

The Major Autolysin of *Streptococcus gordonii* Is Subject to Complex Regulation and Modulates Stress Tolerance, Biofilm Formation, and Extracellular-DNA Release[∇]

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A gene, designated *atlS*, encoding a major autolysin from *Streptococcus gordonii*, was identified and characterized. The predicted AtlS protein is 1,160 amino acids and 127 kDa and has a conserved β 1,4-*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.

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 *Streptococcus 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 coworkers showed that a complex posttranscriptional 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 H₂O₂ but independent of cell lysis (30). By computer analysis of the genome of *S. gordonii* strain Challis at <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.

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TABLE 1. Strains used in this study

Strain	Relevant characteristics	Source or reference
SgWT UA159	<i>S. gordonii</i> DL1/ <i>P_{atIS}-cat</i> Wild type	This study University of Alabama, Birmingham
AtIS	<i>ΔatIS S. gordonii</i> / <i>P_{atIS}-cat</i>	This study
SglytT	<i>ΔlytT S. gordonii</i> / <i>P_{atIS}-cat</i>	This study
SglytS	<i>ΔlytS S. gordonii</i> / <i>P_{atIS}-cat</i>	This study
SglytST	<i>ΔlytST S. gordonii</i> / <i>P_{atIS}-cat</i>	This study
SgvicK	<i>ΔvicK S. gordonii</i> / <i>P_{atIS}-cat</i>	This study
SgvicR	<i>ΔvicR S. gordonii</i> / <i>P_{atIS}-cat</i>	This study
SgvicRK	<i>ΔvicRK S. gordonii</i> / <i>P_{atIS}-cat</i>	This study
SgWT2	<i>S. gordonii</i> DL1/ <i>P_{lytT}-cat</i>	This study
SgvicR2	<i>ΔvicR S. gordonii</i> / <i>P_{lytT}-cat</i>	This study
SgvicK2	<i>ΔvicK S. gordonii</i> / <i>P_{lytT}-cat</i>	This study
SgvicRK2	<i>ΔvicRK S. gordonii</i> / <i>P_{lytT}-cat</i>	This study
SgWT-pDL278	SgWT/pDL278	This study
SgWT-clytST	SgWT/pDL-lytST	This study
SgWT-cvicRK	SgWT/pDL-vicRK	This study
SglytST-clytST	SglytST/pDL-lytST	This study
SgvicRK-cvicRK	SgvicRK/pDL-vicRK	This study

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*), eryth-

romycin (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 1/4-strength BHI medium (BHI medium diluted 1:3 with distilled water [dH₂O]) 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 *atIS* promoter (SGO.2013), a 624-bp fragment immediately 5' to the start codon of *atIS* was amplified by PCR with primers *P_{SG2013}-5'-1* 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 BamHI recognition sequences. The products harboring *P_{SG2013}* and *P_{lytT}* were fused with the promoterless *cat* gene derived from pC194 (14, 27). After the correct sequence of the promoter fusion was confirmed, 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 BamHI sites designed into each primer set, and cloned into the pGEM-T Easy vector (Promega, Madison, WI). These plasmids were digested with BamHI, and a nonpolar 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 of the flanking regions to ensure that no undesired mutations were inadvertently

TABLE 2. Primers used in this study

Primers	Sequence ^a	Application	Source
AtIS-5'	5'-CCAACGAGAGGGTCTTAG-3'	Deletion of <i>atIS</i>	This study
AtIS-BamHI-3'	5'-CTGTCTGGATCCAGACATTATAGCATG-3'	Deletion of <i>atIS</i>	This study
AtIS-BamHI-5'	5'-CAGCCTGGATCCAGCTTAGTAAAG-3'	Deletion of <i>atIS</i>	This study
AtIS-3'	5'-CGGTCGTGACCGCCATGATCGAAG-3'	Deletion of <i>atIS</i>	This study
<i>P_{atIS}</i> -5'-biotin	5'-/5Biosg/CGATGGCAGTTATAGGGAC-3'	Amplification of <i>atIS</i> promoter probe with biotins for EMSA	This study
LytT-5'	5'-CAAGCCGCTACCCTGTTATTGATGC-3'	Deletion of <i>lytT</i>	This study
LytT-BamHI-3'	5'-CCCTGTCTTACCGATCCTCATCCTCTAC-3'	Deletion of <i>lytT</i>	This study
LytT-BamHI-5'	5'-GTGGTAGGGATCCCGCTAGACAG-3'	Deletion of <i>lytT</i>	This study
LytT-3'	5'-GGCTTTGAGACTATTGTAGCCGATATCCA-3'	Deletion of <i>lytT</i>	This study
LytS-5'	5'-GGCTTTTCAACTAGGATTTAGTCCATC-3'	Deletion of <i>lytS</i>	This study
LytS-BamHI -3'	5'-CGATCAACTGGATCCGAAGCGGATAGCG-3'	Deletion of <i>lytS</i>	This study
LytS-BamHI-5'	5'-TCTGCCAGGATCCAATGATAAATAGTCG-3'	Deletion of <i>lytS</i>	This study
LytS-3'	5'-TCCCATAACATAGCGGAACCAAG-3'	Deletion of <i>lytS</i>	This study
ClytST-BamHI-5'	5'-GCCAAGGATCCCCCGTCCATCAAGTCCTGAT-3'	Construction of <i>lytST</i> complementation	This study
ClytST-EcoRI-3'	5'-CTTTCCGAATTCATGTCCCAGTATATATGTC-3'	Construction of <i>lytST</i> complementation	This study
VicR-5'-1	5'-GATGGTCGTGAAGCTCTTGA-3'	Deletion of <i>vicK</i>	This study
VicR-BamHI-3'	5'-GGCGAGGATCCCTTCGATTCTC-3'	Deletion of <i>vicK</i>	This study
VicK-BamHI-5'	5'-GGAGGATCCCTGGGAAAGTGAAG-3'	Deletion of <i>vicK</i>	This study
VicK-3'	5'-CCGATAAAATTGTGGTGGCCGCCGC-3'	Deletion of <i>vicK</i>	35
VicR-5'-2	5'-CAAGGGTGCCTTCCCAACATGGC-3'	Deletion of <i>vicR</i>	35
VicR-BamHI-3'-2	5'-GCCGGATCCACTTCATAGCCCTC-3'	Deletion of <i>vicR</i>	35
VicR-BamHI-5'	5'-CTCGTCTGGATCCCGCTAC-3'	Deletion of <i>vicR</i>	35
VicR-3'	5'-GAACAGCTACCAAACCAGAG-3'	Deletion of <i>vicR</i>	35
CvicRK-BamHI-5'	5'-GCTACGGATCCAAAGAAACACATACGGTAGC-3'	Construction of <i>vicRK</i> complementation	This study
CvicRK-SphI-5'	5'-CCACTAGCATCGGATGCTAAAATGCTATAC-3'	Construction of <i>vicRK</i> complementation	This study
LytT-MBP-5'	5'-TCCAGTATAAATAAGGATCCAATCTTGTG-3'	Construction of LytT-BMP protein	This study
LytT-MBP-3'	5'-GGATAAGAAGTCGACAGCGTTTCATTCTCC-3'	Construction of LytT-BMP protein	This study
<i>P_{lytT}</i> -5'	5'-GCCAAGGATCCCCCGGGCTA-3'	Construction of <i>lytT</i> promoter fusion	This study
<i>P_{lytT}</i> -3'	5'-GAATACACGGATCCCAACTCC-3'	Construction of <i>lytT</i> promoter fusion	This study
16S rRNA-S	5'-CACACCGCCGTCACACC-3'	eDNA quantification	This study
16S rRNA-AS	5'-CAGCCGCACCTTCCGATACG-3'	eDNA quantification	This study

^a Boldface indicates engineered restriction sites.

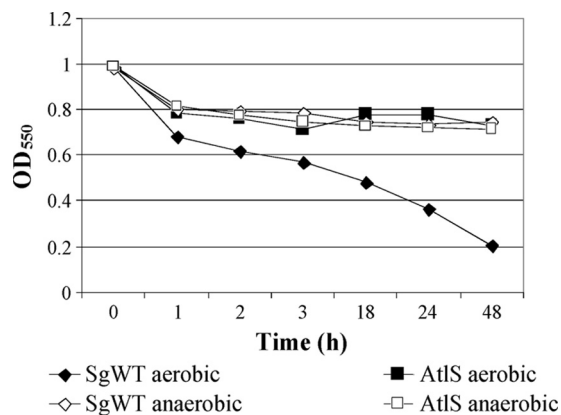


FIG. 1. Comparison of autolysis by the SgWT and *atlS* mutant strains. An autolysis assay was carried out as described in Materials and Methods.

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_{autS} -*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 multiwell disposable microtiter plates. An aliquot (3 μ l) from an overnight culture was inoculated in at least triplicate into wells containing 300 μ l of BHI or BHI/HCl. All inocula were adjusted to the same optical density at 600 nm (OD_{600}) before dilution. To assess the ability of cells to grow in the presence of oxidative stressors, cells from overnight cultures were transferred to prewarmed BHI and grown at 37°C in a 5% CO_2 aerobic atmosphere to an OD_{600} of 0.5. The cells were then diluted into fresh BHI containing 0.003% H_2O_2 (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 an OD_{600} of 0.9. Cell pellets were resuspended in 500 μ l of 4% SDS and incubated for 60 min at room temperature with agitation, followed by centrifugation at 13,000 $\times g$ 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 four times with distilled H_2O (dH_2O). The pellet was suspended in 60 ml of 4% SDS and boiled for 30 min. The heat-killed cells were washed five times in dH_2O , and the pellet was saved. The pellet was resuspended in dH_2O , and the suspension was added to a polyacrylamide gel at a final concentration of 1% (vol/vol) (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 (OD_{600} , 0.5 to 0.6) were centrifuged and washed twice with Tris-buffered saline (10 mM Tris, 0.9% NaCl,

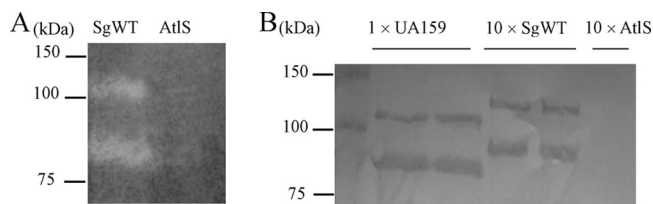


FIG. 2. (A) Zymographic assay: autolysin profiles of the SgWT and *AtIS*-deficient strains determined by renaturing SDS-PAGE using a 7.5% polyacrylamide gel containing 1% (wet weight) of cell walls prepared from *S. gordonii*. (B) Western blot analysis of proteins extracted from SgWT and *atlS* mutant strains after incubation in 4% SDS extracts. Of note, in order to detect similar signals for the *AtIA* and *AtIS* proteins with the anti-*AtIA* antibody, it was necessary to apply 10-fold more surface protein extract from *S. gordonii* to the gel than is required for *S. mutans* (Fig. 2B). Thus, duplicate lanes labeled 1 \times UA159 contain proteins extracted from *S. mutans* UA159. Duplicate lanes labeled 10 \times SgWT and lanes labeled 10 \times *AtIS* indicate that 10-fold more protein from the wild-type strain or *atlS* mutant of *S. gordonii*, respectively, was 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-*AtIA* polyclonal antiserum at a dilution of 1:500. See Materials and Methods for more details.

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 2,000 $\times g$ for 10 min (15). Protein samples were separated by SDS-PAGE, blotted onto polyvinylidene difluoride (PVDF) membranes, and incubated with affinity-purified (33) *AtIA* 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).

EMSA. An electrophoretic mobility shift assay (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- μ l reaction mixture containing 10 mM HEPES (pH 7.9), 50 mM KCl, 10 mM EDTA, 5 mM $MgCl_2$, and 2 μ g poly(dI-dC). After incubation at room temperature for 1 h, the DNA-protein samples were resolved in a nondenaturing, low-ionic-strength polyacrylamide gel and blotted onto hybridization transfer membranes (Perkin-Elmer). Signals were developed using the Chemiluminescent nucleic acid detection module (Thermo Scientific).

RESULTS

Characterization of SGO2013 (*AtIS*) activity. To determine whether SGO.2013 encoded an autolysin, the autolytic activity of an 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* *AtIA* autolysin (3), which were affinity purified using a 6-His-tagged *AtIA* protein (3), also disclosed proteins of 130 and 90 kDa (Fig. 2B). Thus, similar to *atIA* of *S. mutans* (Fig. 2B), SGO.2013 encodes a gene product, heretofore designated *AtIS*, which is present as a higher-molecular-mass spe-

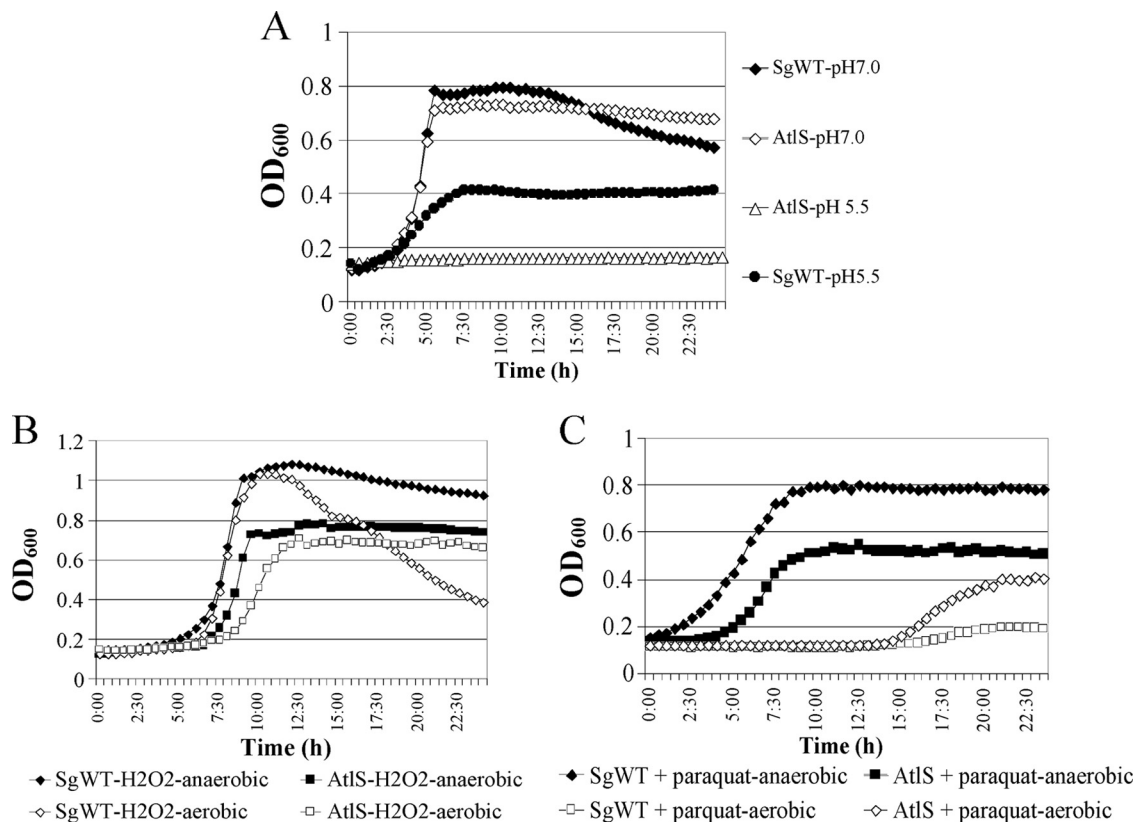


FIG. 3. Growth curves of SgWT and *atIS* mutant strains under aerobic or anaerobic conditions in BHI (pH 7.0) or BHI that was acidified to pH 5.5 with HCl (A); in BHI broth with or without 0.003% hydrogen peroxide (B); and in BHI broth with or without 25 mM paraquat (C). Optical density at 600 nm was determined every 30 min for 24 h using a Bioscreen C.

cies and as a lower-molecular-weight protein that likely arises from proteolysis of full-length AtIS.

AtIS affects stress tolerance by *S. gordonii*. The *atIS* mutant strain formed longer chains of cells than the wild-type strain and clumped extensively in broth culture (data not shown). To examine whether AtIS of *S. gordonii* affected acid tolerance, SgWT and the *atIS* 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 *atIS* mutant was similar to that of SgWT (Fig. 3A) and the *atIS* 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 *atIS* mutant was not able to grow in BHI/HCl broth (Fig. 3A).

To examine whether AtIS of *S. gordonii* was required for oxidative stress tolerance, the SgWT and *atIS* 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 and a modestly lower growth rate and attained a maximum optical density similar to that obtained when the strain is cultured in anaerobic conditions (Fig. 3B). However, lysis of the cells was much more rapid and extensive in cells grown in aerobic con-

ditions than in those growing under anaerobic conditions (Fig. 3B). The *atIS* mutant consistently displayed a longer lag phase and 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 *atIS* mutant. Similarly, in the presence of 25 mM paraquat, the *atIS* 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 AtIS deficiency and provide more evidence that the cells lacking AtIS are hyperresistant to autolysis.

AtIS-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 (OD₆₀₀, 0.2~0.3) to stationary phase (OD₆₀₀, 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

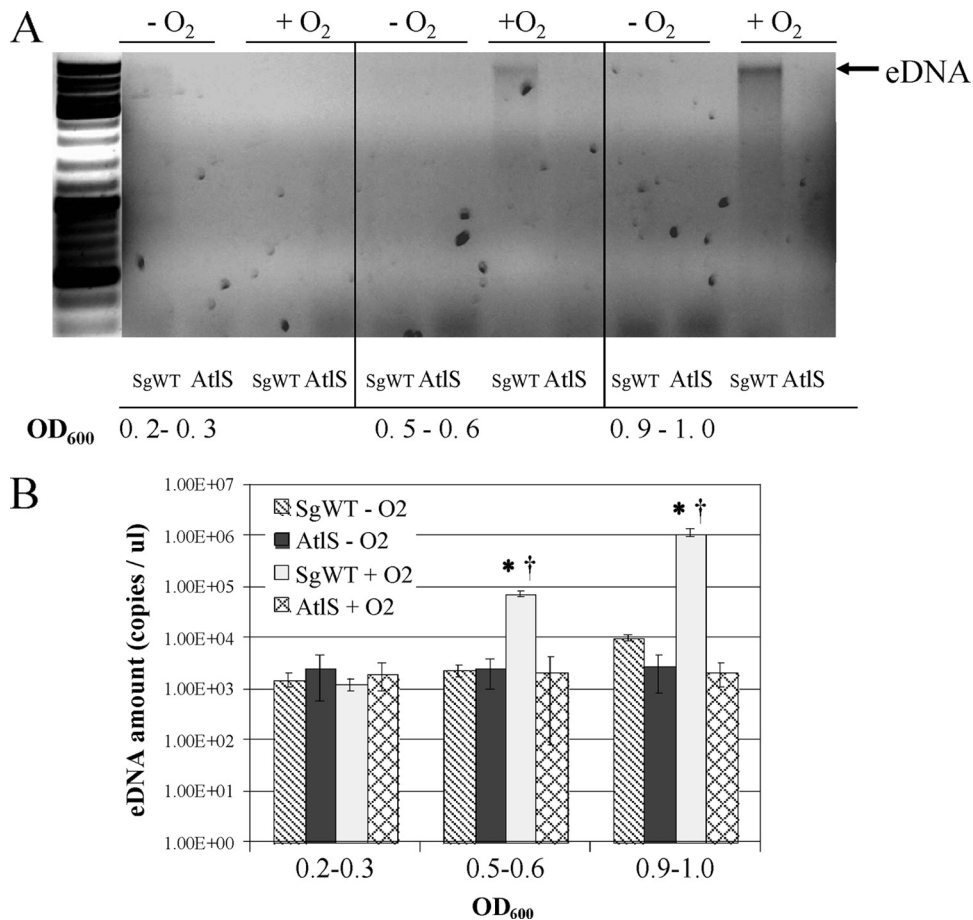


FIG. 4. eDNA release by SgWT and AtIS-deficient *S. gordonii* as a function of growth domain. Cells were cultured in BHI broth under aerobic or anaerobic conditions. (A) Agarose gel electrophoresis (0.8%) of eDNA stained with ethidium bromide as described in the Materials and Methods section. The optical density (OD₆₀₀) 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 were normalized as detailed in Materials and Methods and represent the means and standard deviations of results 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]).

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 *atIS* mutant in all growth phases under aerobic or anaerobic conditions, providing strong evidence that AtIS and cell lysis contribute in major ways to eDNA production by *S. gordonii*.

Regulation of *atIS* expression. The SgWT strain, which carries a *cat* gene fused to the *atIS* promoter, was grown in TY medium containing 25 mM galactose under aerobic or anaerobic conditions. CAT activity arising from P_{*atIS*} expression was examined at different growth phases. From the early exponential phase (OD₆₀₀, 0.2~0.3) to midexponential phase (OD₆₀₀, 0.5~0.6), *atIS* expression of cells was at a basal level, under both aerobic and anaerobic conditions (see Fig. 6A). However, P_{*atIS*} expression was increased more than 10-fold after cells entered stationary phase (OD₆₀₀, 0.9~1.0), with aerobically

grown cells showing 2.5-fold-higher *atIS* expression than anaerobically grown cells in stationary phase (see Fig. 6A). Thus, *atIS* expression is both growth phase and oxygen dependent in *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 *S. pneumoniae*, *S. aureus*, and *S. mutans* (4, 24, 50, 57). To determine whether these signal transduction systems also affected autolysis in *S. gordonii*, the entire *lytST* or *vicRK* operon was replaced by a nonpolar kanamycin cassette (10). To evaluate the function of individual components of the TCS, the *lytS*, *lytT*, *vicR*, and *vicK* genes were disrupted individually by nonpolar insertions (Table 1). All *vic* and *lyt* mutants formed long chains and clumped when growing in BHI (data not shown). With the exception of the strain carrying only the *vicK* mutation, SgvicK, all *vic* and *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

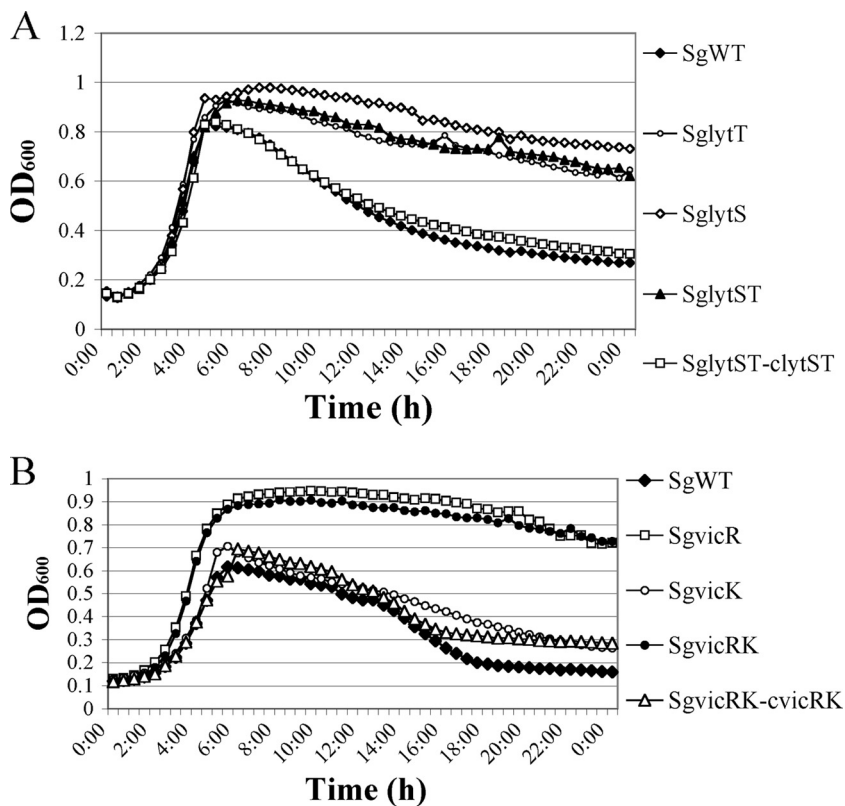


FIG. 5. Growth and lysis of strains SgWT and *lyt* mutants (A) and SgWT and *vic* mutants (B) in BHI broth cultured under aerobic conditions. Optical density at 600 nm was determined every 30 min after shaking using a Bioscreen C.

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 *atIS*, 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_{atIS}* under aerobic conditions than under anaerobic conditions (Fig. 6B), whereas the *lyt* mutants displayed a basal level of *P_{atIS}* expression and no induction in aerobic conditions was evident (Fig. 6). A phenotype similar to that of the *lyt* mutants was observed in the SgvicR and SgvicRK mutants, whereas about a 2-fold induction of *P_{atIS}* 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 *atIS* gene expression by oxygen. Also of note, the complemented strains, SgvicRK-cvicRK and SglytST-clytST, and *lytST* or *vicRK* overexpressing strains (SgWT-cvicRK and SgWT-clytST) had higher levels of expression from the *atIS* 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 *atIS* in response to oxygen and possibly other factors.

Purified LytT binds to the promoter region of *atIS* in vitro.

To explore whether the LytT response regulator could interact with the *atIS* 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 *atIS* 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_{atIS}* was retarded and increasing concentrations of LytT-MBP caused more *P_{atIS}* to shift (Fig. 7). Unlabeled *P_{atIS}* 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 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_{atIS}* 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*

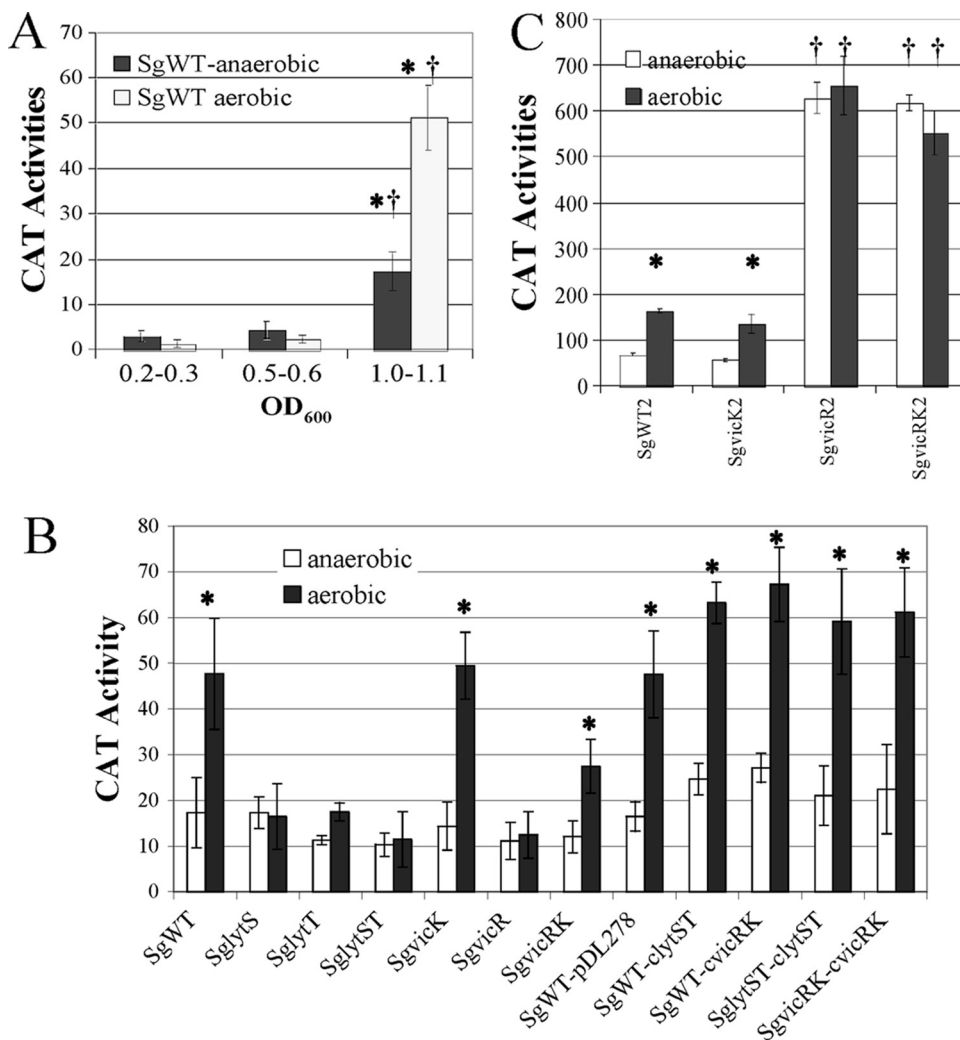


FIG. 6. CAT activities of SgWT carrying P_{atls} -*cat* cultured in TY medium containing 25 mM galactose at different growth phases, under aerobic or anaerobic conditions (A), SgWT and its derivatives cultured in TY medium containing 25 mM galactose and collected at stationary phase, under aerobic or anaerobic conditions (B), and SgWT2 carrying P_{lytT} -*cat* and its derivatives cultured in BHI broth, under aerobic or anaerobic conditions (C). Cells were collected at the midexponential 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 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]).

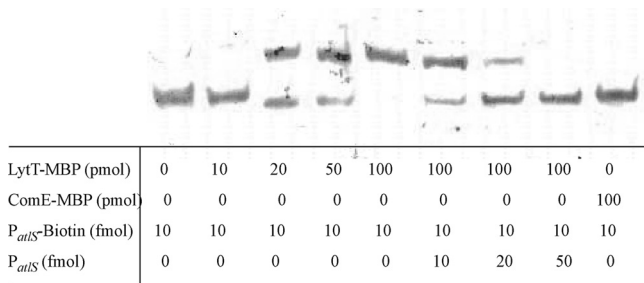


FIG. 7. Electrophoretic mobility shift assays using LytT-MBP and a biotin-labeled fragment containing the *atls* promoter region. Biotin-labeled P_{atls} probe was incubated with increasing concentrations of LytT-MBP at room temperature for 60 min and then analyzed on a low-ion-strength polyacrylamide gel as detailed in Materials and Methods.

or *vicRK* mutant strains in terms of *atls* responsiveness to oxygen, we investigated the possibility that expression of the *vicRK* or *lytST* TCS 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 an OD_{600} of 0.5~0.6, and CAT activities were measured. Approximately 2-fold-higher expression of P_{lytT} was detected under aerobic conditions than under 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

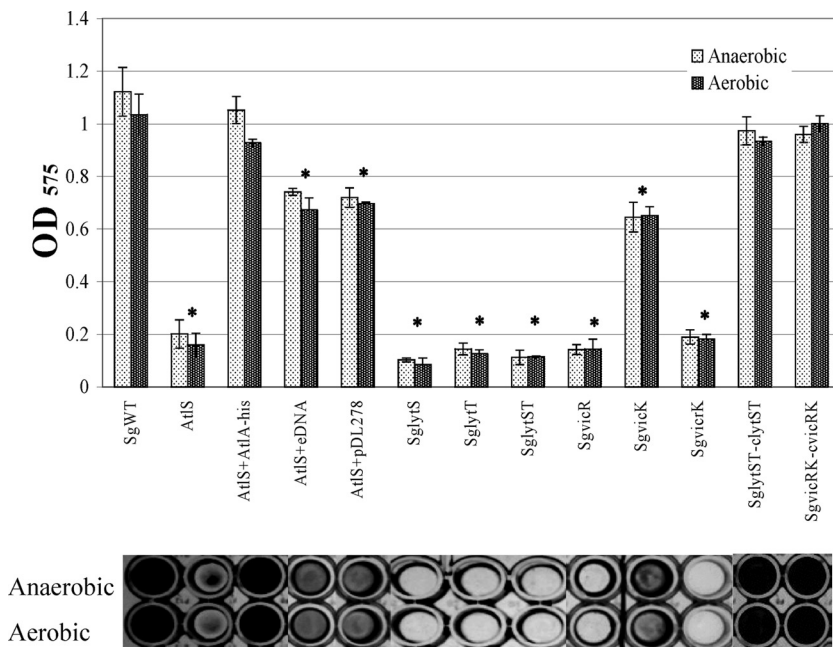


FIG. 8. Biofilm formation assay. Cultures were grown in 1/4-strength BHI medium supplemented with 10 mM sucrose under aerobic or anaerobic conditions for 48 h. The top panel is quantification of the crystal violet-stained biofilms as detailed in the text. Data are representatives of the means of results 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.

profound effect on biofilm formation (3), so we assessed the capacity of the SgWT and *atIS* mutant strains to form biofilms in 96-well microtiter plates (Fig. 8). The strains were cultured in 200 μ l 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 *atIS* 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 that for the *atIS* 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 AtIS for efficient *in vitro* biofilm formation by *S. gordonii*, purified 6-His-tagged, full-length AtIA protein from *S. mutans* (4) was added into the biofilm culture medium at a final concentration of 2.5 μ M. Similar to what was reported for AtIA-deficient *S. mutans* (4), purified AtIA could restore biofilm formation by the *atIS* 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 AtIS-dependent lysis played a role in biofilm formation by *S. gordonii*, *atIS* mutants were cultured in biofilm medium with 50 ng/ μ l of eDNA extracted from aerobically grown wild-type *S. gordonii* (Fig. 4). Under these conditions, the *atIS* 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 *atIS* mutant to form biofilms may be only partially attributable to decreased release of

eDNA. Plasmid DNA (pDL278, 50 ng/ μ l) (32) was also used as the eDNA source with the *atIS* mutant, and biofilm biomass was formed at a level similar to that formed 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 diet-related 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 AtIA autolysin of *S. mutans* is tremendously important to cellular homeostasis and is highly regulated at the transcriptional and posttranscriptional 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 abun-

TABLE 3. Comparison of AtlA and AtlS^a

Parameter	Result for:	
	AtlA	AtlS
Origins	<i>S. mutans</i>	<i>S. gordonii</i>
Encoding genes	SMu0630	SGO2013
Gene locus	Multigene operon	Single-gene operon
Protein characteristics	977 amino acids, 107 kDa, and has a conserved β 1,4- <i>N</i> -acetylmuramidase domain located at C-terminal (3)	1,160 amino acids, 127 kDa, and has a conserved β 1,4- <i>N</i> -acetylmuramidase domain located at N-terminal
Involvement in the cell physiology	Normal cell wall biosynthesis, stress tolerance, autolysis, competence, and biofilm formation (3, 4)	Normal cell wall biosynthesis, stress tolerance, autolysis, and biofilm formation
Contribution to eDNA release	NR	Major
Processing for protein maturation	Cleavage (3)	Cleavage
Inducible conditions	Oxidative stress (4)	Oxidative stress and CSP
Transcriptional regulation		
Promoter	At least three promoters (3)	One proximal promoter
Regulator	NR	VicRK and LytST
Direct regulator	NR	LytT
Posttranscription regulation	ThmA, Smu0629, and VicRK (4)	ND

^a NR, not reported; ND, not determined.

dant 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 (these data are 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 (3, 11, 59), is very complex. For example, the *atlA* gene is in an operon with at least four additional genes, and transcription of *atlA* is influenced by at least three promoters (3, 11, 59). Aeration is a major trigger for AtlA-dependent autolysis in *S. mutans*, and the response to aerobiosis is controlled by the VicRK two-component system (4). While regulation of autolysis by *S. gordonii* has some properties in common with that by these organisms, important differences exist.

The cross-reaction of anti-AtlA antibody with the AtlS protein by Western blot analysis (Fig. 2B) confirmed the prediction from computer algorithms that AtlS and 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, AtlA could function to restore biofilm formation by an *atlS* mutant of *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 AtlA had a profoundly negative impact on the development of genetic competence in *S. mutans* (4), whereas inactivation of *atlS* did not adversely affect the transformation efficiency of *S. gordonii* (data not shown). In addition, compromised tolerance of acid stress was strongly associated with a deficiency of AtlS in *S. gordonii* (Fig. 3A), but strains of *S. mutans* lacking AtlA did not show a similar phenotype (4).

Substantial variations in transcriptional and posttranscriptional control mechanisms for *atlA* and *atlS* are also apparent. Transcriptional studies revealed that *atlA* is part of a multigene operon under the control of at least three promoters (3), but AtlS appears to be encoded as a single cistron (Fig. 6A) (10) driven from a proximal promoter. Proper maturation of AtlA (reference 3 and Fig. 2) of *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 TCS were required for the induction of *atlS* transcription in response to oxygen (Fig. 6B). The effect 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 may

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 *atIS*. Collectively, then, it appears that *S. gordonii* modulates *atIS* 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 led 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 *atIA* 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 *atIS* gene of *S. gordonii* (data not shown). These findings highlight additional fundamental differences between *S. gordonii* and *S. mutans* in control of autolysin expression, maturation, and localization.

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 the 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 μ M) of its cognate CSP through pathways distinct from those in *S. pneumoniae* (24), although we found that *atIA* 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 *atIS* 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 in-

terconnection 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 AtIS 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, Zheng and coworkers 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 *atIS* expression (data not shown) when cells were cultured anaerobically, suggesting that oxygen itself or a metabolite of oxygen other than H₂O₂ 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 AtIS 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 AtIA 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 AtIS on the biogenesis of a normal cell surface, which was a striking phenotype of the *atIA* 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 *atIS* 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 *atIS* mutant could be restored to a level comparable to that of the wild-type strain by the addition of 2.5 μ M AtIA protein to the cultures (Fig. 8), which implies that AtIS is able to recognize *S. mutans* cell walls as a suitable substrate. Interestingly, addition of relatively high concentrations of *S. mutans* AtIA protein caused obvious cell lysis of *S. gordonii* (data not shown). We are presently examining whether a similar antagonistic impact of AtIS 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 redox potential in early biofilms are 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 AtIA and AtIS 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 under way to identify the signals regulating autolysin gene expression, processing, and activity.

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