Quorum-Sensing Systems LuxS/Autoinducer 2 and Com Regulate *Streptococcus pneumoniae* Biofilms in a Bioreactor with Living Cultures of Human Respiratory Cells

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Streptococcus pneumoniae forms organized biofilms in the human upper respiratory tract that may play an essential role in both persistence and acute respiratory infection. However, the production and regulation of biofilms on human cells is not yet fully understood. In this work, we developed a bioreactor with living cultures of human respiratory epithelial cells (HREC) and a continuous flow of nutrients, mimicking the microenvironment of the human respiratory epithelium, to study the production and regulation of *S. pneumoniae* biofilms (SPB). SPB were also produced under static conditions on immobilized HREC. Our experiments demonstrated that the biomass of SPB increased significantly when grown on HREC compared to the amount on abiotic surfaces. Additionally, pneumococcal strains produced more early biofilms on lung cells than on pharyngeal cells. Utilizing the bioreactor or immobilized human cells, the production of early SPB was found to be regulated by two quorum-sensing systems, Com and LuxS/AI-2, since a mutation in either *comC* or *luxS* rendered the pneumococcus unable to produce early biofilms on HREC. Interestingly, while LuxS/autoinducer 2 (AI-2) regulated biofilms on both HREC and abiotic surfaces, Com control was specific for those structures produced on HREC. The biofilm phenotypes of strain D39-derivative $\Delta comC$ and $\Delta luxS$ QS mutants were reversed by genetic complementation. Of note, SPB formed on immobilized HREC and incubated under static conditions were completely lysed 24 h postinoculation. Biofilm lysis was also regulated by the Com and LuxS/AI-2 quorum-sensing systems.

Streptococcus pneumoniae (the pneumococcus) colonizes the mucosal surface of the human nasopharynx in early childhood (1–3). Bacteria can persist in this niche for months or can cause severe illnesses, such as otitis media and pneumonia, which can be rapidly transmitted to other children (1, 4). After colonization, the pneumococcus forms highly organized structures called biofilms on the epithelial surface of the nasopharynx. *S. pneumoniae* biofilms (SPB) may aid in competition against other pneumococci and the native flora, as well as providing a means to evade both the host immune response and the action of antibiotics (5, 6). Thus, biofilms are important for persistence in the human nasopharynx and may be considered a risk factor for pneumococcal disease (3, 7).

Recent investigations reveal that both invasive and carriage isolates of *S. pneumoniae* are capable of producing biofilms on abiotic surfaces, such as glass and polystyrene (5, 8). Perhaps more importantly, biofilm structures have been detected on the surface of adenoid and mucosal epithelial cells from biopsy specimens collected from children with chronic otitis media (9), on the sinus mucosa of human subjects with chronic rhinosinusitis (10), and in the middle-ear mucosa of chinchillas experimentally infected with *S. pneumoniae* (11). *S. pneumoniae* also produces biofilms in the nasopharynx, trachea, and lungs of mice (12–14). SPB may contribute to the increasing rates of antibiotic resistance among pneumococci (15–17).

We and others have independently discovered that a quorum-sensing (QS) system, LuxS/autoinducer 2 (AI-2), regulates *S. pneumoniae* biofilm production on abiotic surfaces (i.e., glass and polystyrene), a finding consistent with the observation that this system regulates pneumococcal persistence in the mouse nasopharynx (18–20). In this signaling network, the enzyme LuxS synthesizes AI-2, which is required for QS-regulated gene expression

(21). In *S. pneumoniae*, the *luxS* gene is carried by both invasive and carriage strains, and its maximum expression is observed in early log phase of planktonic cultures (18). The accumulation of secreted AI-2 in the external milieu stimulates planktonic bacteria to initiate early formation of the biofilm structure, since cultures of a D39-derived *luxS* null mutant remained planktonic and this phenotype was reversed by adding purified AI-2 and by genetic complementation (18). The LuxS/AI-2-controlled regulatory cascade impacts the transcription of genes involved in cellular processes and virulence factors, including the mRNA levels of *ply*, encoding pneumolysin (Ply) (18, 20), another putative hemolysin (SP1466, TIGR4 annotation) (20), and the *cbpD* gene, encoding choline-binding protein D (CbpD). Of these, a role for Ply in SPB is being investigated in our laboratory (J. R. Shak, H. P. Ludewick, K. E. Howery, K. P. Klugman, and J. E. Vidal, unpublished data), whereas Trappetti et al. recently demonstrated that CbpD is required for biofilm formation (19).

An essential attribute of most streptococcal species is the natural propensity for genetic transformation (i.e., DNA release and uptake) mediated by the Com QS system (22). This system is

Received 8 October 2012 Returned for modification 8 November 2012

Accepted 1 February 2013

Published ahead of print 12 February 2013

Editor: B. A. McCormick

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/IAI.01096-12>.

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doi:10.1128/IAI.01096-12

encoded by the operon *comCDE*, where *comC* encodes a secreted 17-amino-acid peptide pheromone (the competence-stimulating peptide [CSP]), *comD* encodes the CSP histidine kinase receptor, and *comE* encodes the response regulator (23, 24). CSP-induced transcriptional regulation affects more than 180 genes that have been classified based on timing of expression as early, late, or delayed genes, of which only 23 are required for competence (25, 26). Natural transformation is spontaneously activated in laboratory broth cultures when an early-log-phase culture of planktonic cells reaches an optical density at 555 nm (OD_{555}) of 0.15 to 0.2, at which a critical concentration of secreted CSP is present in the external milieu (e.g., ~ 1 ng/ml) (23). In terms of pathogenicity, a number of investigators have demonstrated attenuated virulence of *S. pneumoniae* bacteria with mutations in competence genes using mouse models of pneumococcal infection (27, 28).

The biological role for these two QS systems (LuxS/AI-2 and Com) during colonization, carriage, and disease is still not fully understood. A recent publication by Wei and Hvarstein demonstrated that gene transference among streptococci is more efficient among competent biofilm cells, grown on nonbiotic surfaces, than in planktonic cultures (29). The transformation efficiency of *S. pneumoniae* biofilms peaked in early structures (~ 4 h postinoculation) and declined 8 h postinoculation (29). While transformability of planktonic cells lasts no longer than 30 min, competent biofilm cells grown on polystyrene can take up foreign DNA for ~ 4 h (29). Similarly, *Streptococcus mutans* biofilms remain transformable between 4 and 24 h postinoculation (30).

Some evidence links the switch from planktonic to biofilm form with the induction of competence. For example, culture media that favor competence in *S. mutans* also promote biofilm formation (30). In the case of *S. pneumoniae*, the addition of CSP to planktonic cultures has been shown to increase early formation of biofilms on plastic surfaces (31). However, whether cultivated on polystyrene under static conditions or in a continuous culture system, the biofilm formation of an *S. pneumoniae* D39 or TIGR4 *comC* null mutant was not affected (32).

A number of pathogenic bacteria cause human disease related to their ability to form biofilms on different substrates, including human tissues and epithelia. For instance, *Vibrio cholerae* biofilms, which are produced on the human intestine, are excreted in the stool of cholera patients (33). *Pseudomonas aeruginosa* biofilms have been imaged from cystic fibrosis sputum (34), and *Haemophilus influenzae* and *S. pneumoniae* biofilms have been detected in the middle ear mucosa of children with otitis media (9). Although significant advances in the study of these structures have been made using abiotic substrates (e.g., glass and polystyrene), current *in vitro* models for biofilms on eukaryotic cells are scarce. A more realistic *in vitro* technology for the evaluation of biofilms made on biotic surfaces should include living cultures of human cells and a continuous flow of nutrients, such as would physiologically occur when biofilms are formed on the human nasopharynx, lungs, or intestines.

A study by Parker et al. demonstrated that previous exposure of *S. pneumoniae* strain D39 to airway epithelial cells enhanced the ability of the pneumococcus to form biofilms on polystyrene plates (35). A more recent study by Marks et al. demonstrated that pneumococcal biofilms are more robust when they are produced on paraformaldehyde (PFA)-fixed bronchial NCI-H292 cells and primary cultures of human bronchial epithelial cells (14). In this study, we developed a biofilm reactor (bioreactor) with living cul-

tures of human lung cells and also used immobilized human respiratory epithelial cells (HREC) to evaluate the production, regulation, and autolysis of SPB. Our data demonstrate that invasive isolates produce more-robust early biofilms on lung cells than on pharyngeal cells. Furthermore, SPB produced on human cells are regulated by two different QS systems, Com and LuxS/AI-2. Com control of SPB was, however, specific to those structures produced on HREC. We also describe a mechanism involved in the regulation of biofilm autolysis.

MATERIALS AND METHODS

Strains, plasmids, and bacterial culture media. *S. pneumoniae* strains, plasmids, and derivative strains utilized in this study are listed in Table 1. Strains were cultured on blood agar plates (BAP) or grown in Todd Hewitt broth containing 0.5% (wt/vol) yeast extract (THY). Where indicated below, 2% maltose (wt/vol), ampicillin (100 μ g/ml), tetracycline (1 μ g/ml), or erythromycin (0.5 μ g/ml) was added to the culture medium.

Cell cultures. Human-derived lung A549 cells (ATCC CCL-185) and HEP-2 cells (ATCC CCL-23) were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with heat-inactivated 10% fetal bovine serum (FBS) (Atlanta biologicals), 1% nonessential amino acids (Sigma), 1% glutamine (Sigma), penicillin (100 U/ml), and streptomycin (100 μ g/ml), and the pH was buffered with HEPES (10 mM) (Gibco). Human pharyngeal Detroit 562 cells (ATCC CCL-198) were cultured in EMEM (Eagle's minimum essential medium; Lonza, Walkersville, MD) supplemented as described above, except that non-heat-inactivated FBS was used. Each cell line was normally harvested with 0.25% trypsin (Gibco), resuspended in the cell culture medium, and incubated at 37°C in a 5% CO₂ humidified atmosphere.

Preparation of the inoculum for biofilm assays. The inoculum was prepared as previously described (18). Briefly, an overnight BAP culture was used to prepare a cell suspension in THY broth to an OD_{600} of 0.05. This suspension was incubated at 37°C in a 5% CO₂ atmosphere until the culture reached an OD_{600} of ~ 0.2 (early log phase), and then glycerol was added to a final 10% (vol/vol) and the culture stored at -80°C until used.

Production of biofilms on immobilized HREC. Cells were grown until confluent (~ 5 days) on 8-well glass slides (Lab-Tek), tissue culture-treated 6-well polystyrene plates, or CellBIND surface 24-well polystyrene plates (Corning) and immobilized by fixation with 2% paraformaldehyde (Sigma) for 15 min. After extensive washes with sterile phosphate-buffered saline (PBS), immobilized HREC were supplemented with either cell culture medium without antibiotics or THY. Immobilized respiratory cells (biotic surfaces) were then infected with an aliquot containing $\sim 7 \times 10^5$ CFU/ml of each strain. Where indicated below, strains were also inoculated onto wells containing no cells (abiotic surfaces). At the end of incubation, biofilms were washed twice and analyzed as described in the following sections. To collect biofilm cells (e.g., for biofilm counts), plates were sonicated for 15 s in a Branson ultrasonic water bath (Branson, Danbury, CT), followed by extensive pipetting to remove all attached bacteria. When possible, detachment was verified using a fluorescence microscope (i.e., for green fluorescent protein [GFP]-expressing bacteria).

Bioreactor with living cultures on human lung cells. A549 cells were grown on Snapwell filters (Corning); these filters have a polyester membrane (0.4 μ m) supported by a detachable ring. Once confluent (4 to 5 days), cells were inoculated as detailed above and immediately placed in sterile vertical diffusion chambers. Both the basolateral and apical sides (inner chamber) were perfused with sterile DMEM with no antibiotics using a Master Flex L/S precision pump system (Cole-Parmer, Vernon, IL). To avoid the accumulation of toxic substances but allow biofilm formation, a low flow rate (0.20 ml/min) was applied. The bioreactor's inner chamber was filled with ~ 5 ml of medium, whereby complete replacement of the cell culture medium was achieved approximately every 25 min. Bioreactor chambers containing *S. pneumoniae*-infected lung cells were incubated at 37°C under a sterile environment. At the end of the

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
<i>S. pneumoniae</i> strains		
D39	Avery strain, clinical isolate capsular serotype 2	60, 61
R6	D39-derivative unencapsulated laboratory strain	61
TIGR4	Invasive clinical isolate, capsular serotype 4	37
ATCC 33400	Invasive isolate, serotype 1, other designation NCTC 7465	ATCC/NCTC ^a
ATCC 49619	Invasive isolate, serotype 19F, other designation NCTC 12977	ATCC/NCTC
Sp23F	Invasive isolate, serotype 23F	Laboratory collection
SPJV01	D39 encoding pMV158GFP, Tet ^r	18
SPJV05	D39-derivative <i>luxS</i> null mutant, Ery ^r	18
SPJV06	SPJV05 containing pJVR6, Ery ^r Spec ^r	18
SPJV08	SPJV05 containing pMV158GFP, Ery ^r Tet ^r	18
SPJV09	TIGR4 containing pMV158GFP, Tet ^r	This study
SPJV10	D39-derivative <i>comC</i> null mutant, Ery ^r	This study
SPJV11	SPJV01-derivative <i>comC</i> null mutant, Ery ^r Tet ^r	This study
SPJV12	SPJV10 containing pKH <i>comC</i>	This study
<i>E. coli</i> TOP10	Cloning host	Invitrogen
Plasmids		
pCR2.1 TOPO	Cloning vector	Invitrogen
pPP2	Integrative plasmid for <i>S. pneumoniae</i> strains	62
pJVR6	pReg-derivative containing the <i>luxS</i> wt gene from strain D39	18
pMV158GFP	<i>S. pneumoniae</i> mobilizable plasmid encoding green fluorescent protein	63
pKH <i>comC</i>	pPP2 containing the <i>comC</i> wt gene from strain D39	This study

^a ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures.

incubation period, inserts containing biofilms were removed and the biomass was analyzed as described below.

Quantification of biofilm biomass. Biofilm development was evaluated by fluorescence as previously reported (18) or by obtaining biofilm cell counts (CFU/ml) by dilution plating. Briefly, the fluorescence of biofilms produced on immobilized HREC was quantified directly from GFP-expressing bacteria or by fluorescence staining as follows: after PBS washes, biofilms were fixed with 2% paraformaldehyde for 15 min, made permeable by adding 0.5% Triton X-100 (Roche), and incubated for 5 min at room temperature. After washing three times with PBS, biofilms were blocked by adding 2% bovine serum albumin (BSA) and stained for 1 h at room temperature with a polyclonal anti-*S. pneumoniae* antibody (~40 µg/ml) coupled to fluorescein isothiocyanate (FITC; ViroStat, Portland, ME). To quantify biofilm biomass, fluorescence readings (arbitrary units) were obtained using a Victor X3 multilabel plate reader (Perkin-Elmer). The fluorescence arbitrary units obtained under a chosen experimental condition (e.g., wild-type [wt] biofilms 8 h postinoculation) were set to 100% of biofilm biomass and used to calculate the percentage of biofilm biomass of all other conditions tested.

Quantification of extracellular DNA (eDNA). Strains were inoculated into 24-well plates containing immobilized A549 cells and incubated for the times indicated below at 37°C with 5% CO₂. The supernatant was removed, centrifuged for 5 min at 12,000 × g in a refrigerated centrifuge (Eppendorf), and filter sterilized using a 0.2 µm syringe filter. This supernatant was mixed with a 0.5 volume of ethanol and vortexed for 10 s, and the DNA was purified using the QIAamp DNA minikit (Qiagen) following the manufacturer's instructions. To quantify amounts of DNA, a quantitative PCR (qPCR) assay targeting the *lytA* gene was utilized. Reactions were performed with IQ SYBR green super mix (Bio-Rad), 300 nM each primer (*lytA*-Fwd and *lytA*-Rev) (Table 2), and 2 µl of DNA template. Reactions were run in duplicate using a CFX96 real-time PCR detection system (Bio-Rad) with the following conditions: 1 cycle at 55°C for 3 min, 1 cycle at 95°C for 2 min, and 40 cycles of 95°C for 15 s, 55°C for 1 min, and 72°C for 1 min. Melting curves were generated by a cycle of 95°C for 1 min and 65°C for 1 min and 80 cycles starting at 65°C with 0.5°C increments. For quantification purposes, standards (1, 10, 100, and 1,000 pg of DNA per reaction) were run in parallel to generate a standard curve

and the amounts of eDNA were calculated using Bio-Rad CFX Manager software.

Confocal microscopy studies of SPB produced in the bioreactor. *S. pneumoniae* biofilms produced on human lung cells were fixed with 2% paraformaldehyde and washed three times with PBS. Sialic acid residues present on plasma membranes were first stained with wheat germ agglutinin (WGA; 5 µg/ml) conjugated with Alexa Fluor 555 (Molecular Probes, Invitrogen) for 30 min (36). Cells were then washed twice with PBS, permeabilized with 0.5% Triton X-100 for 5 min, and washed again with PBS, and SPB were fluorescence stained as described above. Nucleic acids were stained with TO-PRO-3 (1 µM), a carbocyanine monomer nucleic acid stain (Molecular Probes), for 15 min. Staining procedures were done at room temperature. The preparations were finally washed three times with PBS, mounted with Vectashield mounting medium (Vector Laboratories), and analyzed with a Zeiss LSM 510 confocal microscope. Confocal images were analyzed with LSM Image Browser, version 4.0.2.121.

Electron microscopy studies. Human lung A549 cells were grown on Thermanox coverslips (Fisher Scientific) that had been installed in 6-well plates (Corning) until fully confluent (~5 days). Cells were fixed with 2% PFA, washed, inoculated with strain D39, and incubated for 8 or 24 h at

TABLE 2 Primers used in this study

Primer	Sequence ^a
Ery-L-XbaI	CAGTCTAGAAAAAATTTGTAATTAAGAAGGAGT
Ery-R-XhoI	CAGCTCGAGCCAAATTTACAAAAGCGACTCA
ComC5-L	GATTTGCTAAGTTTGAAATGATTGA
ComC5-R-XbaI	CAGTCTAGACCTTCAAAGCTACAAACTGTTC
ComC3-L-XhoI	CAGCTCGAGTTGTCAAATTTCTCCGTGATTT
ComC3-R	GCAATCTCTTCTCCATTTGAA
comC-L	GGGGTACCTTTACGATTACAGCAGGGATTC
comC-R	GGTCTAGATCTGTTAGTAATGTATAAGCACCA
<i>lytA</i> -Fwd	GTGAGCAGTTTAAGCATGATATTG
<i>lytA</i> -Rev	CAAAAGACAAGTTTGAGAAAATCA

^a Italics show restriction enzyme sites added for cloning purposes.

37°C with 5% CO₂. Pneumococcal biofilms produced on lung cells were then washed three times with PBS and fixed with a 2.5% glutaraldehyde solution in 0.1 M cacodylate buffer, pH 7.4, overnight and then washed with the same buffer. For scanning electron microscopy (SEM), preparations were post fixed with 1% osmium tetroxide and 1.5% potassium ferrocyanide in 0.1 M cacodylate buffer for 1 h. They were subsequently rinsed with deionized water, followed by dehydration through an ethanol series ending with three exchanges of absolute ethanol. The samples were then placed into individual ventilated processing vessels in fresh absolute ethanol and placed into a Polaron E3000 critical-point drying unit, where the ethanol was exchanged for liquid CO₂. This liquid CO₂ was eventually brought to its critical point of 1,073 lb/in² at 31°C and allowed to slowly vent. Dried samples were then secured to labeled aluminum SEM stubs and coated with approximately 15 nm of chromium with a Denton DV-602 turbo magnetron sputter coater. Samples were then viewed with a Topcon DS130F field emission scanning electron microscope using 5-kV accelerating voltage.

For transmission electron microscopy (TEM), pneumococcal biofilm samples were fixed and dehydrated as described above. Biofilm preparations for TEM were infiltrated and embedded in Eponate 12 resin. Ultra-thin sections were cut on an RMC PowerTome XL ultramicrotome at 70 nm, stained with 5% aqueous uranyl acetate and 2% lead citrate, and examined on a JEOL IEM-1400 transmission electron microscope equipped with Gatan UltraScan US1000.894 and Orius SC1000.832 charge-coupled-device (CCD) cameras.

Preparation of D39-derived *comC* null mutant and complemented strain. To inactivate the *comC* gene, a DNA cassette containing the *ermB* gene (which confers resistance to erythromycin) flanked by 5' and 3' regions (~720 bp each) of *comC* was prepared. Briefly, *ermB* was PCR amplified using Platinum PCR supermix high fidelity (Invitrogen) and primers Ery-L-XbaI and Ery-R-XhoI (Table 2). The PCR product was purified using a QIAquick PCR purification kit (Qiagen) and digested with XbaI. Then, the 5' or 3' sequence of *comC* was PCR amplified using primers ComC5-L and ComC5-R-XbaI or ComC3-L-XhoI and ComC3-R, respectively. The 5' fragment was digested with XbaI and ligated to the digested *ermB* gene using T4 DNA ligase (Promega). The ligation product (~1,515 bp) was used as a template for PCRs utilizing primers ComC5L and Ery-R-XhoI, purified, digested with XhoI, and ligated to a previously XhoI-digested *comC* 3' fragment. The final ligation product (~2.35 kb) was PCR amplified with primers ComC5-L and ComC3-R and purified.

This ComC-Ery-ComC cassette was transformed (100 ng) into competent cells of wt strain D39 by standard procedures (23). The transformation reaction mixture was incubated for 2 h at 37°C, plated onto BAP containing 0.5 µg/ml erythromycin, and incubated for 48 h at 37°C in a 5% CO₂ atmosphere. Colonies were screened by PCR using primers ComC5-L and ComC3-R, and a transformant, named SPJV10, was obtained. SPJV11 was obtained similarly. The mutation was verified by sequencing. A single insertion of the Com-Ery-Com cassette was confirmed by Southern blotting (not shown).

To prepare a *comC*-complemented strain (SPJV12), the *comC* gene, including its promoters (6), was PCR amplified (~500 bp) with primers comC-L and comC-R, purified, and digested simultaneously with KpnI and XbaI. This gene was ligated into a previously KpnI-XbaI-digested pPP2 integrative plasmid (7) using T4 DNA ligase (Promega) and transformed into One Shot TOP10 chemically competent *Escherichia coli* (Invitrogen). Transformants were selected on LB agar plates containing 100 µg/ml ampicillin and screened by colony PCR with primers comC-L and comC-R, and *comC*-bearing clones were confirmed by sequencing. Plasmid pPP2 carrying *comC* (pKHcomC) was further purified using the QIAprep spin miniprep kit (Qiagen), and 4-µg amounts were transformed into competent SPJV10 cells as described above. Transformants were selected on BAP containing 0.5 µg/ml erythromycin and 3 µg/ml tetracycline, and a transformant named SPJV12 was obtained. In SPJV12, the presence of both the insertionally inactivated *comC* gene (comC-Ery-

FIG 1 Production of *S. pneumoniae* biofilms on human cells. (A and B) Immobilized HEp-2 cells (A) or lung A549 cells (B) were inoculated with strain SPJV01 and incubated for the indicated times. Another set of wells with no cells (abiotic) were also inoculated. Biofilm biomass was quantified by fluorescence, the biomass under the no-cells condition was set as 100%, and amounts of biomass under the other conditions were calculated. Experiments were repeated at least three times. *, statistical significance ($P < 0.05$) was demonstrated in comparison with results for abiotic surfaces as calculated using a nonparametric *t* test. (C to F) Biofilms produced by GFP-expressing SPJV01 strain on lung cells were imaged by fluorescence 2 h (C), 4 h (D), or 6 h (E) postinoculation or 6 h postinoculation under the no-cells condition (F). (C) Arrows show *S. pneumoniae* chains attached to lung epithelial cells 2 h postinoculation. (C to E) Cell nuclei were stained with DAPI (4',6'-diamidino-2-phenylindole; 100 nM). Bar is valid for all panels.

comC) and the restored *comC* wt gene was confirmed by PCRs with primers comC-L and Ery-R-XhoI and comC-L and comC-R, respectively, and Southern blotting (see Fig. S2 in the supplemental material; also data not shown). These PCR products were also sequenced.

RESULTS

***S. pneumoniae* produces robust biofilms on HREC.** To investigate whether human lung A549 and pharyngeal Detroit 562 and HEp-2 cells would support SPB and to compare pathogen-relevant biotic surfaces against a widely used abiotic substrate (poly-

FIG 2 *S. pneumoniae* strains produce more-robust biofilms on human lung cells. (A) Immobilized pharyngeal Detroit 562 cells or lung A549 cells were infected with SPJV01, and biofilms were quantified by fluorescence at the indicated times. Biofilms obtained 6 h postinoculation of lung cells were set to 100%, and amounts of biomass under the other conditions were calculated. *, statistical significance ($P \leq 0.05$) was demonstrated in comparison with biomass of lung cells 6 h postinoculation. (B) *S. pneumoniae* strain ATCC 33400, TIGR4, or ATCC 41619 or clinical isolate 23F was inoculated into immobilized pharyngeal cells (black bars) or lung A549 cells (gray hatched bars) and incubated for 6 h. Biofilms were removed, serially diluted, and plated onto BAP to obtain biofilm cell counts (CFU/ml). *, statistical significance ($P \leq 0.05$) was demonstrated in comparison with biofilm biomass on lung cells. (C and D) Lung (C) or pharyngeal (D) cells were inoculated with GFP-expressing *S. pneumoniae* SPJV01 and incubated for 6 h. Preparations were fixed with PFA and sialic acid residues (sa), and nuclei (nuc) were fluorescence stained. Preparations were analyzed by confocal microscopy. Top, optical *xy* sections; bottom, optical *xz* sections. Arrows and dotted line point out Sp and sa colocalization. Bar is valid only for the *xz* sections.

styrene), cells were immobilized by fixation with PFA and inoculated with a GFP-expressing *S. pneumoniae* strain D39 (SPJV01). Compared to biofilms produced on polystyrene 6 h postinoculation, biofilm biomass increased significantly, ~2-fold or ~5-fold, on pharyngeal HEp-2 or lung cells, respectively (Fig. 1A and B). A similar increase in biofilm biomass was observed when Detroit 562 cells were utilized (not shown) or when those immobilized HREC were supplemented with bacterial culture medium (see Fig. S1 in the supplemental material). Fluorescence microscopy images show *S. pneumoniae* chains attached to lung epithelial cells 2 h postinoculation (Fig. 1C, arrows). The attachment was more robust at 4 h postinoculation (Fig. 1D), and at 6 h postinoculation, biofilms covered nearly the entire surface (Fig. 1E). In contrast, at 6 h postinoculation, biofilms produced on polystyrene showed only a few dispersed cells attached (Fig. 1F). The biofilm biomass of another invasive strain, TIGR4 (37), and of R6 also increased when produced on HREC (data not shown).

***S. pneumoniae* strains preferentially produce early biofilms on immobilized human lung cells.** The results described above suggest that invasive strains produce more biofilms on lung cells than on pharyngeal cells. Our subsequent experiments demonstrated that initial interactions with host cells (e.g., adhesion bacteria-cells or/and bacterium-bacterium interactions) were similarly obtained 2 and 4 h postinoculation, whereas at 6 h

postinoculation, early biofilms on lung cells had a statistically significant ~2-fold increase compared to biofilms on pharyngeal cells (Fig. 2A). Biofilms produced by other invasive strains, e.g., TIGR4, ATCC 33400, and a clinical isolate of serotype 23F, but not ATCC 49619, also increased significantly on lung cells compared to the biofilms of these strains on pharyngeal cells (Fig. 2B).

To better visualize the early biofilm structure, sialic acid-containing cell membranes and nuclei were stained by fluorescence and biofilms were visualized by means of SPJV01's GFP expression. Confocal microscopy studies (*xy* optical sections) revealed that the biofilms produced by strain SPJV01 6 h postinoculation were homogeneously distributed on lung cells (Fig. 2C). An *xz* optical section further showed a thick layer of biofilm cells (~5 μ m) stacking up on the eukaryotic cell surface (Fig. 2C, bottom). Conversely, *S. pneumoniae* biofilms produced on pharyngeal cells were mainly localized in specific foci (Fig. 2D). An *xz* optical section of these foci revealed a thin layer (~2 μ m) of biofilm cells (Fig. 2D).

Confocal *xy* and *xz* optical sections also revealed strong colocalization of biofilm cells in close contact with sialic acid residues on the cell membrane (Fig. 2C, arrows and bottom), whereas GFP fluorescence of biofilm top layers (*xz* optical section) remained unchanged. Colocalization was also observed, to a lesser extent, with biofilms produced on pharyngeal cells (Fig. 2D). Thus, early

FIG 3 Production of *S. pneumoniae* biofilms in a bioreactor with living cultures of human lung cells. (A) Schematic diagram of the biofilm bioreactor; see the text for details. (B) The bioreactor inner chamber was inoculated with wt strain D39 (left) as described in Materials and Methods and incubated for the indicated times or inoculated with D39 (WT), SPJV05 ($\Delta luxS$), or SPJV10 ($\Delta comC$) (right) and incubated for 24 h. Biofilms were then detached and serially diluted, and biofilm counts obtained (CFU/ml). (C to E) In another set of experiments, lung cells in the bioreactor were inoculated with GFP-expressing SPJV01 and incubated for 8 h. Cells and biofilms were fixed with PFA, and sialic acid residues (red) and nuclei (blue) were fluorescence stained. The preparations were imaged from top to bottom (*xy* focal plane) with a confocal microscope. Images are of optical sections ($\sim 1 \mu\text{m}$) from the top (C), middle (D), and bottom (E) of the lung cells. The image under panel D shows optical *xz* sections; arrow points out colocalization of *S. pneumoniae* with sialic acid residues.

biofilms are preferentially produced on lung cells by invasive strains. This increased affinity might be related to the presence of specific, still-unknown pneumococcal receptors on the lung epithelium.

Development of a bioreactor with living cultures of human lung cells. *S. pneumoniae* biofilms have not yet been reported on living cultures of human respiratory cells. This is in large part due to the cell cytotoxicity induced by the pneumococcus under static incubation (38). To avoid this cytotoxic effect, we developed a bioreactor with a continuous flow of nutrients. The bioreactor utilizes a chamber designed for biological studies in which cells or tissues are exposed to an air interface as may occur in the normal *in vivo* environment in the nose and lungs (Fig. 3A). While biofilms are produced in the bioreactor's inner chamber, planktonic cells can also be collected from the influx coming off the apical perfusion path. We first demonstrated that simultaneous inoculation at the start of continuous flow (0.2 ml/min) produced biofilm biomass 8 h postinoculation that was similar to the biomass of biofilms produced on lung cells that had been preincubated for 1 or 2 h under static conditions before starting to pass fresh medium (not shown). Longer incubation under static conditions (e.g., >3 h) induced detachment of lung cells (not shown). Similar counts

of viable pneumococcal cells ($P = 0.199$) were successfully obtained 8 and 24 h postinoculation (Fig. 3B, left). An average of $\sim 5.6 \times 10^8$ CFU of biofilm cells/cm² of cultured human lung A549 cells was obtained.

S. pneumoniae strains can invade cultures of human pharyngeal, lung, and brain cells within 4 h postinoculation (39, 40). To identify internalized bacteria, confocal optical sections were collected from the top, middle, and bottom (total sectioned, $\sim 10 \mu\text{m}$) of the infected lung cells. These optical sections ($\sim 1 \mu\text{m}$) demonstrate that *S. pneumoniae* biofilms were mainly detected in the top (~ 1 to $2 \mu\text{m}$) (Fig. 3C) and middle sections (~ 5 to $6 \mu\text{m}$) (Fig. 3D), whereas bacteria were almost absent from optical sections imaged from the bottom (~ 8 to $9 \mu\text{m}$) (Fig. 3E). The absence of internalized bacteria was also confirmed in an optical *xz* section (Fig. 3D, *xz* panel). Membrane and nuclear staining of lung cells additionally confirmed that inoculated lung cells remained morphologically similar to noninfected cells. Our experiments demonstrate that early biofilms (~ 8 h) and mature viable biofilms (~ 24 h) can be produced on living cultures of human lung cells.

The LuxS/AI-2 quorum-sensing system regulates *S. pneumoniae* biofilms on HREC. We and others have independently demonstrated that the LuxS/AI-2 QS system regulates early bio-

FIG 4 Quorum-sensing control of *S. pneumoniae* biofilms on HREC. (A to C) GFP-expressing strains SPJV01 (WT), SPJV08 ($\Delta luxS$), and SPJV11 ($\Delta comC$) were inoculated into immobilized pharyngeal (A) or lung (B) cells or into 24-well plates with no cells (abiotic) (C) and incubated for the indicated times. Biofilm biomass was obtained by fluorescence. Biomass of SPJV01 8 h postinoculation was set as 100% and used to calculate the biomass from all other conditions. (D and E) At 8 h postinoculation, biofilms produced on immobilized lung cells (D) or in the bioreactor (E) were detached, serially diluted, and plated to obtain biofilm viable counts (CFU/ml). Error bars represent the standard errors of the means calculated using data from at least four independent experiments. Statistical significance ($P \leq 0.05$) was calculated using a nonparametric *t* test, in comparison with the results for wt D39 (*) or the $\Delta comC$ strain (#).

films produced by strain D39 on polystyrene (18, 19). To assess whether this QS system also controls biofilms produced on human lung and pharyngeal cells, cells were infected with a D39-derived *luxS* mutant (SPJV08) and biofilms were evaluated. In comparison to the wt strain, SPJV08 produced statistically significantly less biofilm biomass, as quantified by fluorescence, on pharyngeal and lung cells 4, 6, and 8 h postinoculation (Fig. 4A and B). Biofilm biomass, however, was restored to wt levels on pharyngeal and lung cells inoculated with the complementing strain SPJV06 (not shown). As expected, SPJV08 biofilm counts were significantly reduced 8 h postinoculation in comparison to those of the wt strain whether biofilms were produced under static conditions (Fig. 4D) or in the bioreactor with living cultures of lung cells (Fig. 4E). In contrast with the thick layer of biofilms produced by the wt strain or SPJV06, confocal images revealed few SPJV08 bacteria attached to lung cells 8 h postinoculation (Fig. 5).

The Com quorum-sensing system regulates production of early biofilms on human cells but not on polystyrene. A role for the Com system in biofilms produced by *S. pneumoniae* on abiotic surfaces has recently been suggested (31, 32), as competence genes were upregulated during biofilm formation (31). To gain further insights into the role of the Com system in biofilm formation, we prepared a $\Delta comC$ mutant (SPJV10), as well as a derivative expressing GFP (SPJV11), and evaluated their biofilm phenotypes. Consistent with a previous report (31), early attachment of

SPJV11 to polystyrene and biofilm biomass (Fig. 4C) showed wt levels. This confirms that the Com system does not regulate the first 8 h of biofilm production on abiotic surfaces. Furthermore, the initial interaction between *S. pneumoniae* and human cells was not affected by a mutation in the *comC* gene, since SPJV11 biomass quantified 2 and 4 h postinoculation showed wt levels on both pharyngeal and lung cells (Fig. 4A and B). In sharp contrast, the biofilm biomass of SPJV11 did not increase after 6 to 8 h of growth on immobilized lung and pharyngeal cells (Fig. 4A, B, and D) or in the bioreactor with living cultures of lung cells 8 h postinoculation (Fig. 4E). The biofilm counts of SPJV11 on lung cells were significantly higher than those produced by the *luxS* mutant SPJV08 at 8 h postinoculation (Fig. 4D), whereas the biomass had a clear increase when it was quantified by fluorescence (Fig. 4B).

Confocal images of SPJV11 showed few bacteria attached to lung cells (Fig. 5). This phenotype was fully reversed when we integrated a single copy of the *comC* gene into the $\Delta comC$ genome (Fig. 5; see also Fig. S2 and S3 in the supplemental material). Altogether, our results indicate that the Com system is necessary to produce early biofilms on human cells but not on abiotic surfaces.

Static incubation of *S. pneumoniae* biofilms induces autolysis. Using fluorescence to quantify the biomass of SPJV01, we observed that the biofilm levels were almost completely reduced 16 and 24 h postinoculation. Similar trends were observed in bio-

FIG 5 Confocal images of *S. pneumoniae* biofilms produced on lung cells. GFP-expressing *S. pneumoniae* strains SPJV01 (WT), SPJV08 ($\Delta luxS$), SPJV11 ($\Delta comC$), SPJV06 ($\Delta luxS/luxS$), and SPJV12 ($\Delta comC/comC$) were inoculated into immobilized human lung A549 cells and incubated for 8 h. Cells were then fixed, and sialic acid residues (sa) or nuclei (nuc) were fluorescence stained. SPJV06 and SPJV12 were stained with an FITC-labeled anti-*S. pneumoniae* antibody. Preparations were analyzed by confocal microscopy. The bottom panels show the merge of the three channels (green, red, and blue). Except where optical *xz* sections are indicated, *xy* sections are shown. Bar (20 μ m) in left middle panel is valid for all *xy* sections.

films produced on polystyrene or lung cells and whether supplemented with THY or DMEM (not shown). Fluorescence microscopy showed a clear decay in GFP expression by SPJV01 24 h postinoculation (Fig. 6A). Biofilms were observed, however, when the structure was stained with an FITC-labeled anti-*S. pneumoniae* antibody (Fig. 6A). These structures lacked viability, as the biofilm counts decreased significantly between 8 and 16 h postinoculation and no growth was obtained at 24 h (Fig. 6B).

Electron microscopy studies were conducted to analyze the ultrastructure of those biofilms. Scanning electron microscopy (SEM) first showed healthy diplococci held together within the biofilm structure 8 h postinoculation. The cell envelope of these diplococci was seen to be intact, showing no apparent membrane alterations (Fig. 6C, left). In contrast, 24-h biofilms showed a flattened structure characterized by contracted biofilm cells with membrane disruption (Fig. 6C, right). Transmission electron microscopy (TEM) allowed us to visualize, at 8 h postinoculation, diplococci with abundant homogenous electrodense material, whereas at 16 h postinoculation, the electrodense material appeared condensed, indicating increased permeability but reten-

tion of the heavy metal staining (Fig. 6D, left and middle). Twenty-four hours later, heavy metal staining had disappeared from inside those bacteria, with some cells showing areas of membrane disruption (Fig. 6D, right). Together, these findings confirm that under static conditions, *S. pneumoniae* biofilms undergo cell death. The signal(s), if any, might be concentrated under static conditions, since biofilms produced in the bioreactor with a unidirectional flow of fresh medium were viable 24 h postinoculation (Fig. 3B, left).

Autolysis of *S. pneumoniae* biofilms is regulated by QS. To investigate whether QS regulates biofilm autolysis, lung cells were infected with SPJV05 ($\Delta luxS$) or SPJV10 ($\Delta comC$) and biofilms were obtained 16 and 24 h postinoculation. While the results in Fig. 4 demonstrate that SPJV05 and SPJV10 have a defect in the production of early biofilms at 8 h, at 16 h postinoculation, both QS mutants were able to form biofilms on lung A549 cells (Fig. 7A). Biofilms of the *comC* mutant SPJV10 showed counts similar to those seen in the wt, whereas biofilms of the *luxS* mutant were significantly different (Fig. 7A). In contrast to the nonviable wt biofilms observed 24 h postinoculation, those produced by

FIG 6 Autolysis of *S. pneumoniae* biofilms is triggered under conditions of static incubation with human cells. Strain SPJV01 was inoculated into immobilized human lung A549 cells and incubated for the indicated times. Where indicated, biofilms were stained with an FITC-labeled anti-*S. pneumoniae* antibody (FITC-anti-Sp). (A and B) Fluorescence images were obtained (A) or biofilms were detached, serially diluted, and plated to obtain viable counts (CFU/ml) (B). Error bars represent the standard errors of the means calculated using data from three independent experiments. *, statistical significance ($P \leq 0.05$) in comparison with the results for SPJV01 biofilms at 8 h postinoculation was demonstrated using a nonparametric t test. (C) Strain D39 was inoculated on top of immobilized lung cells and incubated for 8 h or 24 h. Preparations were processed for electron microscopy, and SEM micrographs obtained. Scale bar is valid for both panels. (D) TEM micrographs were also obtained at the indicated times. Middle, arrows show contracted and permeable pneumococci; right, arrows point out empty pneumococci with disrupted cell walls.

SPJV05 and SPJV08 were still culturable (Fig. 7B). However, after 48 h of incubation, the biofilms produced by QS mutants SPJV05 and SPJV10 were completely lysed (i.e., no bacteria could be recovered from those cultures; not shown).

EM imaging (Fig. 6) suggested that lysis-induced membrane disruption leads to loss of bacterial DNA, and therefore, nucleic acid staining could be used to verify the structural integrity of biofilms. In other words, DNA will be stained in viable biofilms, while lysed biofilms will not retain the DNA-binding fluorescent dye. As hypothesized, wt biofilms were stained with a GFP-labeled anti-*S. pneumoniae* antibody but bacterial DNA was barely detected (Fig. 7C to E). However, nucleic acids and *S. pneumoniae*

cells were observed in biofilms produced by SPJV05 (not shown) and SPJV10 (Fig. 7F to H). Biofilm viability was not related to nutrient starvation, osmolality, or pH, as culture conditions were similar for all strains. The growth rates and the autoaggregation phenotype of planktonic cells were also similar (see Fig. S2 in the supplemental material; also data not shown). Thus, biofilm autolysis appears to be controlled by these two QS mechanisms.

To investigate the release of DNA due to the observed autolysis phenotype, eDNA was purified from the supernatants of biofilms produced on lung cells. As we would expect since the wt cells had undergone autolysis, the results shown in Fig. 8 confirm the presence of released DNA in the supernatant of the wt strain 16 h

FIG 7 Autolysis of *S. pneumoniae* biofilms is controlled by quorum sensing. *S. pneumoniae* strains D39 (WT), SPJV05 ($\Delta luxS$), and SPJV10 ($\Delta comC$) were inoculated into immobilized cultures of human lung A549 cells and incubated for 16 h (A) or 24 h (B). Biofilms were detached, serially diluted, and plated to obtain viable counts (CFU/ml). *, statistical significance ($P \leq 0.05$) in comparison with the results for D39 biofilms was demonstrated using a nonparametric t test. Error bars show standard errors of the means. (C to H) Lung cells inoculated with wt D39 (C to E) or SPJV10 (F to H) were incubated for 24 h and then fixed. Eukaryotic and prokaryotic DNA was fluorescence stained, *S. pneumoniae* cells were stained with an FITC-labeled anti-*S. pneumoniae* antibody, and cells were visualized by staining sialic acid residues (sa). Arrows (D, F, and G) point out *S. pneumoniae* biofilms. Bar is valid for all panels.

postinoculation. The DNA levels were significantly lower in the supernatants of SPJV05 and SPJV08. Twenty-four hours postinoculation, when complete autolysis has occurred, with the subsequent release of nucleases (41), the presence of eDNA decreased in the supernatant of wt biofilms, whereas the levels of eDNA increased in SPJV05 biofilms and remained the same in SPJV10 biofilms (Fig. 8).

DISCUSSION

We have developed a bioreactor to produce viable SPB on living cultures of human respiratory cells. This bioreactor simulates the environment of the respiratory epithelium, as it includes a unidirectional flow of nutrients and the presence of human respiratory cells. This is the first study, to our knowledge, describing the pro-

duction of viable pneumococcal biofilms on living cultures of human respiratory cells. Unlike the cell detachment observed when the pneumococcus and human cells are incubated under static conditions (38), in the bioreactor, viable SPB were stable on top of human cell cultures for up to 48 h (not shown). Using the bioreactor and immobilized cell cultures, we identified two QS regulators of SPB (Com and LuxS/AI-2). While LuxS/AI-2 regulated SPB on abiotic surfaces and HREC, Com specifically regulated early biofilm formation on human cells.

There are two other systems that allow the formation of bacterial biofilms on cultures of human cells (42, 43). One such *in vitro* culture system utilizes a rotating-wall vessel that forms 3-dimensional aggregates of lung A549 cells in about 15 days. When inoculated with *P. aeruginosa* strains, however, biofilms induce de-

FIG 8 Quantification of extracellular DNA. Strains D39 (WT), SPJV05 ($\Delta luxS$), and SPJV10 ($\Delta comC$) were inoculated into immobilized cultures of human lung A549 cells and incubated for 16 h or 24 h. DNA release (pg/ml) into the supernatant was quantified as described in Materials and Methods. Error bars represent the standard errors of the means calculated using data from three independent experiments. *, statistical significance ($P \leq 0.05$) in comparison with the results for wt D39 was demonstrated using a nonparametric *t* test.

tachment of those 3-dimensional cell aggregates after 8 h of incubation (42). A flow chamber system has also been used to study *Neisseria gonorrhoeae* and *Neisseria meningitidis* biofilms (42, 44). The chamber system is inoculated and perfused with medium, and biofilms are produced without loss of cell viability. Compared to these biofilm culture systems, the bioreactor developed here has additional advantages, including (i) a perfusion path for both the apical and basolateral sides of the cells and (ii) inlets from which cells can be directly inoculated into the inner chamber, avoiding contamination of the perfused medium. The inoculation inlets also allow, at any time during biofilm development, the addition of reagents (i.e., antibiotics, inhibitors, proteases, etc.) or reinoculation of monospecies biofilms with different bacterial species or genera.

Our studies also revealed that early biofilms formed on HREC contained more biofilm cells than those formed on abiotic surfaces. Similar discoveries have been published recently by Marks et al. (14). For example, our bioreactor produced $\sim 5.6 \times 10^8$ CFU of biofilm cells/cm² of cultured human lung A549 cells, whereas Donlan et al., using a reactor with abiotic surfaces (e.g., germanium coupons), obtained 1×10^5 biofilm cells/cm² in 21-h-old biofilms (45). The culture medium was not the stimulating factor for the increased biofilm biomass, since the biomass of pneumococci statically incubated in either cell culture medium or bacterial culture medium increased similarly when strains were inoculated onto immobilized HREC (Fig. 1; see also Fig. S1 in the supplemental material). This is a clear indication that early bacterium-host cell interactions play an important role in the building of the nascent biofilm structure. Future high-throughput genetic analysis and proteomic studies of different stages of pneumococcal biofilms (i.e., early, mature, and old) formed on HREC may reveal the

repertoire of molecules utilized by the pneumococcus to produce biofilms on the human nasopharynx and lungs.

Our findings emphasize the need for studying pneumococcus-host cell interaction in a more natural scenario (i.e., using cultures of human cells). Previous work has demonstrated that the presence of eukaryotic cells stimulates enteropathogenic *E. coli* to up-regulate the transcription of genes involved in intimate adherence (46) and toxin secretion (47). In the presence of human enterocytes, *Clostridium perfringens* strains upregulate the production of toxins linked to enteric disease (48), while *Salmonella* and *Shigella* make a functional type III secretion system and translocate invasion proteins into host cells (49, 50). We have further learned in this work and from other recently published studies (2, 17) that human respiratory cells stimulate the pneumococcus to produce biofilms. Moreover, the studies whose results are presented in Figure 2 demonstrated the importance of the type of epithelium for early biofilm formation, as the biomass of biofilms formed by invasive strains increased when they were produced on lung cells rather than pharyngeal cells. Whether this behavior has implications for pathogenesis or carriage remains to be investigated.

An interesting observation made in this work was that autolysis of biofilms was triggered 16 h postinoculation when bacteria were incubated under static conditions (Fig. 6B). Similar findings were recently described by Wei and Håvarstein, utilizing abiotic surfaces as the substrate (29). In contrast to the biofilm population, planktonic cultures of wt strain D39 begin the autolytic process during the exponential phase of growth (6 to 8 h postinoculation; data not shown). Autolysis, also called fratricide, is a competence-driven mechanism that induces lysis of noncompetent bacteria (51, 52). As a result, DNA is released from lysed bacteria and taken up by competent ones (51, 53). It is possible that autolysis of planktonic cells provides eDNA required for biofilm assembly (29). This eDNA has recently been visualized on the biofilm matrix (54). Biofilm autolysis seen 16 h postinoculation was, in part, controlled by the LuxS/AI-2 and Com QS systems. Accordingly, the eDNA levels in the supernatants of wt biofilms were significantly higher than those observed in the supernatants of the *comC* or *luxS* mutant (Fig. 8). Biofilms produced in the bioreactor, however, remained viable (Fig. 3B), further confirming that biofilm autolysis is mediated by a QS-controlled secreted factor(s). The factor(s) was perhaps diluted or removed when biofilms were produced in the bioreactor, as complete turnover of culture medium in the bioreactor's inner chamber occurred in ~ 25 min.

This study is also the first to describe that competence regulates biofilms produced on human respiratory cells and biofilm autolysis. The initial attachment of the pneumococcus to pharyngeal or lung cells appears not to be controlled by Com, as the biomass of the $\Delta comC$ mutant was not affected at early time points, i.e., 2 and 4 h. Despite this normal attachment phenotype, the biofilm biomass of this mutant did not reach wt levels at 6, 8, or 12 h postinoculation. The requirement of an intact Com system to produce early biofilms on HREC was demonstrated when we integrated a single copy of the *comC* gene into our $\Delta comC$ mutant, restoring wt levels (see Fig. S2 and S3 in the supplemental material). A regulatory role for Com in early biofilm formation is therefore evident, as this QS system requires specific timing and culture conditions to become activated and regulate the transcription of more than 180

target genes (25, 26). Some of those, for example, *lytA* (55) and *cpbD* (19), have been implicated in biofilm formation.

Similarly, the LuxS/AI-2 QS system was found to control SPB on both abiotic surfaces (18, 19) and human cells. LuxS/AI-2 also controls the biofilm formation of other streptococci found in the human oropharynx and nasopharynx, including *S. intermedius*, *S. oralis*, *S. gordonii*, and *S. mutans* (56–59). Molecular interaction between these two QS systems, Com and LuxS/AI-2, has not been experimentally confirmed. Perhaps LuxS/AI-2 is necessary to control gene expression and allow the attachment of early biofilms, as the signal can be provided from a variety of species in the nasopharynx. Then, mature pneumococcal biofilms would only be properly assembled once the Com system is activated, controlling the transcription of genes implicated in biofilm formation.

ACKNOWLEDGMENTS

This research was supported in part by PHS grant UL1 RR025008 from the Clinical and Translational Science Award program, NIH, National Center for Research Resources (J.E.V.).

We thank Hong Yi, Elizabeth Wright, and Jeannette Taylor for their assistance with electron microscopy; they were supported in part by the Robert P. Apkarian Integrated Electron Microscopy Core. We thank Joshua R. Shak and Gideon Matzkin for critical reading of the manuscript. We also thank Reinhold Brückner from the Department of Microbiology, University of Kaiserslautern, for providing pPP2, and Manuel Espinoza from the “Centro de Investigaciones Biológicas,” Madrid, Spain, for his kind gift of plasmid pMV158GFP.

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