The LuxS-Dependent Quorum-Sensing System Regulates Early Biofilm Formation by *Streptococcus pneumoniae* Strain D39

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*Streptococcus pneumoniae* is the leading cause of death in children worldwide and forms highly organized biofilms in the nasopharynx, lungs, and middle ear mucosa. The luxS-controlled quorum-sensing (QS) system has recently been implicated in virulence and persistence in the nasopharynx, but its role in biofilms has not been studied. Here we show that this QS system plays a major role in the control of *S. pneumoniae* biofilm formation. Our results demonstrate that the luxS gene is contained by invasive isolates and normal-flora strains in a region that contains genes involved in division and cell wall biosynthesis. The luxS gene was maximally transcribed, as a monocistronic message, in the early mid-log phase of growth, and this coincides with the appearance of early biofilms. Demonstrating the role of the LuxS system in regulating *S. pneumoniae* biofilms, at 24 h postinoculation, two different *D39ΔluxS* mutants produced ~80% less biofilm biomass than wild-type (WT) strain D39 did. Complementation of these strains with luxS, either in a plasmid or integrated as a single copy in the genome, restored their biofilm level to that of the WT. Moreover, a soluble factor secreted by WT strain D39 or purified Al-2 restored the biofilm phenotype of *D39ΔluxS*. Our results also demonstrate that during the early mid-log phase of growth, LuxS regulates the transcript levels of *lytA*, which encodes an autolysin previously implicated in biofilms, and also the transcript levels of *ply*, which encodes the pneumococcal pneumolysin. In conclusion, the luxS-controlled QS system is a key regulator of early biofilm formation by *S. pneumoniae* strain D39.

*Streptococcus pneumoniae* (the pneumococcus) is a Gram-positive pathogen that causes severe illnesses such as otitis media, pneumonia, and meningitis mainly in young children (<5 years old), immunocompromised patients, and the elderly (20, 25, 53). The pneumococcus colonizes the mucosal surface of the upper respiratory tract, the nasopharynx, in early childhood (22, 51). After colonization, this bacterium can cause disease and be rapidly transmitted to other children by coughing or may persist for months, thereby maintaining noninvasive strains in the human population (53). Colonization of the nasopharynx and persistence are important risk factors for both pneumococcal disease and carriage (20, 22).

The mechanism by which *S. pneumoniae* strains colonize and persist in the nasopharynx is still incompletely understood. During this transition, however, pneumococci must be able to adapt to their new niche by competing against other pneumococci, as well as other native flora, and evade the host immune response. Recently, the persistence of opportunistic pathogens, such as *Haemophilus influenzae*, *Bordetella*, or *Moraxella catarrhalis*, at different anatomic sites has been linked to highly specialized biofilms (3, 45, 47).

All of the *S. pneumoniae* strains investigated so far, whether they are invasive isolates or normal-flora strains, are capable of producing biofilms *in vitro* (2, 11, 13, 28, 34). More importantly, *S. pneumoniae* biofilms have been detected on the surface of adenoid and mucosal epithelial cells from biopsy specimens collected from children with chronic otitis media (16). Further studies with a chinchilla model support a role for biofilms in pneumococcal otitis media (56). While such structures have not been studied or described in patients with pneumonia, detailed work by Sanchez et al. (44) demonstrated that a virulent *S. pneumoniae* strain produced biofilms in the nasopharynx, trachea, and lungs of mice experimentally infected with strain TIGR4. Biofilms may also contribute to the increasing rates of antibiotic resistance among *S. pneumoniae* strains. For example, *in vitro* studies show that *S. pneumoniae* biofilms, in comparison to their planktonic cultures, have a profile of increased resistance to all of the classes of oral antibiotics widely used to treat pneumococcal infections, namely, β-lactams, macrolides, and fluorquinolones (11, 13).

Several molecular factors, including virulence factors, have been implicated in *S. pneumoniae* biofilm formation. Studies by Moscoso et al. (32) found that the amidases LytA, LytC, and LytB and adhesins such as CbpA, Ppa, and PspA play some role in *S. pneumoniae* biofilms. Another study by Muñoz-Elias et al. (34) identified 23 genes (encoding adhesins, choline binding proteins, and cell wall components) implicated in biofilm formation and colonization in a mouse model. Two other important virulence factors implicated in the production of biofilms are PsrP (44) and the neuraminidase NanA (40). While it is clear that all of these factors may play a role in the development of the biofilm structure, the specific mechanism by which *S. pneumoniae* biofilms are built remains to be elucidated.

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Biofilm formation requires a concerted mechanism regulated, in part, by numerous environmental signals (24). In S. intermedius, S. oralis, S. gordonii, and S. mutans strains, regulation of biofilm formation has been linked to LuxS (1, 7, 31, 42), an enzyme that synthesizes autoinducer 2 (AI-2), which is used to regulate competence state (50). Besides S. pneumoniae, S. oralis, and S. intermedius, autoinducer regulation of differential genes involved in some metabolic processes, including the expression of proteins in strain D39 (48) and regulation of LytA-dependent autolysis (43). In terms of pathogenicity, LuxS was implicated in virulence and persistence in the nasopharynx (49), an enzyme that synthesizes autoinducer 2 (AI-2), which is required for quorum sensing (QS). The phenomenon of QS is regulated, in part, by numerous environmental signals (24). S. pneumoniae strains isolated from blood or cerebrospinal fluid (n = 53) and normal-flora strains (n = 50) belong to our laboratory collection. These strains were isolated in different geographic regions, including the United States, Spain, Taiwan, Peru, and Brazil. Identification and serotyping were performed by standard procedures (8). S. pneumoniae strains were cultured on blood agar plates (BAP) or Todd-Hewitt broth containing 0.5% (wt/vol) yeast extract (THY). When indicated, 2% (wt/vol) maltose, ampicillin (100 μg/ml), tetracycline (1 μg/ml), erythromycin (0.5 μg/ml), or spectinomycin (110 μg/ml) was added to the culture medium.

**MATERIALS AND METHODS**

**Strains and bacterial culture media.** The S. pneumoniae reference and derivative strains used in this study are listed in Table 1. All of the other invasive S. pneumoniae strains isolated from blood or cerebrospinal fluid (n = 53) and normal-flora strains (n = 50) belong to our laboratory collection. These strains were isolated in different geographic regions, including the United States, Spain, Taiwan, Peru, and Brazil. Identification and serotyping were performed by standard procedures (8). S. pneumoniae strains were cultured on blood agar plates (BAP) or Todd-Hewitt broth containing 0.5% (wt/vol) yeast extract (THY). When indicated, 2% (wt/vol) maltose, ampicillin (100 μg/ml), tetracycline (1 μg/ml), erythromycin (0.5 μg/ml), or spectinomycin (110 μg/ml) was added to the culture medium.

**DNA extraction.** Genomic DNA from the S. pneumoniae strains in Table 1 was purified from overnight cultures on BAP using the QiAamp DNA minikit (Qiagen) by following the manufacturer’s instructions. DNA-containing supernatant from invasive isolates and normal-flora strains was extracted by the Chelex method (10).

**PCRs.** Reactions were performed with genomic DNA (~100 ng) or DNA-containing supernatant (3 μl) as the template, the indicated pair of primers at 1 μM (Fig. 1A and Table 2), 1× Taq master mix (New England BioLabs), and Taq master mix (New England BioLabs), and DNA grade water. PCRs were run in a MyCycler Thermal Cycler System (Bio-Rad). Products were run in 2% agarose gels, stained with ethidium bromide, and photographed using a ChemiDoc XRS gel documentation System (Bio-Rad).

**RNA extraction.** Total RNA was extracted as previously described (54, 55). Briefly, a cell suspension was prepared using 200 μl of acetate solution (20 mM sodium acetate [pH 5], 1 mM EDTA, 0.5% sodium dodecyl sulfate [Bio-Rad]) with 200 μl of saturated phenol (Fisher Scientific) added. This was incubated at 60°C in a water bath with vigorous shaking for 5 min and centrifuged at 17,000 × g at 4°C for 5 min. Cold ethanol was added to the supernatant obtained and mixed well by inverting the tube, and the mixture was centrifuged at 17,000 × g at 4°C for 5 min to obtain the RNA pellet. The pellet was resuspended in 20-μl aliquots at –80°C.

**qRT-PCR analysis.** Quantitative reverse transcription (qRT)-PCR was performed using the iScript One-Step RT-PCR kit with SYBR green (Bio-Rad) and the CFX96 Real-Time PCR Detection System (Bio-Rad). qRT-PCRs were performed in triplicate with 20 ng of total RNA and a 500 nM concentration of each primer. The first QS mechanism described involved a 17-amino-acid peptide (competence-stimulating peptide [CSP]) secreted by S. pneumoniae that regulates its competence state (50). Besides this QS system, S. pneumoniae reference strains (e.g., D39 and TIGR4) contain a luxS gene and produce AI-2 (21, 48). Some evidence suggests that this LuxS-controlled QS system might be part of the regulatory network controlling competence and LytA-dependent autolysis (43). In terms of pathogenicity, LuxS was implicated in virulence and persistence in the nasopharynx by using mouse models of pneumococcal infection (21, 48). The LuxS-generated signal has been implicated in differential expression of proteins in strain D39 (48) and regulation of genes involved in some metabolic processes, including the pneumolysin (Ply) gene (ph) (21). In this work, we demonstrate, for the first time, that LuxS plays a major role in controlling biofilm formation by S. pneumoniae strain D39. Two different D39-derivative luxS mutants were unable to produce early biofilms, while this phenotype was reversed by genetic complementation or physical complementation. Together, these results shed light on a new and important regulatory network of one of the most important human bacterial pathogens, S. pneumoniae.
primer (Table 2) under the following conditions: 1 cycle of 50°C for 20 min, 1 cycle of 95°C for 10 min, and 40 cycles of 95°C for 15 s and 55°C for 1 min. Melting curves were generated by a cycle of 95°C for 1 min and 55°C for 1 min and 80 cycles of 55°C with 0.5°C increments. The relative quantitation of mRNA expression was normalized to the constitutive expression of the 16S rRNA housekeeping gene and calculated by the comparative CT method (27).

Preparation of D39-derivative luxS mutant SPJV05 and complemented strains. To inactivate the luxS gene in strain D39, a DNA cassette containing the ermB gene (which confers resistance to erythromycin) flanked by the 5′ and 3′ regions of luxS was prepared. Briefly, ermB was PCR amplified using primers Ery-L and Ery-R (Table 2) and cloned into pCR2.1-TOPO. The 5′ and 3′ regions of luxS were PCR amplified using primers Lux5-L and Lux3-R, and a transformant named SPJV05 was obtained. The luxS gene was amplified using QiAamp gel extraction (Qiagen) and digested with KpnI and BamHI (5′ luxS fragment) or XhoI and XbaI (3′ luxS fragment). The digested fragments were again purified and ligated, using T4 DNA ligase (Promega), upstream or downstream, respectively, of pCR2.1-TOPO containing ermB to create plasmid pLuxS-ery-LuxS. The whole luxS-ery-luxS cassette (~1.1 kb) was amplified and then purified.

The luxS-ery-luxS cassette was transformed into competent cells of wild-type (WT) strain D39 by standard procedures (18). This transformation reaction mixture was incubated for 2 h at 37°C, plated onto BAP containing erythromycin, and incubated for 48 h at 37°C in a 5% CO2 atmosphere. Colonies were screened by PCR using primers Lux5-L and Lux3-R, and a transformant named SPJV05 was obtained. The luxS mutation was also verified by sequencing. Another D39ΔluxS mutant strain, EJ3, previously prepared and characterized (21), was kindly provided by Elizabeth Joyce of the Department of Microbiology and Immunology, Stanford University School of Medicine.

To prepare complemented strains, the luxS WT gene was amplified using primers LuxReg-L and LuxReg-R or LuxP2-L and LuxP2-R (Table 2) and cloned into Gram-positive plasmid pReg696 (14) or the S. pneumoniae integrative vector pPP2 (15) to create pJVR6 and pJVPP9 (Table 1). Plasmid pJVR6 or pJVPP9 was extracted from ECJV10 or ECJV11 and used to transform complemented strains of SPJV05 to create SPJV06 or SPJV07, respectively (Table 1). EJ3, resistant to spectinomycin, was only complemented with pJVPP9 or pPP2 (the empty vector).

Preparation of the inoculum for biofilm assays. An overnight BAP culture was used to prepare a cell suspension in THY broth to an optical density at 600 nm (OD600) of 0.05 and incubated at 37°C in a 5% CO2 atmosphere until the culture reached an OD600 of ~0.2. An aliquot (7 × 107 CFU/ml) was inoculated in triplicate into either an 8-well glass slide (Lab-Tek) or a polystyrene-treated 96- or 24-well microtiter plate (Corning) containing THY with no antibiotics and incubated at 37°C with 5% CO2 for the indicated times.

Quantification of biofilm biomass by crystal violet. Biofilms were washed three times with phosphate-buffered saline (PBS) and then allowed to dry for 15 min. Crystal violet (0.4%) was then added, and the biofilms were incubated for 15 min. After washing, crystal violet-stained biofilms were further dried at room temperature for 15 min. To quantify biofilm biomass, crystal violet was removed by adding 33% acetic acid solution. The absorbance of solubilized dye was obtained in an Epoch microplate spectrophotometer (Biotek).

Quantification of biofilm biomass by a fluorescence-based assay. Biofilms were fixed with 2% paraformaldehyde (Sigma) for 15 min and made permeable by the addition of 0.5% Triton X-100 (Roche) and incubation for 5 min at room temperature. After being washed three times with PBS, biofilms were blocked by adding 2% bovine serum albumin and stained for 1 h at room temperature with a polyclonal anti-S. pneumoniae antibody (~40 μg/ml) coupled to fluorescein isothiocyanate (FITC; Virostar, Portland, ME). To quantify biofilm biomass, FITC fluorescence readings (arbitrary units) were obtained using a VICTOR X3 Multilabel Plate Reader (Perkin-Elmer). The number of arbitrary fluorescence units of 24-h biofilms of WT strain D39 was set to a biofilm biomass of 100% and used to calculate the biofilm biomass percentages of all of the other S. pneumoniae strains tested or at different time points.

Physical complementation of the biofilm phenotype and AI-2 studies. To assess physical complementation, a mixture of two strains was incubated in the same biofilm assay but the biomass of only one strain was quantified. Briefly, WT strain D39 or strain SPJV05 was transformed with plasmid pMV1580 GFP, which contains the gfp gene under the control of the inducible maltose promoter (36), to create D39JVR6 or SPJV05 JVR6 (Table 1). The resulting mixture of these fluorescent versions was similar to that of its parent strain (data not shown). A mixture of WT strain D39 and SPJV07 or WT strain D39 and SPJV01 was then inoculated into the same biofilm bioassay. As a control, SPJV01 or SPJV08 was also inoculated into individual wells. After 6 h of incubation at 37°C, biofilms were washed and fluorescence readings or images were immediately obtained. For these experiments, the number of arbitrary fluorescence units obtained from SPJV01 biofilms was set to 100% of the biofilm biomass and the biofilm biomass of all of the other experimental conditions were calculated.

To physically separate the bacteria within the same biofilm assay, two chambers were created within the same well by installing a Transwell filter device (Corning, Corning, NY). The Transwell membrane (0.4 μm) creates a physical barrier that is impermeable to bacteria but allows the passage of small molecules between the two chambers (top and bottom). The WT D39 strain was inoculated into the top chamber (Transwell filter), while SPJV08 was inoculated into the bottom chamber. To further confirm whether LuxS mediates this secreted QS signal, luxS mutant strain SPJV05 was inoculated into the top chamber and SPJV08 was inoculated into the bottom chamber. These Transwell biofilm bioassays were incubated for 6 h at 37°C in a 5% CO2 atmosphere, and the biofilms produced by SPJV08 in the bottom chamber were quantified and photographed as described above.

For AI-2 studies, D39 or SPJV05 was inoculated in triplicate into 96-well plates with or without different concentrations (0.1 to 100 nM) of chemically synthesized AI-2 (dihydroxycetandelone; Omm Scientific) as used elsewhere (4, 9, 42) and incubated for 24 h. Biofilms were stained with fluorescent antibody, and biomass was calculated as described earlier. A concentration of 10 nM induced a statistically significantly different biofilm biomass when incubated along with D39 or SPJV05.

Statistical analyses. All data were analyzed using the nonparametric two-tailed Student t test and the Minitab 15 software.

RESULTS

The luxS gene is located in the same chromosomal region in S. pneumoniae isolates. Bioinformatic analysis of S. pneumoniae strains whose genomes have been sequenced indicated that the luxS gene is located in a region that contains genes
involved in division and cell wall biosynthesis. Specifically, luxS is situated upstream (~6.4 kb) of the gene cps4A (encoding a capsule polysaccharide biosynthesis protein) and ~4 kb downstream of the pbp2X gene, whose encoded protein is involved in cell wall biosynthesis and is a target of β-lactam antibiotics (30, 57), followed by mraY (encoding a phospho-N-acetylmuramoyl-pentapeptide-transferase [~3 kb]) (29) and, ~294 bp downstream, the clpL gene, which encodes a putative ATP-dependent Clp protease (Fig. 1A).

To investigate the presence of the luxS gene among S. pneumoniae strains, PCR analyses were performed with DNA extracted from invasive isolates and strains isolated from the nasopharynges of healthy children. Those PCR analyses revealed that all of the isolates surveyed (n = 103) contained the

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* When indicated (in parentheses), the position of the primer is relative to the hypothetical first ATG of the indicated gene of WT strain D39 (GenBank accession number CP000410).

* Italics show restriction enzyme sites added for cloning purposes.
luxS gene (Fig. 1B). To further study the location of the luxS gene, primers were designed so that a series of overlapping PCRs mapped its chromosomal location. This overlapping-PCR approach demonstrated that the luxS gene is always located downstream of an open reading frame (ORF) annotated as Sp0341 (TIGR4 annotation), which encodes a protein of unknown function. The size of the PCR product obtained was always the same and so included, as predicted by bioinformatic analysis, an intergenic region of ~94 bp between Sp0341 and the luxS gene (Fig. 1A and B).

Downstream of luxS in all of the isolates surveyed, overlapping PCR identified a gene annotated as clpL, encoding a putative ATP-dependent protease (Fig. 1B). For some invasive isolates (n = 23) or normal-flora strains (n = 19), the size of the PCR product correlated with that observed when using as the template the DNA of reference strain ATCC 33400, which belongs to serotype 1 (~1,200 bp). All of the other strains allowed the amplification of a product (~960 bp) similar to that of TIGR4 (serotype 4). The size of those PCR products could not be correlated with serotypes of the strains surveyed (not shown). Overlapping PCR detected the genes mraY and pbp2X downstream of luxS in 17 normal-flora strains and 22 invasive isolates (Fig. 1B). PCR analysis targeting mraY or pbp2X further clarified that these genes were not located in this position by the rest of the strains (data not shown). Taken together, these results indicate that the luxS gene is located near the capsule locus (cps) in all of the S. pneumoniae isolates surveyed.

Transcription of the luxS gene. In S. bovis and S. pyogenes, a homologous luxS gene is transcribed during the early mid-log phase of growth as a monocistronic message (5, 46). Unlike S. bovis, whose direction of transcription is the opposite of that of both its upstream and downstream genes (5), in S. pneumoniae, the direction of transcription of luxS is the same as that of its upstream gene (Sp0341) and the opposite of that of the downstream clpL gene (Fig. 1A).

To evaluate whether luxS mRNA is cotranscribed with Sp0341 by S. pneumoniae strain D39, qRT-PCR analyses were performed. These analyses showed that at 4 h postinoculation, levels of luxS transcripts increased ~20-fold with respect to the RNA from BAP (Fig. 2). While expression of Sp0341 mRNA also increased ~16-fold, almost no change (~0.5) was obtained for that of Sp0341-luxS. As expected, the transcription of a housekeeping gene used as an internal control (gyrB) did not change appreciably. Similar results were obtained by RT-PCR with S. pneumoniae strains TIGR4 and ATCC 33400 (not shown). Overall, these results indicate that the luxS gene of S. pneumoniae is transcribed as a monocistronic message during the mid-log phase of growth.

Development of a new fluorescence-based assay to quantify S. pneumoniae biofilm biomass. Before addressing the role of LuxS in S. pneumoniae biofilms, we quantified the biofilm biomass produced by reference strains D39 and TIGR4 and strain R6, a nonencapsulated variant of strain D39. As shown in Fig. 3A, the crystal violet assay demonstrated biofilm formation by all of the strains. The TIGR4 biofilm biomass was low (A630 <0.3), as previously described by Muñoz-Elias et al. (34). S. pneumoniae strain D39 produced more robust biofilms than either TIGR4 or R6 in 96-well plates (Fig. 3A) or 24-well plates (data not shown).

To optimize and improve the biofilm assay for S. pneumoniae strains, we developed a new fluorescence-based assay using an anti-S. pneumoniae antibody which is coupled to fluorescein. This fluorescent antibody will bind to the pneumococcus cell wall and stain the biofilm structure. Once formed, 24-h biofilms were fixed with 2% paraformaldehyde, permeabilized with 0.5% Triton X-100, and stained. We found that permeabilizing the biofilms with Triton X-100 produces a stable and reproducible quantification of fluorescence (data not shown). Arbitrary fluorescence units confirmed the tendency of strain D39 to form more biofilm biomass at 24 h postinoculation than strain TIGR4 or R6 (Fig. 3B). This new biofilm assay was more sensitive than the crystal violet assay. While biofilms stained with crystal violet began to be detectable at 6 h postinoculation (Fig. 3C), at the same time point, ~50% of the biofilm biomass was already detected by the fluorescence assay (Fig. 3D).

The LuxS-controlled QS mechanism regulates S. pneumoniae biofilms. To study the role of LuxS in pneumococcus biofilms, WT strain D39 and isogenic derivative luxS mutants SPJV05 and EJ3 were assessed using our newly developed fluorescence-based biofilm assay. In comparison with D39, SPJV05 or EJ3 produced only ~20% of the biofilm biomass at 24 h postinoculation (Fig. 4A). Epifluorescence images of biofilms produced by WT strain D39 showed bacteria attached to the bottom, covering the entire surface of the well (Fig. 4B). A higher magnification (~63) showed that the structure formed by D39 is highly organized, forming compact layers of bacteria that create aggregates where the fluorescence signal is more intense (not shown). In contrast, fluorescence images of bio-

![FIG. 2. Monocistronic transcription of the luxS gene during the mid-log phase of growth. qRT-PCRs were performed with RNA extracted from D39 grown overnight in BAP or THY broth at 4 h postinoculation. Primers amplified ORF Sp0341, Sp0341 and the luxS gene (0341-luxS), the luxS gene, or the gyrase B subunit gene. Average CT values were normalized to the 16S rRNA housekeeping gene, and n-fold differences were calculated by the comparative CT (2−ΔΔCT) method (27). The values above the bars indicate the calculated n-fold changes relative to the overnight BAP culture. Error bars represent the standard error of the mean calculated using data from three independent experiments.](image-url)

![FIG. 3. Development of a new fluorescence-based assay to quantify S. pneumoniae biofilm biomass.](image-url)
films produced by SPJV05 or EJ3 clearly show few bacteria attached to the bottom of each well (Fig. 4B). Indeed, both mutants appear to form small aggregates in the bottom of the well that do not progress to form a mature biofilm (Fig. 4B).

To confirm the role of luxS in S. pneumoniae biofilms, complemented strains SPJV04 and SPJV06 were assessed for biofilm production. As shown in Fig. 4A, complementation of luxS mutants with a copy of luxS integrated either in bgaA or in a plasmid, SPJV04 and SPJV06, respectively, restored the biofilm biomass to the WT level. Production of biofilm biomass by SPJV02, containing the pPP2 empty vector, was similar to that of EJ3 (Fig. 4A), demonstrating that disruption of bgaA did not alter the biofilm phenotype. Epifluorescence images of SPJV04 and SPJV06 biofilms show structures similar to that of WT strain D39 (Fig. 4B).

LuxS regulates early events in biofilm formation. To begin exploring the mechanism by which LuxS controls biofilm formation, a time course study was conducted that evaluated the early production of biofilms. Figure 5A shows that at 3 h postinoculation, ~10% of the biofilm biomass had already been produced by the WT strain. The biofilm biomass reached ~25%, ~45%, and ~80% after 4, 6, and 8 h of incubation, respectively (Fig. 5A), while the biofilm biomass at 10 or 12 h postinoculation was similar to that produced in a 24-h period.

FIG. 3. Quantification of the biofilm biomass of S. pneumoniae strains. (A and B) An aliquot of the indicated strain was inoculated in triplicate into 96-well plates and incubated for 24 h at 37°C in a 5% CO₂ atmosphere. In panels C and D, S. pneumoniae D39 was inoculated and incubated for the indicated time. Biofilms were stained with crystal violet (A and C) or with an anti-S. pneumoniae polyclonal antibody coupled to FITC (B and D). The biofilm biomass (arbitrary fluorescence units) of WT strain D39 at 24 h postinoculation was adjusted to 100%, and the biomass percentages of the other strains or time points were calculated.

FIG. 4. Biofilm formation by S. pneumoniae strain D39 is regulated by the luxS gene. (A) The indicated strain was inoculated into 96-well plates and incubated for 24 h. Biofilms were stained by a fluorescent antibody, and the biomass of WT strain D39 was adjusted to 100% to calculate all of the others. In all of the panels, the error bars represent the standard error of the mean calculated using data from at least four independent experiments. Asterisks indicate values statistically significantly different from that of WT strain D39 (P ≤ 0.05, calculated using a nonparametric t test). (B) Biofilms were imaged using an inverted fluorescence microscope. The bar in the bottom right panel is valid for all of the panels.
In contrast, biofilms produced by SPJV05 were undetectable at early time points (i.e., 2 or 4 h postinoculation) while at 8 h postinoculation, only 15% of the 24-h biofilm biomass was found (Fig. 5A).

As shown in Fig. 5B, small pneumococcus aggregates could be detected as early as 2 h postinoculation with the WT strain. Those D39 aggregates became more evident after 4 and 6 h of incubation and covered almost the entire surface after 8 h (Fig. 5C, D, and E). Bacterial aggregates produced by SPJV05, however, were smaller and covered only 20% of the surface at 8 h postinoculation (Fig. 5F), and by complemented strain SPJV06 at 8 h postinoculation (G) were imaged using an inverted fluorescence microscope. The bar in the bottom left panel is valid for all of the panels.

Evidences that LuxS-mediated AI-2 regulates early biofilm formation. To confirm that a QS signal was responsible for the biofilm defect of the luxS mutants, the WT strain D39 and SPJV05 were inoculated into 96-well plates and incubated for 1 to 8 or 24 h at 37°C in a 5% CO₂ atmosphere. (A) Biofilms were stained with an anti-S. pneumoniae antibody coupled to FITC and quantified using a fluorometer. The biofilm biomass of WT strain D39 at 24 h postinoculation was set to 100%, and all of the others were calculated. Error bars represent the standard error of the mean calculated by using data from three independent experiments. The asterisk indicates a value statistically significantly different from that of WT strain D39 at 8 h postinoculation (P \leq 0.05, calculated using a nonparametric t test). Biofilms formed by WT strain D39 at 2 h (B), 4 h (C), 6 h (D), or 8 h (E) postinoculation, by strain SPJV05 at 8 h postinoculation (F), and by complemented strain SPJV06 at 8 h postinoculation (G) were imaged using an inverted fluorescence microscope. The bar in the bottom left panel is valid for all of the panels.

FIG. 5. Time course study of biofilm formation. Strain D39 or SPJV05 was inoculated into 96-well plates and incubated for 1 to 8 or 24 h at 37°C in a 5% CO₂ atmosphere. (A) Biofilms were stained with an anti-S. pneumoniae antibody coupled to FITC and quantified using a fluorometer. The biofilm biomass of WT strain D39 at 24 h postinoculation was set to 100%, and all of the others were calculated. Error bars represent the standard error of the mean calculated by using data from three independent experiments. The asterisk indicates a value statistically significantly different from that of WT strain D39 at 8 h postinoculation (P \leq 0.05, calculated using a nonparametric t test).

Biofilms formed by WT strain D39 at 2 h (B), 4 h (C), 6 h (D), or 8 h (E) postinoculation, by strain SPJV05 at 8 h postinoculation (F), and by complemented strain SPJV06 at 8 h postinoculation (G) were imaged using an inverted fluorescence microscope.
of the WT at all time points (data not shown). Complemented stains showed transcript levels similar to that of the WT strain D39 was set to 100%, and all of the others were calculated. The symbol * or # indicates a value statistically significantly different (P ≤ 0.05, calculated using nonparametric t test) from that of WT strain D39 or SPJV05, respectively.

In contrast, the bottom of the well and SPJV05 in the Transwell system was not significantly different from that of SPJV08 alone (Fig. 6A).

To confirm the role of secreted AI-2 in the biofilm phenotype, strain D39 or SPJV05 was inoculated along with chemically synthesized AI-2. As shown in Fig. 7, AI-2 allowed the production of a biofilm biomass greater than that produced by strains grown with no purified AI-2.

Temporal expression of the luxS gene and evidence that LuxS regulates lytA mRNA and ply mRNA levels. Since we had demonstrated that LuxS regulates early biofilm formation, a time course study was conducted to evaluate levels of luxS mRNA expression during the early mid-log phase of growth. The luxS transcript was found to be maximally expressed (~28-fold increase) at 4 h postinoculation (Fig. 8A). At that time point, the changes in luxS mRNA in some invasive isolates and normal flora ranged from ~6 to ~300-fold and expression could not be correlated to the subset of strains (data not shown). A clear decline in the levels of luxS mRNA in strain D39 was obtained at 6 h (5-fold difference) and 8 h (3.8-fold difference) postinoculation. As expected, gyrB mRNA levels did not significantly change after 2, 4, 6, or 8 h of incubation (Fig. 8A). These results indicate that the transcription of the luxS gene, and therefore its activity, is maximal during the early mid-log phase of growth.

Previous studies have demonstrated that LytA, the capsular polysaccharide, the neuraminidase NanA, and choline binding proteins such as PspA play a role in S. pneumoniae biofilm formation (32, 40). Increased levels of Ply have also been detected in S. pneumoniae biofilms during the last few years, the biofilm controlled QS system regulates luxS. Strain D39 or SPJV05 was inoculated along with chemically synthesized AI-2. As shown in Fig. 7, AI-2 allowed the production of a biofilm biomass greater than that produced by strains grown with no purified AI-2.

DISCUSSION

S. pneumoniae strains usually colonize the nasopharynges of healthy children during the first months of life and either leave the children asymptomatic or go on to cause diseases such as pneumonia, meningitis, and otitis media (17, 37). It has been postulated that the pneumococcus resides in the human nasopharynx, forming biofilms (33). Despite the increasing importance of S. pneumoniae biofilms during the last few years, the regulatory network behind these structures has not been completely elucidated. The present study demonstrates, for the first time, that the luxS-controlled QS system regulates S. pneumoniae early biofilm formation. This AI-2-mediated regulatory network appears to be specific for a subset of biofilm effectors, since in this research and elsewhere (21), LuxS was found to regulate a particular set of genes in planktonic cultures.

Recent experimental evidence indicates that the LuxS QS system is implicated in the persistence, virulence, and dissem-
ination of *S. pneumoniae* (3, 21, 48). A previous study by Stroehler et al. (48) demonstrated that a D39-derivative *luxS*-null mutant was less able to spread to the lungs or the blood than was WT strain D39, suggesting that the QS signal might be important for dissemination within the host. This *luxS* mutant was also less virulent for mice than WT strain D39 was (48). Another study, by Joyce et al. (21), showed that this QS system is implicated in persistence in the mouse nasopharynx.

Our results extend these observations by demonstrating that LuxS is absolutely required for the establishment of early biofilm structures. While *luxS* mutants were unable to form early biofilms, the phenotype was fully restored in the complemented strains. In an attempt to verify that a secreted QS signal was controlling early biofilms, we used chemically synthesized AI-2 or AI-2 containing supernatants from WT strain D39 that demonstrated statistically significantly more biofilm biomass when AI-2 was incubated along with the *luxS* mutant or the WT (Fig. 7).

Recent publications have also shown that the competence QS system (Com), which is regulated by the secreted CSP, controls biofilm production by *S. pneumoniae* strains (38, 52). An investigation by Oggoni et al. (38) showed that supplementing strain D39 with exogenous CSP produces more biofilm biomass, and in a more recent publication, Trapetti et al. (52) demonstrated that a TIGR4-derivative *comC* mutant was unable to form biofilms, while the phenotype could be restored by adding exogenous CSP. Specific CSP-regulated biofilm effectors and potential LuxS-Com synergism for biofilm development, if any exist, remain to be investigated.

The present study shows that the *luxS* gene is located near the capsular locus in *S. pneumoniae* strains and transcribed as a monocistronic unit. Expression of the capsular polysaccharide has been implicated in virulence (35) and biofilm formation (32); however, the LuxS system does not seem to regulate capsule genes, since our qRT-PCR studies showed that the *luxS* mutant contained a level of csp4A mRNA similar to that of the WT. Other proteins previously implicated in biofilms, such as the neuraminidase NanA (40) and choline binding protein PspA (32), were also found not to be regulated by this system. The expression of *lytA*, encoding the autolysin involved in cell wall degradation (20) and production of biofilms (32), however, was found to be regulated by LuxS during the mid-log phase of growth (Fig. 8B). Whereas our experiments showed that *lytA* transcripts were higher in the WT during the early mid-log phase (2 to 4 h) of growth, the mutant had reduced *lytA* mRNA levels at 4 h postinoculation. In line with these results, evidence indicating that LuxS may play a role in LytA-dependent autolysis in the stationary phase of growth (at 5 to 7 h postinoculation) has been published (43). Therefore, LuxS appears to regulate levels of *lytA* mRNA in exponentially growing cultures that results in a defect in the autolysis phenotype during the stationary phase. A complete characterization of the biology of this phenomenon is under way in our labs.

Evidence now directly links the production of pneumococcal biofilms with LuxS regulation. For instance, a previous report found that a *luxS* mutant had a protein expression profile (cytosolic and membrane proteins) different from that of WT strain D39 (48). Joyce et al. (21), using microarrays, detected 46 genes down- or upregulated by the LuxS system only when they used RNA extracted from WT strain D39 and the *luxS* mutant growing in the early mid-log phase of growth. Since levels of the *luxS* transcript are also higher during the early mid-log phase of growth (Fig. 8A) and the *luxS* mutant is unable to produce biofilms (Fig. 4A), regulation of genes encoding proteins implicated in early biofilm formation, such as *lytA*, should be a main target of the LuxS-generated signal.

The regulation of proteins present in early biofilms has been previously investigated (2). A particular association between early biofilm-produced proteins and the LuxS system was the discovery that levels of Ply, a protein implicated in colonization and virulence in animal models (19, 39), increase during early stages of biofilm formation (2). The gene that encodes it (*ply*) has also been shown by microarray analyses to be regulated by the LuxS system (21). Our studies extend this observation by demonstrating, by qRT-PCR, that LuxS dramatically impacts levels of *ply* transcripts during the early mid-log phase of growth (Fig. 8C). The contribution of Ply to *S. pneumoniae*-produced biofilms is unclear and, given that D39Δ*ply* has a reduced ability to colonize the mouse nasopharynx (39), requires further elucidation. It may have a role in initial attachment, since Ply is not secreted by pneumococcus but rather located in the cell wall (41).

Our study also introduced a new fluorescence-based biofilm assay that is specific for *S. pneumoniae* strains. While other nonspecific fluorescence methods have been used to visualize *S. pneumoniae* biofilms in vitro (2, 12, 32), our newly developed assay uses an anti-*S. pneumoniae* antibody coupled to FITC that permits the quantification of biofilm biomass and direct visualization of those structures in a single assay. This assay may also be useful for studies where a heterogeneous population of bacteria (i.e., normal-flora strains) are present with or within *S. pneumoniae* biofilms, for example, in samples collected from children with pneumococcal diseases, animal studies, and *in vitro* studies dissecting the contribution of other bacteria to pneumococcal biofilms.

An important feature of our assay is that the permeabilization of the biofilm structure with Triton X-100 may allow those anti-*S. pneumoniae* antibodies to reach most of the pneumococci within the biofilm matrix. Levels of arbitrary fluorescence units were consistently higher only when biofilms were made permeable before staining with the fluorescent antibody. Since *S. pneumoniae* biofilm structures have been calculated to be ~25 µm thick (32), it is possible that the crystal violet dye does not reach all of the pneumococci cells within the matrix. The new fluorescence assay is more sensitive, being able to both quantify and visualize early biofilm structures (bacterial aggregates) within 2 h postinoculation (Fig. 3). In contrast, our experiments and those reported by Muñoz-Elias et al. (34), who used crystal violet, detected *S. pneumoniae* biofilms at 6 to 8 h postinoculation.

Using this new assay to gain insights into biofilm biology has shown that pneumococcal biofilm formation involves three stages, (i) initial attachment between 2 and 4 h, (ii) formation of bacterial aggregates occupying ~50% of the surface between 4 and 6 h, and (iii) biofilm development. Similar stages have been previously discerned using a continuous-flow biofilm reactor, although in that study the stages were observed between 1 and 6 days (2). Once bacterial aggregates were produced (stage 2), biofilms continued growing exponentially, even in the absence of planktonic cells (R. M. Kunkel et al.,
unpublished data). In fact, with the sole exception of *cps4A*, whose mRNA levels remained similar, biofilm cells showed levels of *napA*, *ply*, *pssA*, and *lta4* transcripts at 6 h postinoculation that were higher than those of their counterpart planktonic cultures (data not shown), clearly demonstrating active metabolism within biofilm cells. Attempts to determine the levels of these transcripts in a luxS background failed because of the absence of biofilm cells at that time point.

In summary, we have demonstrated that the *S. pneumoniae* LuxS-controlled QS system regulates early biofilm formation. Biofilm structures might be important for *S. pneumoniae* strains to persist and possibly cause important diseases such as otitis media or pneumonia. Findings in the present study may have implications for developing new targets to reduce pneumococcal carriage and therefore pneumococcal disease.

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