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Biofilm Formation by *Streptococcus pneumoniae*: Role of Choline, Extracellular DNA, and Capsular Polysaccharide in Microbial Accretion[▽]

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Streptococcus pneumoniae **colonizes the human upper respiratory tract, and this asymptomatic colonization is known to precede pneumococcal disease. In this report, chemically defined and semisynthetic media were used to identify the initial steps of biofilm formation by pneumococcus during growth on abiotic surfaces such as polystyrene or glass. Unencapsulated pneumococci adhered to abiotic surfaces and formed a three-dimensional structure about 25 μm deep, as observed by confocal laser scanning microscopy and low-temperature scanning electron microscopy. Choline residues of cell wall teichoic acids were found to play a fundamental role in pneumococcal biofilm development. The role in biofilm formation of choline-binding proteins, which anchor to the teichoic acids of the cell envelope, was determined using unambiguously characterized mutants. The results showed that LytA amidase, LytC lysozyme, LytB glucosaminidase, CbpA adhesin, PcpA putative adhesin, and PspA (pneumococcal surface protein A) mutants had a decreased capacity to form biofilms, whereas no such reduction was observed in Pce phosphocholinesterase or CbpD putative amidase mutants. Moreover, encapsulated, clinical pneumococcal isolates were impaired in their capacity to form biofilms. In addition, a role for extracellular DNA and proteins in the establishment of *S. pneumoniae* biofilms was demonstrated. Taken together, these observations provide information on conditions that favor the sessile mode of growth by *S. pneumoniae*. The experimental approach described here should facilitate the study of bacterial genes that are required for biofilm formation. Those results, in turn, may provide insight into strategies to prevent pneumococcal colonization of its human host.**

It is now widely recognized that biofilm formation is the natural form of growth for most microbial species in their natural habitats (27). A biofilm is a highly structured, sessile microbial community characterized by bacterial cells attached to a surface or interface and embedded in a matrix of extracellular polymeric substances (10, 17). Biofilm development is a multistep process, and the study of the genetic basis of colonization of abiotic surfaces has become an important aspect in the search for genes involved in biofilm formation and maintenance in gram-positive and gram-negative bacteria (6, 40, 55). The importance of biofilms is best illustrated by the finding that more than 60% of bacterial infections are considered to involve biofilms, and these communities exhibit an inherent tolerance to antibiotic therapies and host immune attack (11, 56). The dynamics of biofilm formation constitute a simple strategy of microbial survival that facilitates the transmission of pathogens by providing a stable protective environment and by acting as a reservoir for the dissemination of a great number of microorganisms to new surfaces (28).

Streptococcus pneumoniae (pneumococcus) is a major gram-positive human pathogen and currently the leading cause of community-acquired pneumonia, meningitis, and bloodstream infections in the elderly, the young, and patients with immunosuppressive illness and chronic diseases; it is also the main causative agent of middle-ear infections in children. More than 500,000 cases of pneumococcal pneumonia occur each year in the United States, causing almost 50,000 deaths in adults and children. Moreover, since the introduction of *Haemophilus influenzae* type Ib conjugate vaccine, *S. pneumoniae* has become the principal cause of bacterial meningitis (5). The global importance of *S. pneumoniae* as the cause of illness, sequelae, and death, together with the emergence of strains resistant to β-lactam antibiotics and quinolones, has conferred upon this microorganism the status of "superbug" (30).

Members of the genus *Streptococcus* are important human pathogens, and their ability to reach a high density may be due to the formation of biofilm-like populations in confined areas of the body, such as the heart valves, tonsillar crypts, and implanted medical devices. *Streptococcus mutans*, the major etiological agent of dental caries, provides a notable example of the formation of complex biofilms by the oral microbiota. However, it has been suggested that in contrast to the very dense dental plaques formed by *S. mutans* and other oral streptococci, pathogenic streptococci that infect mucosal tissues colonize environments where bacterial microcolonies are usually less dense due to the bathing effects of secretions and epithelial desquamation (12).

Until now, few studies have documented the potential ability of pneumococci to produce biofilms in vitro (8, 18, 75). However, it was shown that pneumococcal growth on Sorbarod filters in a continuous culture-like system mimics nasopharyngeal carriage. In that model, type 3 pneumococci generated tandem duplications within the *cap3A* gene of the capsule locus, leading to the formation of unencapsulated mutants

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TABLE 1. Characteristics of the streptococcal strains used in this study

Strain[a]	Relevant characteristics	Source or reference[b]
S. pneumoniae		
Unencapsulated strains		
R6	D39 derivative; *lytA*$^+$	32
M11	*lytA*$^+$	23
M31	Δ*lytA*	61
M31B	M31 but *lytB*::*ermC*; Em^r	24
M31BC	M31B but *lytC*::*tet*; Em^r Tc^r	B. de las Rivas; CSIC
M31BCD	M31 but *lytB*::*neo lytC*::*tet pce*::*ermC*; Kan^r Tc^r Em^r	B. de las Rivas
M32	Δ*lytA32*	47
R6B	R6 but *lytB*::*ermC*; Em^r	24
R6BC	R6B but *lytC*::*tet*; Em^r Tc^r	B. de las Rivas
R6C	R6 but *lytC*::*ermC*; Em^r	52
R6D	R6 but *pce*::*ermC*; Em^r	13
R924	R6 but *lytA*::*kan* by *mariner* mutagenesis[c]; Kan^r	51
R1582	R6 but *cbpD*::*spc* by *mariner* mutagenesis[c]; Spc^r	J. P. Claverys; CNRS
P040	R6 (pMV158GFP) by transformation with plasmid DNA; Tc^r	This study
P042	R6 but *lytA*::*kan lytC*::*tet*; R924 transformed by chromosomal DNA from R6DC; Kan^r Tc^r	This study
P046	R6 but *lytA*::*kan lytC*::*ermC*; R924 transformed by chromosomal DNA R6C; Kan^r Em^r	This study
P049	R6 but *pce*::*ermC* (pMV158GFP); R6D transformed by pMV158GFP; Em^r Tc^r	This study
P050	R6 but *lytA*::*kan lytC*::*ermC* (pMV158GFP); P046 transformed by pMV158GFP; Kan^r Em^r Tc^r	This study
P064	R6 but *cbpA*::*spc* by *mariner* mutagenesis[d]; Spc^r	This study
P065	R6 but *pcpA*::*cat* by *mariner* mutagenesis[d]; Cm^r	This study
P066	R6 but *pspA*::*kan* by *mariner* mutagenesis[c]; Kan^r	This study
ST344	*lytA*$^+$	4
Encapsulated strains		
13868	S1$^+$	52
P005	M11 but S1^{+e}	D. Llull; CSIC
D39	S2$^+$	G. Pozzi; US
P006	M11 but S2^{+e}	D. Llull
TIGR4	S4$^+$	67
P008	M11 but S4^{+e}	D. Llull
Spain^{6B}-2	S6B$^+$	A. Fenoll; ISCIII
P010	M11 but S6B^{+e}	D. Llull
SSISP9V/1	S9V$^+$	Staten Seruminstitut
P019	M11 but S9V^{+e}	D. Llull
Spain^{14}-5	S14$^+$	A. Fenoll
P021	M11 but S14^{+e}	D. Llull
G54	S19F$^+$	19
P015	M11, but S19F^{+e}	D. Llull
SSISP23F/1	S23F$^+$	Staten Seruminstitut
P022	M11 but S23F^{+e}	D. Llull
7077/39	S37$^+$	42
P018	M11 but S37^{+e}	D. Llull
Mitis group streptococci		
Streptococcus mitis^T		NCTC
Streptococcus oralis^T		CECT
Streptococcus gordonii^T		CECT
Streptococcus pseudopneumoniae^T		CCUG

[a] ST, sequence type; superscript T, type strain.
[b] CSIC, Consejo Superior de Investigaciones Científicas, Spain; CNRS, Centre National de la Recherche Scientifique, France; US, Università di Siena, Italy; ISCIII, Instituto de Salud Carlos III, Spain; NCTC, National Collection of Type Cultures; CECT, Colección Española de Cultivos Tipo; CCUG, Culture Collection, University of Göteborg, Sweden.
[c] Cotranscribed orientation of the antibiotic resistance cassette of the minitransposon with respect to the targeted gene.
[d] Antitranscribed orientation of the antibiotic resistance cassette of the minitransposon with respect to the targeted gene.
[e] S1$^+$ to S37$^+$ indicate that the M11 transformant synthesizes a capsular polysaccharide of the corresponding serotype.

(75). Capsular polysaccharide is considered a fundamental genetic trait for pneumococcal virulence, and these observations of changes in the expression of genes involved in capsule biosynthesis were later extended to the capsular genes of pneumococcal serotypes 8 and 37 (74). The ability of pneumococci to regulate capsule expression, as demonstrated on Sorbarod biofilms, could play a fundamental role in the transition from the carrier state to invasive disease (75). Recently, Allegrucci

TABLE 2. Characteristics of the plasmids and primers used in this study

Plasmid or primer	Relevant characteristics[a]	Source or reference
Plasmids		
pEMcat	ColE1 derivative; Ap^r Cm^r; carries a 1,303-bp-long minitransposon containing the IRs of the *Himar1* transposon and ~100 bp of *Himar1* transposon sequences flanking the *cat* Cm^r gene (*magellan2*)	1
pMV158GFP	pMV158 derivative; Tc^r; harbors the gene encoding GFP under the control of a maltose-inducible promoter	53
pR410	pEMcat derivative; Ap^r Kan^r; carries a 1,337-bp-long minitransposon containing the IRs of the *Himar1* transposon and ~100 bp of *Himar1* transposon sequences flanking the *kan* gene	J. P. Claverys
pR412	ColE1 derivative; Ap^r Spc^r; carries a 1,145-bp-long minitransposon containing the IRs of the *Himar1* transposon and ~100 bp of *Himar1* transposon sequences flanking the *spc* gene	48
Oligonucleotide primers		
MP127	CCGGGGACTTATCAGCCAACC; *mariner* cassette universal primer; internal to IRs; outward orientation	
MP128	TACTAGCGACGCCATCTATGTG; *mariner* cassette universal primer; adjacent to left IR; outward orientation	
cbpAmar3	TCCGTACTGTCCAAGAAGCCA; downstream of *cbpA* (*pspC*) (accession no. AE008564)	
cbpAmar5	GCAATGGCGGGAAAGAATTTGG; upstream of *cbpA* (*pspC*); (accession no. AE008564)	
pcpAmar3	CAGAAGAAAAACGCTTGATCAGC; downstream of *pcpA* (accession no. AE008558)	
pcpAmar5	GCAAATGCTGAGTAGGTTTCCC; upstream of *pcpA* (accession no. AE008558)	
pspAmar3	TCCCAGTTCTTCATGAAGATACC; downstream of *pspA* (accession no. AE008396)	
pspAmar5	CAAGTTGTTGCATCGTAGCTAAG; upstream of *pspA* (accession no. AE008396)	

[a] IR, inverted repeat.

and coworkers reported that the biofilm development process correlates not only with differential production of proteins but also with a dramatic increase in the number of detectable proteins (2).

Additional evidence for a link between the pneumococcus and the biofilm mode of growth comes from study of the asymptomatic carrier state associated with *S. pneumoniae* colonization of the throat or nasopharynx. This condition is found mainly in children, 60% of whom may be colonized (3), but progression to a disease state occurs only under appropriate conditions (50). It may thus be the case that, in the carrier state, *S. pneumoniae* constitutively adopts the sessile mode of growth in order to survive for long periods of time. To ascertain whether *S. pneumoniae* benefits from forming an interactive community in which sessile growth is not the result of a random accretion of bacterial cells but, rather, of the formation of a bacterial community that cooperates to form a well-defined structure, laboratory conditions were established that favored biofilm formation, and the structural peculiarities of these biological entities were studied.

Current evidence suggests that pneumococci are able to form biofilms on abiotic surfaces. In the present study, this ability provided the basis for applying a genetic approach to studying biofilm accretion, i.e., the obligatory attachment of *S. pneumoniae* to a surface before an ordered three-dimensional (3D) structure can be constructed and the conditions that lead to defective biofilm formation. Our analyses revealed a role for several gene products in biofilm development and suggested that DNA and certain proteins contribute to the formation of an extracellular matrix in this system. The experimental data are important steps in furthering our understanding of the putative role of biofilms in some stages of the pneumococcal infection.

MATERIALS AND METHODS

Strains, media, plasmid, and growth conditions. The streptococcal strains used in this study are listed in Table 1. Routinely, pneumococcal strains were grown in C medium (38) either supplemented with 0.08% yeast extract (C+Y medium) or nonsupplemented. For assays of biofilm formation, in addition to C medium, the following media were used: Todd-Hewitt broth supplemented with 0.5% yeast extract, casein tryptone (CAT) medium (59), and the chemically defined media Cden (70) and CDM (72) supplemented with choline chloride (5 $\mu g\ ml^{-1}$), asparagine (50 $\mu g\ ml^{-1}$), and sodium pyruvate (250 $\mu g\ ml^{-1}$). CDM was purchased from JRH Biosciences (Lenexa, Kansas). Where indicated, Cden and CDM media were supplemented with either 2% choline chloride or ethanolamine (EA) (50 $\mu g\ ml^{-1}$). Cells were incubated at 37°C without shaking, and growth was monitored by measuring the A_{595}. When used, antibiotics were added at the following concentrations: erythromycin, 0.5 $\mu g\ ml^{-1}$; kanamycin, 250 $\mu g\ ml^{-1}$; tetracycline, 0.5 to 1 $\mu g\ ml^{-1}$. Plasmid pMV158GFP, kindly provided by C. Nieto, is a derivative of the natural streptococcal plasmid pMV158, which harbors the gene encoding green fluorescent protein (GFP) under the control of a maltose-inducible promoter (53).

Transformation procedures. Chromosomal DNA from pneumococcal isolates was prepared as previously described (21). *S. pneumoniae* was transformed with chromosomal or plasmid DNA by treating precompetent cells with 100 ng of synthetic competence-stimulating pheromone 1 (CSP1) ml^{-1} at 37°C for 10 min to induce competence (48, 51), followed by incubation at 30°C during DNA uptake. Transformants were selected by plating in CAT agar supplemented with 3% (vol/vol) defibrinated sheep blood and then by challenge with a 10-ml overlay containing the appropriate antibiotic after phenotypic expression for 120 min at 37°C (51). Capsulated transformants of strain M11 were enriched, when required, by successive transfers of the transformed culture to C medium containing 0.08% bovine serum albumin and supplemented with 1 μg of anti-R antiserum ml^{-1} before plating. Anti-R (antisomatic) antiserum contains group-specific agglutinins, which, at the proper dilution, agglutinate only unencapsulated pneumococci.

***mariner* mutagenesis of the *cbpA*, *pcpA*, and *pspA* genes.** Insertions of the *spc*, *cat*, or *kan* gene cassettes in the *cbpA*, *pcpA*, and *pspA* genes were generated by in vitro *mariner* mutagenesis as previously described (48). Plasmids used as a source for minitransposons are listed in Table 2. Briefly, plasmid DNA (≅1 μg) was incubated in the presence of purified *Himar1* transposase (39) (in a total volume of 40 μl), with a PCR fragment (cbpAmar5-cbpAmar3 for *cbpA*; pcpAmar5-pcpAmar3 for *pcpA*; pspAmar5-pspAmar3 for *pspA*) leading to a

random insertion of the minitransposon into the fragment. Gaps in transposition products were repaired as previously described (1), and the resulting in vitro generated transposon insertion library was used to transform *S. pneumoniae*. Location and orientation of each *mariner* cassette were determined by PCRs using primers MP127 or MP128 (Table 2) in combination with either one of the two primers used to generate each mutagenized PCR fragment. The cassette-chromosome junctions were sequenced using primer MP128. The *cbpA*::*spc2A* (position 1537 with respect to the ATG of *cbpA*) (strain P064), *pcpA*::*cat1A* (position 1323 with respect to the ATG of *pcpA*) (strain P065), and *pspA*::*kan1C* (position 1145 with respect to the ATG of *pspA*) (strain P066) insertions were used for this study (Table 1).

Biofilm formation assay and quantification. Biofilm formation was determined by the ability of cells to adhere to the walls and base of 96-well (flat-bottom) polystyrene (PST) microtiter dishes (Costar 3595; Corning Incorporated), using a modification of a previously reported protocol (55). Unless stated otherwise, cells grown in C+Y medium to an A_{595} of ≅0.5 to 0.6 were sedimented by centrifugation, resuspended in an equal volume of the indicated prewarmed medium, diluted 1/10 or 1/100, and then dispensed at a concentration of 200 μl per well. Plates were incubated at 34°C for 6 h, and bacterial growth was determined by measuring the A_{595} using a plate reader (microplate absorbance reader 2020; Anthos Labtec Instruments GmbH). Fifty microliters of a 1% solution of crystal violet (CV) was added to each well. The plates were then incubated at room temperature for approximately 15 min, rinsed three times with 200 μl of distilled water, and air dried. CV-stained biofilm formation was quantified by solubilizing the biofilm with 95% ethanol (200 μl/well) and then determining the A_{595}. Under these conditions, approximately 2.4×10^8 and 1.5×10^8 CFU per ml grew as planktonic or sessile cells, respectively.

CLSM. For observation of *S. pneumoniae* biofilms by confocal laser scanning microscopy (CLSM), pneumococcal strains containing pMV158GFP (Table 2) were grown on glass-bottom dishes (WillCo-dish; WillCo Wells B. V., The Netherlands) in 2 ml of C medium containing 1% maltose and 1 μg of tetracycline ml^{-1} for 10 to 12 h at 34°C. The culture medium was removed, and the pneumococcal biofilms were rinsed with phosphate-buffered saline (PBS) to remove nonadherent bacteria, followed by submersion in 0.5 ml of PBS. *S. pneumoniae* biofilms were observed using a Leica TCS-SP2-AOBS-UV CLSM equipped with an argon ion laser. The excitation wavelength was 488 nm, and the magnification was ×100. Images were analyzed using the LEICA software LCS. Projections through the *x-y* plane (individual scans at 0.5-μm intervals) and the *x-z* plane (images at 3-μm intervals) were obtained.

A LIVE/DEAD *Bac*Light bacterial viability kit L-13152 (Invitrogen-Molecular Probes) was used for monitoring the viability of bacterial populations as a function of the membrane integrity of the cell (16). Cells with a compromised membrane that are considered to be dead or dying will stain red, whereas cells with an intact membrane will stain green.

LTSEM. Low-temperature scanning electron microscopy (LTSEM) is useful for studying the in vivo organization of cells without being influenced by artifacts due to fixation, dehydration, and drying (33). Also, LTSEM allows the localization of water in the biofilm, and microorganisms can be examined without invasive treatments and without apparently disturbing the biofilm organization. Pneumococcal biofilms formed on glass surfaces were examined by LTSEM as previously described (15). Biofilms of *S. pneumoniae* R6 strain were grown on 14-mm glass coverslips that had been placed in each well of a 24-well PST microtiter dish (Falcon 3047; Becton Dickinson Labware). One milliliter of C medium was added, and the dishes were incubated for 10 to 12 h at 34°C. The coverslips were rinsed with PBS to remove nonattached cells. Small fragments were mechanically cut off, fixed onto the specimen holder of a cryotransfer system, plunged into liquid nitrogen, and then transferred to a preparation unit via an air lock transfer device. The frozen specimens were cryofractured and transferred directly via a second air lock to the microscope cold stage, where they were etched for 2 min at −90°C. After ice sublimation, the etched surfaces were sputter coated with gold in the preparation unit and then transferred onto the cold stage of the scanning electron microscope chamber. Fractured surfaces were observed at −135°C with a DSM 960 Zeiss scanning electron microscope.

RESULTS

Adherence of *S. pneumoniae* to abiotic surfaces and the environmental factors affecting biofilm development. The initial step in biofilm formation is the adherence of cells to a surface where organic nutrients concentrate. This observation can be extrapolated to organisms such as *S. pneumoniae*, which colonizes the human nasopharynx to establish a host carrier state. To determine the optimal conditions for biofilm formation of *S. pneumoniae* on abiotic surfaces, biofilm assays were carried out under various conditions. First, the ability of unencapsulated pneumococcus to form biofilms on polyvinylchloride, PST, or glass was tested following basically the procedure described by O′Toole and Kolter (55). Biofilm formation was measured by staining with CV. The best results were obtained on PST microplates and on glass-bottom dishes. Several culture media and supplements were then tested for biofilm development on PST microplates. Figure 1A and B show that either chemically defined (Cden or CDM) or semisynthetic (C) medium supported strong biofilm formation, whereas growth in complex medium, such as CAT or Todd-Hewitt broth sup-

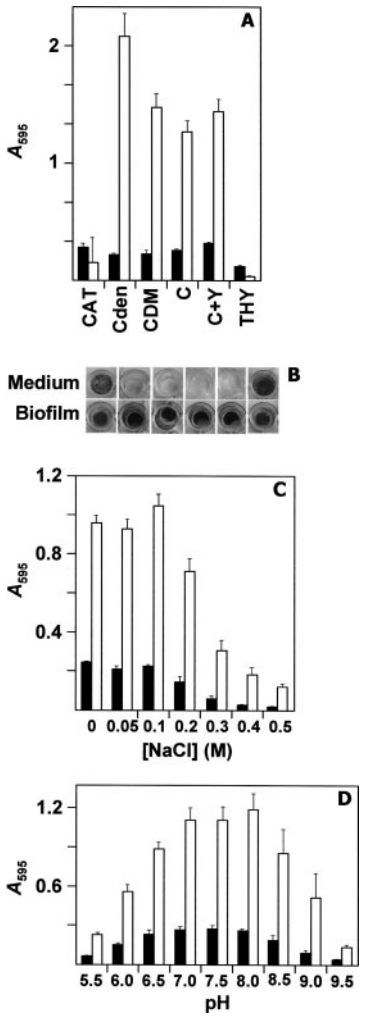

FIG. 1. Some factors influencing biofilm formation in *S. pneumoniae* R6 strain. (A) Effect of the culture medium. (B) Biofilm formation by *S. pneumoniae* on PST microtiter plates in different media for 6 h at 34°C. Cells were stained with CV and the plates were washed to remove unattached cells. As a control, wells with medium but without bacteria were also stained with CV. The results of a representative experiment are shown. (C) Effect of ionic strength. (D) The influence of the initial pH of the medium. In all cases, filled and open bars indicate bacterial growth and biofilm formation, respectively. The data represent the average of six samples. Standard error bars are shown.

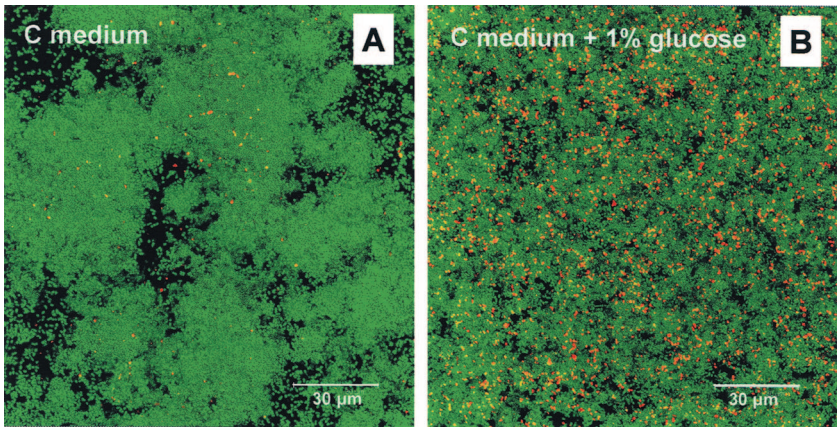

FIG. 2. CLSM image of the viability of biofilm-grown *S. pneumoniae* as a function of the incubation time. *S. pneumoniae* R6 cells were incubated in C medium to an A_{595} value of 0.5. The culture was centrifuged, and the cells were resuspended in an equal volume of prewarmed C medium. A portion of the culture was diluted 1/1,000 in the indicated medium. One percent glucose stimulates cell growth and the pH of the medium drops to below 6.0; this pH is nonpermissive for autolytic activity. After incubation at 34°C for 12 h (A) or 24 h (B), the cells in the biofilms were stained with the *Bac*Light kit showing living (green fluorescence) and dead (red fluorescence) bacteria. Images are horizontal 3D reconstructions of 25 scans in the *x-y* plane.

plemented with 0.5% yeast extract, resulted in weak (if any) biofilms. Moreover, culture of the cells in rich medium alone led to the production of a highly colored background material (Fig. 1B), whereas pneumococci incubated in minimal biofilm medium supplemented with glucose (0.8%, wt/vol) or casamino acids (0.2%, wt/vol), which is optimal for *Streptococcus gordonii* biofilm formation (43), failed to grow and attach to the abiotic surface. In addition, there were no significant differences in growth and biofilm formation in medium supplemented with 0.5% sucrose or 0.1% catalase (data not shown). When the osmolarity of the C medium was varied between 0 and 0.5 M NaCl, biofilm formation was clearly inhibited above 0.2 M (Fig. 1C). In addition, optimal biofilm development was observed when the starting pH of the medium was adjusted to between 7.0 and 8.0 (Fig. 1D).

The influence of inoculum size on the adherence of *S. pneumoniae* to PST plates and C medium or CDM was subsequently investigated (data not shown). In C medium, the number of adherent cells reached a maximum after 8 h of incubation at 34°C, irrespective of the initial cell density, except when the initial inoculum was very small, in which case optimal biofilm formation was only achieved after 16 h of incubation. After 16 h of incubation in C medium, the number of adherent cells diminished, probably for reasons related to the typical autolytic behavior of *S. pneumoniae*. In favor of this hypothesis is the finding that no such decrease in biofilm formation was found when *S. pneumoniae* R6 cells were incubated in CDM instead of C medium (not shown). As the glucose concentration in CDM is eight times higher than in C medium (10 mg ml^{-1} versus 1.24 mg ml^{-1}), it allows higher cell growth. Consequently, the pH of the medium drops below 6.0 at the stationary phase (unpublished observations); at this low pH, the peptidoglycan hydrolytic activity of the major pneumococcal autolysin (LytA) is completely prevented (46), and important biological activities of LytA, such as progeny-phage release, are hindered at nonpermissive pH values (>8.0 or < 5.8) (46, 60). Consequently, we decided to study the viability of pneumococcal biofilms in C medium supplemented with 10 mg of glucose ml^{-1} to prevent the lysis of the culture (Fig. 2). Interestingly, although autolysis was inhibited, the number of dead cells in the biofilm markedly increased after prolonged incubation (Fig. 2).

Biofilm formation in species genetically related to *S. pneumoniae*. The biofilm-forming capacity of several streptococci of the mitis group was assayed and quantified using the optimal conditions determined for pneumococcus (see Materials and Methods). With the exception of *Streptococcus mitis*^{T}, the other three streptococcal type strains were able to form biofilms in C medium (Fig. 3). To the best of our knowledge, this is the first demonstration of biofilm formation by *Streptococcus pseudopneumoniae*. It should be mentioned, however, that variations in the ability to form biofilms among strains of the same species have been previously reported (43).

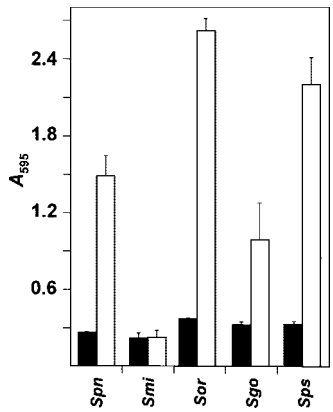

FIG. 3. Biofilm formation capacity of various streptococcal species of the mitis group. *Smi*, *Sor*, *Sgo*, and *Sps* indicate, respectively, the type strain of *S. mitis*, *S. oralis*, *S. gordonii*, and *S. pseudopneumoniae*. The *S. pneumoniae* R6 strain (*Spn*) was used as a control. Cells were grown in C medium for 6 h at 34°C. Filled and open bars indicate growth and biofilm formation, respectively. The results are the average of three independent experiments.

Role of choline and choline-binding proteins in biofilm formation capacity. In most bacteria, surface molecules appear to be important for biofilm development. We were interested in examining the initial stages of the infective process of *S. pneumoniae* by identifying specific microbial components that might affect biofilm formation and, presumably, pneumococcal colonization of the human nasopharynx. In this sense, we paid special attention to the pneumococcal surface proteins, particularly choline-binding proteins (CBPs), involved in adhesion to and invasion of the host cells (as the adhesins CbpA and PcpA) and in the establishment of nasopharynx colonization (as the murein hydrolases LytB and LytC). Choline is an essential component of pneumococcal teichoic and lipoteichoic acids (22) and is required for anchoring the CBPs that play important roles in pneumococcal infection (44). Likewise, it seems that choline mediates bacterial adherence to the host cells, apparently through its interaction with the human platelet-activating factor receptor (22). Some CBPs are released from the surface of the pneumococcus upon incubation with high choline concentrations (>1%). Under these conditions, choline also inhibits those CBPs that have cell wall hydrolase activity (45). Accordingly, there was a notable reduction in biofilm formation by the R6 strain when incubated in C medium containing 2% choline chloride (Fig. 4A). This effect might be attributed to the excess of choline and/or to the inhibition of one or more CBPs. These possibilities were investigated by analyzing the effects on biofilm formation of mutations in several CBPs, namely, the murein hydrolases LytA (SP1937) (an *N*-acetylmuramoyl-L-alanine amidase; the main pneumococcal autolysin), LytB (SP0965) (a possible glucosaminidase involved in cell separation), and autolytic LytC lysozyme (SP2190), as well as the phosphocholinesterase Pce (SP0930) and CbpA major adhesin (SP2190), CbpD putative amidase (SP2201), PcpA possible adhesin (SP2136), and PspA (SP0117) (pneumococcal surface protein A) (for recent reviews, see references 45 and 66). As shown in Fig. 4B, inactivation of LytA, LytB, LytC, CbpA, PcpA, or PspA diminished biofilm formation on PST plates, whereas a mutation in the *pce* or *cbpD* genes had no detectable effect. We also analyzed the capacity of several pneumococcal mutants affected simultaneously in more than one CBP to form biofilms (Fig. 4B). Notably, biofilm formation was inhibited by 70% when a *lytA-lytB-lytC* triple mutant was used (Fig. 4B). Pneumococcal strains lacking an active LytB enzyme (either *lytB* mutants or *lytB*$^+$ strains incubated in 2% choline chloride or in EA-containing medium; see below) grow as long chains of cells (14). Consequently, it was tempting to speculate that chaining may hinder biofilm formation. However, chain formation by itself does not appear to be the determining factor in the reduced adherence of either the *lytB* mutant or the R6 strain cultivated with 2% choline chloride, since several streptococci of the mitis group that grow as long chains (e.g., strains 1078 or 782) (52) formed a biofilm virtually identical to that of the R6 strain (unpublished observations).

When choline is replaced by its structural analogue EA in a chemically defined medium, teichoic acids contain EA instead of choline (69), and, phenotypically, EA-grown cells are identical to those incubated in the presence of 2% choline (7). A near 50% reduction in biofilm formation was found when the R6 strain was incubated in either Cden medium or CDM in the presence of EA (Fig. 4A). Moreover, this inhibition became even more marked when 2% (about 140 mM) choline chloride was used instead of EA. This behavior may be related to the previous finding that an increase in the ionic strength of the medium reduced development of the biofilm (Fig. 1C).

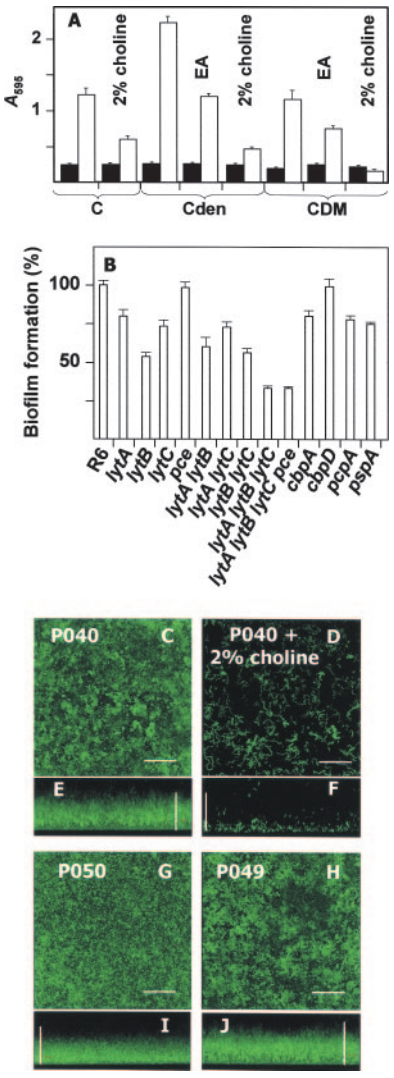

FIG. 4. Influence of choline and CBPs on biofilm formation and CSLMs of *S. pneumoniae* biofilms. (A) Influence of choline and EA on biofilm formation by the R6 strain. Filled and open bars indicate growth and biofilm formation, respectively. (B) Biofilm formation capacity of several mutant strains. In this experiment, the following strains were incubated in C medium for 6 h: R6, M32 (*lytA*), R6B (*lytB*), R6C (*lytC*), R6D (*pce*), M31B (*lytA lytB*), P042 (*lytA lytC*), R6BC (*lytB lytC*), M31BC (*lytA lytB lytC*), M31BCD (*lytA lytB lytC pce*), P064 (*cbpA*), R1582 (*cbpD*), P065 (*pcpA*), and P066 (*pspA*). The values of biofilm formation were normalized for absorbance, and the percentages were calculated in relation to strain R6. Shown are CLSM images of pneumococcal strains harboring pMV158GFP: P040 (R6), P049 (*pce*), and P050 (*lytA lytC*). (C, D, G, and H) Horizontal 3D reconstructions of 55 scans in the *x-y* plane. (E, F, I, and J) Vertical 3D reconstructions of 65 scans in the *x-z* plane. In all of the images, the bar corresponds to 30 μm.

Biofilm architecture of some pneumococcal strains. Biofilms formed by GFP-expressing *S. pneumoniae* cells incubated in C medium for 10 to 12 h at 34°C were examined by CLSM and

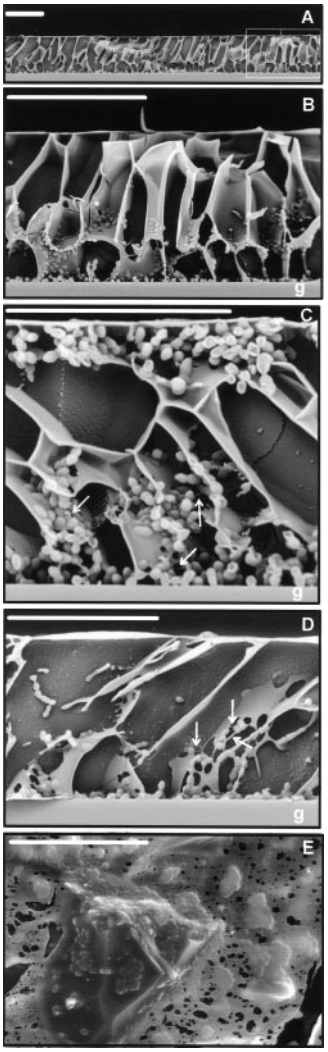

FIG. 5. LTSEM of *S. pneumoniae* R6 biofilm. (A) General view of the biofilm formed on the surface of a glass coverslip. (B) Magnification of the area indicated by a rectangle in panel A. (C and D) Two different views. Filamentous material (indicated by arrows) links pneumococcal cells to each other and to the intercellular matrix. (E) Apical view of the irregular surface of a pneumococcal biofilm. Microcolonies of different sizes can be seen. In all micrographs, the bar indicates 20 μm.

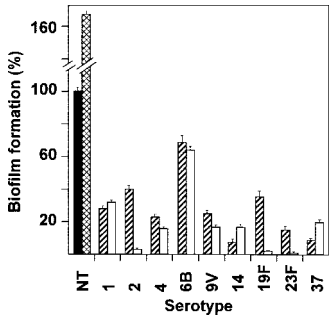

FIG. 6. Biofilm formation by encapsulated *S. pneumoniae* strains. Clinical pneumococcal isolates of the indicated serotypes were grown in C medium for 6 h at 34°C. Biofilm formation (hatched bars) was quantified by staining with CV. Open bars correspond to biofilms developed by encapsulated transformants of the indicated serotype using the unencapsulated M11 strain as recipient. NT corresponds to the M11 strain, which was used as control (filled bar) and to the nontypeable pneumococcal isolate ST344 (cross-hatched bar). The values of biofilm formation by encapsulated strains were normalized for absorbance, and the percentage was presented and normalized for the control strain M11. The percentages shown are the average of three independent experiments.

exhibited the 3D multicellular structure typical of a biofilm (Fig. 4C and E). The adherent cells formed a mat of cells of significant depth (20 to 30 μm), which, as expected, was much more crowded and thick than the mat formed by the same strain incubated in medium containing 2% choline chloride (Fig. 4D and F). It should be mentioned that biofilms formed in the presence of a high choline concentration were not tightly attached to the PST or glass surfaces, and extreme care was employed during washes to avoid a complete loss of the attached cells (not shown). Confocal micrographs also showed that the biofilms formed by a *lytA lytC* mutant (strain P050) (or, simply, by a *lytA* mutant) were consistently thinner (15 to 20 μm) (Fig. 4G and I) than those formed by the P040 (*lytA*$^+$) strain (Table 1). However, and in agreement with the results mentioned above, no detectable differences were found between the biofilms formed by strain P040 and a *pce* mutant (strain P049) (Fig. 4H and J).

The ultrastructure of biofilms formed by *S. pneumoniae* R6 was observed using LTSEM (Fig. 5). The micrographs confirmed that this strain formed a biofilm about 25 μm deep, in which many pneumococcal cells lay on the surface of the coverslip. Large void spaces (originally occupied by water) could be observed on transverse sections of biofilms (Fig. 5B to D). The cells were interconnected by small, thin filaments that linked the cells to each other and/or bound the cells to the intercellular matrix (Fig. 5D). It is well established that, as ice crystals grow during cryofixation, they sweep the solutes from the medium into a eutectic boundary formed between neighboring ice crystals (33). That boundary corresponds to the scaffold-like material, which could also be observed in the absence of cells (not shown). Figure 5E shows an apical view of several microcolonies, revealing an irregular and discontinuous surface that coated the cells and contained holes of different sizes which might represent channels between the cell clusters.

Influence of the capsular polysaccharide in biofilm formation. The definition of a biofilm has evolved during the last 25 years (17), and the current definition takes into account the finding that extracellular polysaccharides are not always required for biofilm development, as recently reported in *Staphylococcus aureus* (68). The great variety of polysaccharide and protein components of the biofilm matrix is an emerging theme of great importance, since the expression of different extracellular molecules affects the surface properties of bacteria and, consequently, promotes (or hinders) the formation and maintenance of different types of multicellular communities (6). These observations are particularly important in the case of pneumococcus, in which 90 different types of capsular polysaccharides have been described (31). Down-regulation of capsular polysaccharide production appears to enhance attachment of *S. pneumoniae* to host cells and bacterial invasion of the nasopharynx during establishment of the host carrier state (77). To gain further information on the role of the capsule in

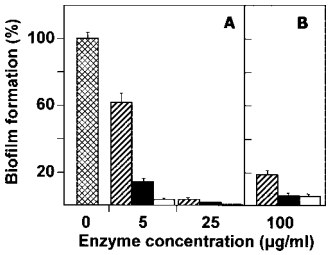

FIG. 7. Inhibition of biofilm development in *S. pneumoniae* cultures in the presence of DNase or proteases. (A) *S. pneumoniae* R6 was grown overnight at 37°C to an A_{595} value of 0.5 to 0.6 in C+Y medium, centrifuged, and adjusted to an A_{595} of 0.6 with fresh medium. Afterwards, the cell suspension was diluted 10-fold, and 200-μl aliquots were distributed in the wells of a microtiter plate, which was then incubated for 6 h at 34°C (cross-hatched bars). Other samples received either DNase I (hatched bars), trypsin (filled bars), or proteinase K (open bars) at the indicated concentrations and were incubated as above. (B) After biofilm development (6 h at 34°C), DNase I (hatched bars), trypsin (filled bars), or proteinase K (open bars) was added at 100 μg ml^{-1}, and incubation proceeded for an additional 1 h at 34°C before staining with CV to quantify biofilm formation.

the initial steps of biofilm formation, the capacities of clinical pneumococcal isolates of various serotypes to develop biofilms were compared. The results shown in Fig. 6 indicated that, with the exception of serotype 6B strains that showed a reduction of approximately 30%, the presence of a capsule reduced biofilm development by more than 60%. To ascertain whether the observed differences in the capacity to form biofilms were due exclusively to the capsular polysaccharide itself and not to other factors (e.g., CBPs) that may vary among different isolates, we constructed a series of strain M11 derivatives that showed growth rates similar to that of their parental M11 strain and expressed comparable amounts of the different capsular polysaccharides. These encapsulated transformants were also impaired in their capacity to form biofilms, and some of them formed significantly less biofilm than the clinical isolates. Again, the transformant of serotype 6B showed only a limited reduction in its capacity to form biofilms (Fig. 6). Furthermore, it should be emphasized that a nontypeable pneumococcal isolate (strain ST344) formed biofilms with an efficiency even greater than that of laboratory strain R6.

Involvement of extracellular DNA and proteins in biofilm development. Extracellular DNA has been implicated as a major structural component for the initial establishment of biofilms in bacteria that specifically release DNA (78). Moreover, it has been recently observed that free DNA plays an important role in biofilm formation by *S. mutans* (58). In *S. pneumoniae*, DNA is spontaneously released into the medium in a process which is triggered by competence induction for genetic transformation and which depends on LytA and LytC (51, 63, 64). We observed that when strain R6 was grown in the presence of DNase I, biofilm formation was greatly impaired (Fig. 7A) although culture growth was not affected by addition of the enzyme (not shown). Similar experiments carried out with proteases again provided evidence of a dramatic reduction in biofilm formation. Finally, incubation of preformed R6 biofilms with either DNase or proteinase K drastically diminished the number of biofilm-associated sessile cells (Fig. 7B).

DISCUSSION

Most microorganisms adopt biofilm formation as a lifestyle in nature, and the unique properties of many such multicellular communities have been characterized. However, molecular approaches to study these sessile forms of growth are lacking. The experiments reported in the present study provide evidence for the influence of several well-defined genetic traits and extracellular macromolecules in biofilm development by *S. pneumoniae*. Under the experimental conditions described here, pneumococcal cells adhered to a surface and adopted a multicellular 3D structure; i.e., they formed a biofilm.

Biofilm formation by pneumococcus cultured in a semisynthetic medium suggests that sessile growth represents a survival strategy in a nutritionally limited environment, assuming that surface colonization provides certain advantages, such as an increased capacity to capture nutrients, and that the oral surface represents a nutritionally limited environment. The environment defined by the human nasopharynx environment is subjected to significant fluctuations in pH, osmolarity, and nutrient availability; it is a well-ventilated zone where the temperature (about 34°C) (36) is cooler than in the rest of the body. Values of 6.4 to 6.9 for pH (34), 82 to 91 mM for Na^+, 82 to 108 mM for Cl^- (73), and 3.9 to 5.8 mM for glucose (20) have been reported for nasopharyngeal and/or airway surface secretions in humans. In our experiments, environmental changes, such as nutrient content of the medium, pH, and osmolarity, affected biofilm formation in vitro (Fig. 1). Thus, the conditions described here, which were analogous to those found in the nasopharynx (see above), may be suitable for studying the cellular factors and molecular mechanisms that influence the formation of pneumococcal biofilms. Furthermore, our experimental approach fulfills the prerequisites for the comprehensive analysis of biofilm formation and complements previous attempts to study the role of capsular production during in steady-state growth on Sorbarod filters (74, 75).

The biofilms formed by pneumococcal cells developed into a 3D structure made up of a mat of cells, as revealed by CLSM and LTSEM (Fig. 4 and 5). Incubation of pneumococcus in the presence of 2% choline chloride (or EA) from the initial steps of growth reduced biofilm development (Fig. 4D). It is well known that when pneumococcal cells are incubated with 2% choline, some CBPs are released into the medium, whereas those having enzymatic activity are completely inhibited (44). For example, the characteristic long-chain growth of cells incubated in a high concentration of choline is due to the inhibition of LytB glucosaminidase (14, 24). Although it could be speculated that biofilm inhibition is a direct consequence of chaining itself, this appears not to be the case in pneumococci, as several closely related streptococcal strains that grow in long chains were perfectly able to form biofilms (see above). Moreover, the use of pneumococcal strains harboring mutations in the other cell wall hydrolases, namely, LytA, LytC, and Pce, showed that these CBPs play a variable role in biofilm formation on abiotic surfaces, since *lytA*, *lytB*, or *lytC* mutants formed, respectively, 20, 45, and 25% less biofilm than the wild-type strain, whereas no effect was observed when a *pce* mutant was employed (Fig. 4B). An additive effect on biofilm inhibition was observed with a *lytA lytB lytC* mutant. Interestingly, a positive correlation between peptidoglycan breaks,

rather than particular molecules, and biofilm-forming capacity in the gram-positive bacterium *Lactococcus lactis* has been established recently (49). Additionally, it should be noted that a reduced colonizing capacity of the nasopharynx was reported for *lytB* and *lytC* mutants (25).

Many aspects of biofilm formation appear to be counterintuitive; e.g., some bacteria preferentially form biofilms in environments characterized by very high shear forces, such as occurs in the heart valves (17). This is also the case for the production of capsular polysaccharides by pneumococcus in that one would expect these compounds to mediate adherence to the nasopharyngeal epithelium, thereby initiating biofilm formation. Interestingly, it has become apparent that downregulation of pneumococcal capsule production enhances host-cell invasion in the asymptomatic carrier state, although the capsular phase must be restored for the bacteria to survive after invasion (29, 76). This observation may have important implications for experiments with *S. pneumoniae* in vitro. Most of the capsular polysaccharides tested appeared to inhibit biofilm formation although, for still unknown reasons, pneumococci synthesizing a type 6B capsule were only partially inhibited in their capacity to grow as a biofilm (Fig. 6). This behavior provides a model to further study the impact of capsular variation on biofilm development in a species with a large number of different capsular types. Recently, it was suggested that this is a route to better analyze the role of the bacterial extracellular matrix in cell accretion (6). Furthermore, our observations are in keeping with previous findings reporting that exopolysaccharides influence initial attachment either positively or negatively, depending upon the exopolysaccharide type and the organism (62). This may be the case for *S. aureus*, which without a capsule was able to better persist in the murine mammary gland (71). However, it is premature to discard the possibility that capsular polysaccharide is not an essential component of biofilm matrix, as recently illustrated in the case of *S. aureus*, in which biofilm-associated protein (Bap) appears to replace the sugar matrix as a cementing material (68).

In microbial biofilms, bacterial cells aggregate on the surface in microcolonies and are embedded in an extracellular matrix whose composition is as variable as that of the biofilm. In general, the sticky matrix is composed of diverse extracellular polymeric substances, including exopolysaccharides, proteins, and DNA, and may contain other noncellular materials, depending on the environment in which the biofilm developed. Biofilm formation requires protein synthesis, and it is thought that extracytoplasmic proteins, as surface-exposed proteins, play a role in bacterial attachment to abiotic surfaces (55). Recently, using a continuous-culture biofilm system, de novo protein biosynthesis was reported to take place during biofilm formation by *S. pneumoniae*; more than 700 proteins that were detected in 9-day-old biofilms were apparently absent in planktonic cells (2). Our results (Fig. 7) showed that extracytoplasmic protein(s) is required for biofilm formation and maintenance. Furthermore, DNA has been identified as a key structural component of the biofilm extracellular matrix (78), and, more recently, biofilm formation was shown to involve a functional DNA-binding uptake system (58). Under the experimental conditions assayed here, DNA appears to have been released spontaneously into the medium, suggesting that it is an important component of the biofilm formed by *S. pneumoniae*. This conclusion is supported by the fact that there was a significant inhibitory effect on biofilm formation when DNase I was added either before or afterwards (Fig. 7). However, when either homologous or heterologous DNA was added to a pneumococcal culture, biofilm formation was not significantly affected (unpublished results). It has been shown that simultaneous inactivation of *lytA* and *lytC* abolished DNA release in liquid culture (51). In addition, Kausmally and coworkers (35) reported that DNA release is strongly attenuated in a *cbpD* mutant. Interestingly, we found that the two autolysins (but not CbpD) are also involved in biofilm formation (Fig. 4B, G, and I). We are currently investigating whether DNA-binding proteins up-regulated by the CSP are involved in biofilm formation in pneumococcus, as already documented for *Streptococcus intermedius* (57).

Given the tremendous metabolic and physiological changes that are required for the switch from a planktonic to a biofilm form of growth, it would be reasonable to assume that gene regulation facilitates this process. This switch, which is based mainly on local environmental cues that alter gene expression, seems to be crucial for microbes. Moreover, the switch serves to determine the virulence of the invading bacteria (54). It has been pointed out that, once attached to a surface, microbial cells initiate long-term interactions with each other (37). Such interactions may facilitate DNA interchange in organisms possessing natural competence, as is the case for pneumococcus.

Finally, it is well documented that competence and DNA release in pneumococcus are activated by a quorum-sensing (QS) circuit, a regulatory mechanism for bacteria to alter gene expression at very specific cell densities and to coordinate a population-type behavior in which dozens of genes are mobilized. CSP is the QS signal that mediates both competence and genetic transformation, and it has been well studied in *S. pneumoniae* (9). Horizontal DNA transfer is one of the major mechanisms driving the evolution of microorganisms, and related species cooperate with each other by sharing genes. This is exemplified by *S. pneumoniae* in its acquisition of penicillin resistance following genetic transformation with DNA released by other streptococci of the mitis group (26). QS systems have also been suggested to influence biofilm formation by *S. mutans* (41), although the molecular and biochemical details of this finding merit further investigation (65).

Studies of the QS system (9) and CBPs (44) are most advanced in *S. pneumoniae*. Thus, the availability of a feasible design for biofilm formation offers a model for genetically approaching questions regarding the social behavior of this human pathogen. In this sense, details on cell-to-cell communication will generate interesting information on survival strategies, such as passive protection against antibiotics, which appears to represent a phenotype rather than the result of genetic alterations. The goal of the results reported, to provide insight into biofilm development by pneumococcus, is thus beginning to take shape.

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