a minimum of nine separate cultures for each strain and condition. ‡, statistically significant differences among cells collected at different growth phases (\( P < 0.05 \) [Student \( t \) test]). *, statistically significant differences between the same strain cultured anaerobically or aerobically (\( P < 0.05 \) [Student \( t \) test]). †, statistically significant differences between the wild-type and mutant strains cultured under the same conditions (\( P < 0.05 \) [Student \( t \) test]).

Figure 7. Electrophoretic mobility shift assays using LytT-MBP and a biotin-labeled fragment containing the \( \text{atlS} \) promoter region. Biotin-labeled \( P_{\text{atlS}} \) probe was incubated with increasing concentrations of LytT-MBP at room temperature for 60 min and then analyzed on a low-ion-strength polyacrylamide gel as detailed in the methods section.

Figure 8. Biofilm formation assay. Cultures were grown in 1/4 BHI medium supplemented with 10 mM sucrose under aerobic or anaerobic conditions for 48 h. The top panel is quantification of the crystal violet stained biofilms as detailed in the text. Data are representatives of the mean of at least two separate experiments that were performed in triplicate, with error bars delineating standard deviations. *, statistically significant differences between the wild-type and mutant strains cultured under the same conditions (\( P < 0.05 \) [Student \( t \) test]). The bottom panel shows representative biofilms corresponding to the sample quantified in the bar graph above.
Fig. 1
Fig. 2

A

B

(kDa)  SgWT  AtlS
150  
100  
75  

(kDa)  1 × UA159  10 × SgWT  10 × AtlS
150  
100  
75  

Fig. 2
Fig. 3

<table>
<thead>
<tr>
<th>Condition</th>
<th>Time (h)</th>
<th>OD 600</th>
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<tbody>
<tr>
<td>SgWT + paraquat</td>
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<td></td>
</tr>
<tr>
<td>AtlS + paraquat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SgWT + paraquat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtlS + paraquat</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3 shows the growth kinetics of different conditions over time.

A. 
- SgWT-pH7.0
- AtlS-pH7.0
- AtlS-pH5.5
- SgWT-pH5.5

B. 
- SgWT-H2O2-anaerobic
- AtlS-H2O2-anaerobic
- SgWT-H2O2-aerobic
- AtlS-H2O2-aerobic

C. 
- SgWT + paraquat-anaerobic
- AtlS + paraquat-anaerobic
- SgWT + paraquat-aerobic
- AtlS + paraquat-aerobic
Fig. 4

OD 600
- O2  + O2  - O2  + O2  - O2  +
0.2-0.3 0.5-0.6 0.9-1.0

SgWT  AtlS  SgWT AtlS  SgWT AtlS  SgWT AtlS  SgWT AtlS

eDNA amount (copies/μl)
SgWT - O2
AtlS - O2
SgWT + O2
AtlS + O2

**  **  †

A

B

Fig. 4
Fig. 5

(A) 

(B)
Fig. 6
Fig. 7

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