RESEARCH ARTICLE

High Levels of Genetic Recombination during Nasopharyngeal Carriage and Biofilm Formation in *Streptococcus pneumoniae*

Laura R. Marks,[a] Ryan M. Reddinger,[a] and Anders P. Hakansson[a,b,c]

Department of Microbiology and Immunology, University at Buffalo, State University of New York, Buffalo, New York, USA[a]; The Witebsky Center for Microbial Pathogenesis and Immunology, University at Buffalo, State University of New York, Buffalo, New York, USA[b]; and New York State Center of Excellence in Bioinformatics and Life Sciences, Buffalo, New York, USA[c]

ABSTRACT **Transformation of genetic material between bacteria was first observed in the 1920s using *Streptococcus pneumoniae* as a model organism. Since then, the mechanism of competence induction and transformation has been well characterized, mainly using planktonic bacteria or septic infection models. However, epidemiological evidence suggests that genetic exchange occurs primarily during pneumococcal nasopharyngeal carriage, which we have recently shown is associated with biofilm growth, and is associated with cocolonization with multiple strains. However, no studies to date have comprehensively investigated genetic exchange during cocolonization *in vitro* and *in vivo* or the role of the nasopharyngeal environment in these processes. In this study, we show that genetic exchange during dual-strain carriage *in vivo* is extremely efficient (10^{-2}) and approximately 10,000,000-fold higher than that measured during septic infection (10^{-9}). This high transformation efficiency was associated with environmental conditions exclusive to the nasopharynx, including the lower temperature of the nasopharynx (32 to 34°C), limited nutrient availability, and interactions with epithelial cells, which were modeled in a novel biofilm model *in vitro* that showed similarly high transformation efficiencies. The nasopharyngeal environmental factors, combined, were critical for biofilm formation and induced constitutive upregulation of competence genes and downregulation of capsule that promoted transformation. In addition, we show that dual-strain carriage *in vivo* and biofilms formed *in vitro* can be transformed during colonization to increase their pneumococcal fitness and also, importantly, that bacteria with lower colonization ability can be protected by strains with higher colonization efficiency, a process unrelated to genetic exchange.**

IMPORTANCE **Although genetic exchange between pneumococcal strains is known to occur primarily during colonization of the nasopharynx and colonization is associated with biofilm growth, this is the first study to comprehensively investigate transformation in this environment and to analyze the role of environmental and bacterial factors in this process. We show that transformation efficiency during cocolonization by multiple strains is very high (around 10^{-2}). Furthermore, we provide novel evidence that specific aspects of the nasopharyngeal environment, including lower temperature, limited nutrient availability, and epithelial cell interaction, are critical for optimal biofilm formation and transformation efficiency and result in bacterial protein expression changes that promote transformation and fitness of colonization-deficient strains. The results suggest that cocolonization in biofilm communities may have important clinical consequences by facilitating the spread of antibiotic resistance and enabling serotype switching and vaccine escape as well as protecting and retaining poorly colonizing strains in the pneumococcal strain pool.**

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Address correspondence to Anders P. Hakansson, andersh@buffalo.edu.

Horizontal gene transfer mediated by transformation is critical to bacterial evolution, where it facilitates and accelerates the emergence of novel virulence traits and enables the spread of antibiotic resistance (1–3). Natural genetic transformation is present in a variety of bacterial organisms from different phyla and occupying various niches, and although the mechanisms appear to be different between Gram-positive and Gram-negative organisms, the endpoint advantage is the same: to adapt to environmental stresses (4, 5). As one example, *Streptococcus pneumoniae* antimicrobial resistance is widely distributed among the 93 different serotypes and is at least partly due to genetic transformation, as strains recovered from molecular epidemiology surveys show identical or highly related restriction fragment length polymorphism (RFLP) patterns for penicillin (Pen) binding protein genes (responsible for resistance to penicillin), with the remaining genetic background being greatly heterogeneous (6–9).

Natural genetic transformation was first observed in 1928 in *Streptococcus pneumoniae* in the influential Griffith experiments (10) and would later enable Avery and coworkers to identify DNA as the biochemical basis of inheritance (11). Since then, the detailed mechanism of competence initiation, DNA uptake and integration, has been well studied in *S. pneumoniae* (12). But impor-

tantly, while natural transforming ability remains a textbook descriptor of the pneumococcus, most studies investigating this process have relied on a small subset of hypercompetent rough laboratory strains descended from the original Avery experiments. Clinical strains have been frequently found to lack natural competence under these same conditions (13, 14). Therefore, attempts to characterize the natural transformability of clinical isolates have uniformly required the artificial induction of competence using exogenous addition of high concentrations of synthetic competence-stimulating peptide (CSP) (14–16). Thus, although natural transformation is known to occur in *S. pneumoniae* based on its ability to acquire genetic elements, such as antibiotic resistance, it remains unknown what specific signals and conditions in the host environment lead to increased natural recombination *in vivo*.

Epidemiological studies suggest that pneumococci colonizing the nasopharynx, rather than bacteria isolated from invasive disease, are the source for much of the horizontal transfer of resistance genes between strains (6, 17–19). This is not unexpected, since *S. pneumoniae* colonizes the mucosal surface of the nasopharynx effectively beginning during the first few months of life. Successive episodes of colonization are common, and by the age of 2 years, greater than 95% of children will be colonized, with individual serotypes colonizing for weeks to months as children sequentially lose and acquire as many as six distinct serotypes (20–22). Additionally, natural transformation in this niche may be facilitated by simultaneous carriage of multiple strains of pneumococci (cocolonization) (7, 8). Cocolonization has been documented since the 1930s (23), but as most epidemiological studies have relied on serotyping individual colonies isolated from culture (22, 24, 25), this has likely led to a significant underreporting of cocolonization in many epidemiological studies, leading to a wide variation in cocolonization rates that ranges from 5 to 30% (21, 25–31).

Although horizontal gene transfer is primarily associated with colonization by pneumococci, little work has been done to address the role of the specific environment and conditions associated with this milieu and how it affects transformation efficiency and pneumococcal adaptation and fitness. *S. pneumoniae* infects and colonizes the human host under vastly different environments, developing as a pathogen to cause invasive disease at temperatures close to 37°C, under oxygen-limiting conditions and in a glucose-rich environment, or existing as a commensal in the upper respiratory tract, where it grows at a lower temperature under a partial pressure of oxygen close to atmospheric pressure and with limited nutrients. During colonization, pneumococci organize into collaborating multicellular communities attached to the mucosal epithelium and encased in a self-produced polymeric matrix (32, 33), and this biofilm environment has been shown to upregulate competence genes (34, 35) as well as protect pathogens from the hostile environment within the host and facilitate persistence (36–38).

The aim of this study was to investigate the natural transformation efficiency in the nasopharyngeal environment during cocolonization or sequential colonization with multiple strains in comparison to invasive disease, to mechanistically address the host and bacterial factors contributing to transformation in this milieu using a novel biofilm model *in vitro*, and to better understand the interaction of dual-strain biofilms *in vitro* and *in vivo*. As of yet, this has not been addressed comprehensively, and information from these studies has the potential to further our understanding of cocolonization *in vivo* to better predict the mechanistic aspects of factors facilitating recombination, spreading antibiotic resistance, and enabling serotype switching and vaccine escape as well as promoting the persistence of less effectively colonizing strains in the population.

RESULTS

Colonizing bacteria show increased natural transformation compared with planktonic growth during sepsis. Although epidemiological studies suggest that colonizing bacteria are the predominant source of horizontal transfer of resistance genes between strains (6, 7, 17–19), previous work investigating *in vivo* pneumococcal transformation focused on this process in the context of sepsis and other disease states, and thus far, no study has comprehensively addressed the genetic exchange during dual-strain colonization of the nasopharynx.

To investigate the transformability of pneumococci during nasopharyngeal colonization, 6-week-old BALB/cByJ mice were inoculated intranasally for 48 h with 2.5×10^6 CFU of SP670 (a clinical Pen^r strain) and 2.5×10^6 CFU of D39-C08P2 (a strain of D39 pneumococci with colonization efficiency similar to that of D39, carrying a chromosomal insertion of the erythromycin resistance [Erm^r] cassette downstream of the dihydrolipoamide dehydrogenase gene *dldh*). Transformation efficiency was then assessed by determining the number of colonies from nasopharyngeal tissue homogenates growing on selection medium containing both penicillin and erythromycin as a ratio of the total recovered population. To compare, mice were also injected intraperitoneally with a mixture of 1×10^5 CFU of SP670 and 1×10^5 CFU of D39-C08P2 and euthanized 36 h later, after which the ratio of double mutants isolated from blood was determined in the same way as for transformation in the nasopharynx. For all *in vivo* assays, pneumococcal growth in tissues was verified by morphology of the bacteria on blood agar plates in combination with testing several isolated clones for optochin sensitivity. Nasopharyngeal tissues from uninfected mice were always sterile.

Pneumococci cultured from nasopharyngeal tissues of infected mice showed a high transformation efficiency with a median of $\sim 1 \times 10^{-2}$, with a median of 7.9×10^3 CFU of double-resistant transformants recovered per mouse (out of a median of 1×10^5 total CFU recovered per mouse) (Fig. 1; see also Fig. 5D). In contrast, the sepsis model generated comparatively fewer transformants (between 1 and 50 CFU out of approximately 4.5×10^9 CFU recovered per mouse) carrying both antibiotic resistance cassettes, with a significantly (approximately 10^7-fold) lower median transformation efficiency of 3×10^{-9} (Fig. 1; $P < 0.001$).

As acquisition of strains during colonization in real life is thought to most often occur sequentially; mice were also colonized in this fashion. Mice that were first colonized with 1×10^5 CFU of D39-C08P2 for 24 h and then colonized with 1×10^5 CFU of SP670 for another 48 h showed a median transformation efficiency of $\sim 1 \times 10^{-2}$, although 3 mice in this cohort failed to generate any transformants and also cleared the entire D39-C08P2 population. Sequential nasopharyngeal colonization thus yielded results similar to those from inoculating strains simultaneously.

These results demonstrate that genetic exchange between strains *in vivo* is more efficient in the murine nasopharynx than in the blood and that transformation efficiencies are equally high

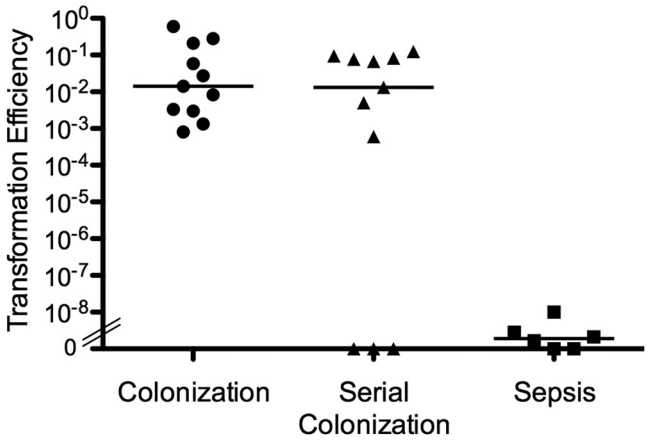

FIG 1 Efficiency of transformation of antibiotic resistance elements between SP670 (Pen^r) and D39-C08P2 (Erm^r) during nasopharyngeal cocolonization, sequential cocolonization, and sepsis.

when strains are acquired sequentially and when they are inoculated simultaneously.

Encapsulated clinical isolates are not naturally transformable during planktonic growth but are easily transformed during growth in biofilm communities. To address the major difference in transformability in different host niches, we investigated the role of different growth conditions encountered during colonization and sepsis. Bacteria in the blood of septic mice have shown gene expression profiles similar to planktonic growth in liquid medium *in vitro*, while tissue-associated bacteria have shown gene expression profiles that match *in vitro* models of pneumococcal biofilms (34). This is made relevant by recent work from our laboratory that demonstrates that pneumococci organize into biofilm communities during nasopharyngeal colonization (32) and work by others showing that the competence system is upregulated during biofilm formation on abiotic surfaces that was either dependent or not on the addition of CSP, a potential prerequisite for biofilm formation (34). Therefore, we hypothesized that biofilm formation may play a role in the elevated gene exchange seen during colonization.

To test this hypothesis, we first measured natural transformation efficiency during planktonic and biofilm growth, first using purified chromosomal DNA. All clinical and laboratory strains used, with the exception of the 23F serotype isolate SP456, could be transformed in broth cultures after exogenous addition of 100 ng/ml synthetic competence-stimulating peptide 1 (CSP1) or CSP2. However, none of the encapsulated clinical isolates showed any natural competence during planktonic growth, which verifies the difficulty experienced with these systems in the field (Table 1).

TABLE 1 Natural transformation efficiency of pneumococcal strains during planktonic and biofilm growth

Strain	Serotype	Natural transformation efficiency	
		Planktonic	Biofilm
D39	2	0	4.4×10^{-4}
SP670	6B	0	6.5×10^{-3}
SP456	23F	0	9.1×10^{-4}
Rx1	Acapsular	1.1×10^{-4}	2.8×10^{-4}

The functionality of the assay was verified by showing that the strain Rx1, a commonly used lab strain that is a rough (unencapsulated) derivative of the R36A Avery strain with increased transformation efficiency due to defects in mismatch repair (*hex* mutant) (39, 40), could be naturally transformed in our assays (Table 1).

To investigate transformation in biofilms, we used our recently developed biofilm model where bacteria form biofilms on a respiratory epithelial cell substratum at the nasopharyngeal temperature of 34°C and in a medium with limited nutrient availability that we have shown mimics the morphology and antibiotic resistance phenotypes of *in vivo* colonization and correlates well with the colonization ability of strains *in vivo* (32). No exogenously added CSP was used in these experiments. These biofilms were exposed to a high concentration (1 μg/ml) of chromosomal DNA harboring heterologous erythromycin or penicillin resistance genes added after initial biofilm seeding. Biofilms were maintained for 72 h with medium being changed twice daily. No additional DNA was added at these medium changes, to allow a straight comparison with transformation in the planktonic cultures, where DNA was added only once. After 72 h, biofilms were sonicated and cell suspensions were vortexed to completely disperse the biofilm prior to plating onto selection plates containing 3 μg/ml erythromycin or 1 μg/ml penicillin G as indicated, to determine the number of transformants by CFU counts. In this assay, the encapsulated strains (including the nontransformable 23F strain SP456) formed biofilms that integrated resistance cassettes with an efficiency of 10^{-3} to 10^{-4}. These results suggest that many "difficult" or "nontransformable" *S. pneumoniae* strains may in fact be capable of natural transformation when grown as biofilms and suggest that biofilm growth confers an optimal environment for genetic exchange.

Lysed bacteria can act as donors for natural transformation in a biofilm. In natural ecosystems, exogenous transforming DNA for bacteria most likely originates from incompletely degraded DNA fragments released from dead cells in the immediate vicinity, which also make up part of the extracellular matrix of many biofilms (41–44). The pneumococcus possesses a unique system to facilitate this process, fratricide, whereby competence development triggers cell lysis and DNA release from a subfraction of the cell population (45).

To test whether a growing biofilm could similarly acquire and incorporate DNA from lysed cells, we grew a planktonic culture of D39-C08P2 pneumococci (Erm^r) through log phase and stationary phase, allowing natural autolysis to take place. To ensure that no viable cells remained, the culture was heat killed and plated on blood agar, revealing no viable organisms. This lysate of D39-C08P2 (Erm^r) was then added either to a culture of SP670 (Pen^r; 1:25 ratio) directly after inoculation of the biofilm or to SP670 cultures grown planktonically to an optical density at 600 nm (OD_{600}) of between 0.05 and 0.15, when natural transformation for this strain, under these experimental conditions, is peaking. Transformation efficiency in biofilms exposed to the dead cell lysates was assayed after 72 h by plating on selection medium, and the cultures showed high levels of natural transformation equivalent to the addition of 1 μg/ml purified DNA, whereas none of the planktonic cultures showed transformants after 6 h of exponential growth (Fig. 2A), when cell density was similar to those of the biofilms.

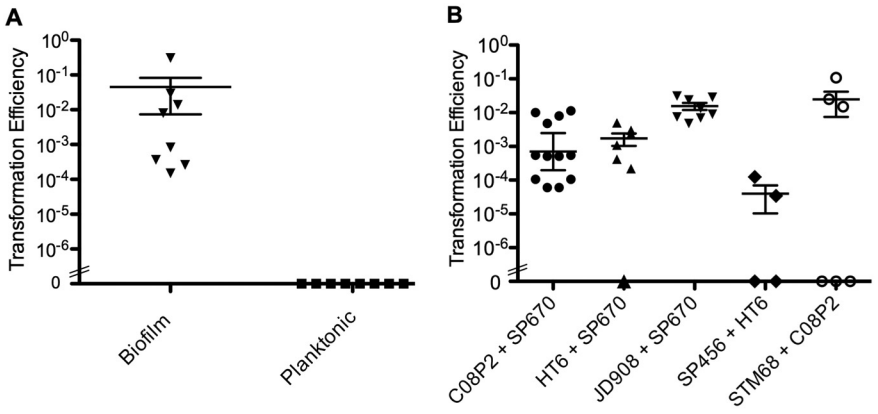

FIG 2 (A) SP670 transformation efficiency during biofilm or planktonic growth after addition of D39-C08P2 natural lysate. (B) Transformation efficiency of dual-strain biofilms. Equal inocula of each strain were cocultured in biofilms on top of prefixed epithelial substrata for 72 h before being plated on selective medium: D39-C08P2/SP670, HT6/SP670, JD908/SP670, and SP456/HT6 were all grown on Pen^r-plus-Erm^r plates, whereas STM68/D39-C08P2 were grown on Cm^r-plus-Erm^r plates.

***In vitro* dual-strain biofilms show increased natural transformation.** To further confirm and expand the *in vivo* transformation results above and the role of biofilm growth in this process, as well as to confirm the ability of biofilms to take up exogenous DNA in a more biologically relevant system, we investigated the transformation efficiency of dual-strain biofilms.

Fixed epithelial cells were inoculated with equal CFU of indicated strains (Table 2) and cultivated for 72 h to produce biofilms. To ensure that this phenomenon was neither serotype nor strain specific, a variety of strains were used in combination with each other. After 72 h, the biofilms were sonicated, removed, vortexed, and plated onto agar plates containing combinations of two antibiotics—one for each strain involved in the coculture (Table 2). Transformation efficiency was determined as the number of double-resistant clones as a ratio of the total colony counts on nonselective agar. The results presented in Fig. 2B show that all strain combinations induced high transformation efficiencies with exchange of the resistance genes between the strains. Taken together, these data clearly show that natural transformation functions optimally when pneumococci live in actively growing biofilms.

Biofilms show increased competence induction compared to that of planktonic growth over time. To address the mechanisms behind the increased transformation efficiency in biofilms, we first investigated the bacterial factors promoting genetic exchange in our biofilm model.

Competence in planktonic pneumococci is a transient physiologic event that is highly dependent on growth phase and cell density. However, upregulation of competence genes has been well documented during pneumococcal biofilm formation and is shown to be more constitutive, although it was recently demonstrated that the expression profile varied depending on the biofilm model used and whether or not exogenous CSP was added initially (34, 35). As our biofilm model is different from any model described in the literature, incorporating aspects of the nasopharyngeal environment and employing a longer incubation time, we were interested in the level of relative gene expression of competence genes in our biofilm model (32).

The level of relative gene expression of competence gene *comD*, which activates the early competence genes, including *comX*, which is a transcriptional activator that activates the 80 or so late competence genes (46, 47), was analyzed by quantitative real-time PCR (qRT-PCR) over 72 h in biofilms and in the early log phase in planktonic organisms, when transformation is known to peak in each system. All data are reported as fold change in gene expression compared with the expression of the same gene in a planktonic log-phase culture of the same strain, and the data in each sample were normalized to the expression of the housekeeping

TABLE 2 Characteristics of strains used in the study

Strain	Serotype	Relevant characteristic(s)	Reference
D39	2	Wild-type Avery strain	11
D39-C08P2	2	D39 with chromosomal insertion of Erm^r cassette, equally as virulent as D39	92
Rx1	Acapsular	Hypercompetent rough D39 derivative	93
AM1000	Acapsular	Unencapsulated D39 derivative	84
JD908	Acapsular	Unencapsulated WU2 (serotype 3) derivative, Erm^r	94
TRE121	2	D39 Δ*pspA* Δ*pspC* Erm^r Tet^r	63
HT6	3	WU2 with chromosomal Erm^r	95
SP670	6B	Clinical Pen^r isolate	96
SP456	23F	Clinical Pen^r isolate	96
STM68	4	TIGR4 with Cm^r transposon insertion	97
EF3030	19F	Clinical isolate	98

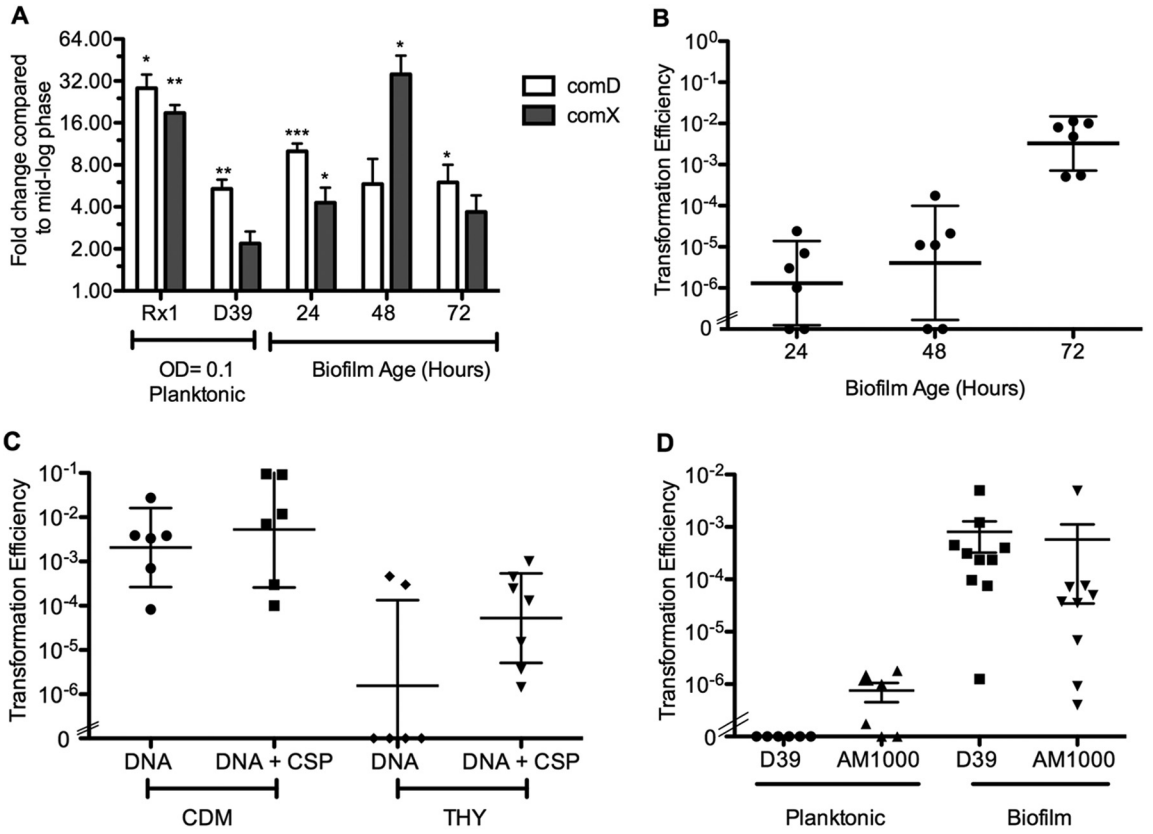

FIG 3 (A) Expression of competence genes during biofilm formation. Levels of gene expression were quantitated by qRT-PCR using the standard curve method and normalized to levels of 16S rRNA as an internal control. All data are reported as fold change in gene expression compared to a mid-logarithmic-phase culture of D39 (for D39 biofilms) or Rx1 (for Rx1 cultures). (B) Transformation efficiency of dual-strain biofilms of SP670 and D39-C08P2 over 72 h. (C) Transformation efficiency of SP670 biofilms after addition of chromosomal D39-C08P2 DNA in chemically defined medium (CDM) or THY complex medium with or without the addition of competence-stimulating peptide (CSP). (D) Transformation efficiency of planktonic cultures of D39 and AM1000 using D39-C08P2 chromosomal DNA compared to transformation efficiency of single-strain biofilms formed by D39 and AM1000, inoculated with D39-C08P2 chromosomal DNA. Transformation efficiency was measured as the CFU/biofilm carrying both Pen^r and Erm^r as a ratio of the total biomass in the biofilm (B and C) or CFU of the total biomass carrying Erm^r (D).

gene 16S RNA (Fig. 3A). Rx1, a strain with increased natural transformation efficiency, showed increased expression of the competence genes *comD* and *comX* during early log phase (OD_{600}, 0.1), peaking at 28.4-fold ($P < 0.05$) and 18.8-fold ($P < 0.005$) above the expression in mid-log phase (OD_{600}, 0.6), respectively. This was expected, as competence expression for these strains under these culture conditions characteristically peaks at an OD_{600} of approximately 0.1 during planktonic growth (48, 49). In comparison, the expressions of *comD* and *comX* in D39 were upregulated during early logarithmic growth (OD_{600}, 0.1) 5.3-fold ($P < 0.005$) and 2.2-fold ($P = 0.13$) above the expression in mid-logarithmic phase, respectively. Although there was an increase in competence gene expression in the D39 strain, it was not enough to induce uptake of genetic material (Table 1), which may be due to either the lower expression level than that of the Rx1 strain and/or the increased ability of the Rx1 strain to incorporate DNA based on its inactivation of mismatch repair systems during homologous recombination. It is also possible that capsule expression of the D39 strain conferred inhibitory activity, as shown below in experiments using the unencapsulated D39 derivative AM1000.

However, during biofilm growth of D39 pneumococci, competence expression increased by 9.9-fold ($P < 0.0005$) and 4.3-fold ($P < 0.05$) for *comD* and *comX*, respectively, after 24 h. At 48 h, *comD* expression remained elevated at 5.8-fold ($P = 0.11$) while *comX* expression peaked at 35.6-fold ($P < 0.05$) above the levels in a mid-logarithmic-phase culture. By 72 h, *comD* expression remained stable at 5.9-fold ($P < 0.05$)-increased expression while *comX* expression declined again to a 3.8-fold ($P = 0.051$) increase. In accordance with these data, the highest frequency of natural transformation was observed with biofilms grown on epithelia for between 48 and 72 h (Fig. 3B). Given the high levels of competence gene expression in the biofilms, we tested if the exogenous addition of competence-stimulating peptide (200 ng/ml) would increase transformation efficiency in the biofilm. We found that the constant presence of CSP did not significantly increase the already elevated biofilm transformation efficiency when biofilms were grown in chemically defined medium (CDM), a synthetic medium promoting optimal biofilm formation (Fig. 3C). It has previously been shown that *S. pneumoniae* forms poor if any biofilms in the rich medium THY (Todd-Hewitt medium containing yeast extract) and that addition of CSP can increase biofilm stability (34, 50). This was verified here as the weak biofilms formed in THY showed both increased biofilm formation and increased transformation efficiency after exogenous addition of CSP

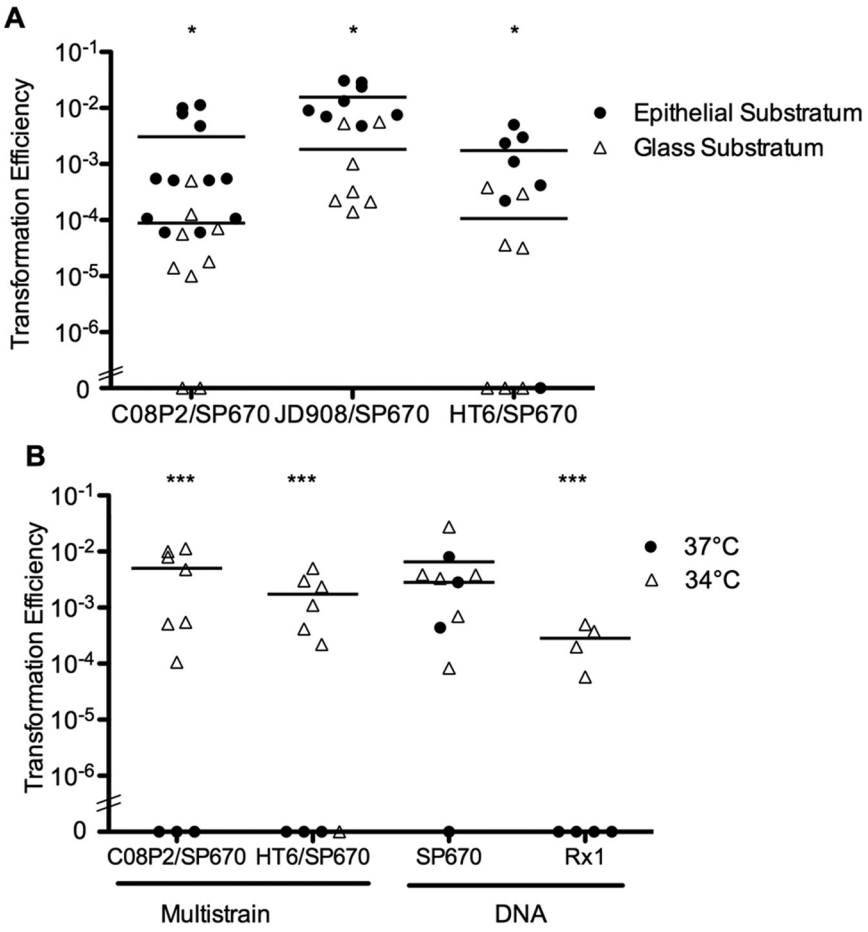

FIG 4 (A) Transformation efficiency of dual-strain biofilms cocultured on an epithelial substratum or on glass for 72 h. (B) Transformation efficiency of dual-strain biofilms SP670/D39-C08P2 and HT6/SP670 or single-strain biofilms SP670 and Rx1 inoculated with D39-C08P2 chromosomal DNA at 34°C and 37°C for 72 h.

(Fig. 3C). In contrast, natural planktonic transformation efficiencies (no exogenous addition of CSP) were the same after growth in THY and that in CDM where planktonic cells were assayed at 10-min intervals over a 2-h window to account for growth differences.

Capsule downregulation during biofilm growth. Phenotypic variation of the polysaccharide capsule occurs as pneumococci regulate capsule expression in the transition from nasopharyngeal carriage, associated with biofilm development, to invasive disease (51). During initial colonization, transparent variants with a thinner capsule layer predominate over opaque variants, expressing increased amounts of capsular polysaccharide that are found during invasive disease (52–54). Interestingly, transformation efficiency is known to decrease with increasing capsule expression (40), leading to the hypothesis that capsule may be partially responsible for the observed lack of natural competence in many clinical strains (13, 14, 40, 55–58).

To evaluate this hypothesis, we measured natural transformation efficiency during planktonic growth of D39 compared to its unencapsulated derivative AM1000. While D39 was not naturally transformable during planktonic growth, the absence of capsule led to the development of a natural transformation efficiency of 1.1×10^{-6}. In contrast, during biofilm growth D39 and AM1000 showed equal, and significantly higher, transformabilities (Fig. 3D). As we had shown that D39 could be transformed during biofilm growth, we measured *cps2* expression using qPCR under planktonic and biofilm growth conditions after 48 h of growth over a prefixed epithelial substratum. Expression of *cps2* was downregulated in all biofilms relative to planktonic growth conditions with an average of 32.7-relative-fold reduction ($P < 0.005$) compared to planktonic cultures. These results are somewhat more pronounced but agree with previous studies showing that *cpsA* is downregulated by up to 10-fold during biofilm growth over an abiotic substratum compared with planktonic cultures (59) and suggest that capsular downregulation during biofilm growth and colonization may contribute to the observed increase in transformation efficiency.

Epithelial interactions enhance biofilm stability, leading to increased transformation. We were next interested in better understanding the specific role of nasopharyngeal host factors, such as temperature, nutrient availability, and epithelial cell interactions, for optimal biofilm formation and transformation efficacy. As shown above, complex media such as THY did not support biofilm formation effectively and required CSP to increase biofilm formation and transformation efficiency (Fig. 3C), whereas our synthetic CDM produces stable biofilms with high transformation efficiency, supporting a role for nutrients in these processes.

Biofilm models are less standardized than the classical growth phases of planktonic bacteria (35). However, none of the model systems evaluated so far have included respiratory epithelial cells, an important aspect of pneumococcal colonization. Therefore, we evaluated the transformation efficiency within dual-strain biofilms formed using our model with a prefixed respiratory epithelial substratum (32) and compared it with the more commonly used static microtiter biofilm model. As shown in Fig. 4A, both models generated high levels of transformation efficiency; however, the presence of a prefixed epithelial substratum led to a significant increase in the rate of recombination ($P < 0.01$). These results indicate that bacterium-host cell interactions are important for the ability to form a biofilm that structurally and functionally supports transformation.

Natural transformation is increased at nasopharyngeal temperatures. Competence development in log-phase cultures of *S. pneumoniae* can be further modulated by changes in growth conditions such as temperature (60, 61). Competence induction has been shown to be dependent on the temperature at which the initial reaction between competent cells and DNA occurs, with the highest efficiency at between 30 and 34°C, and declines linearly as the temperature is either increased or decreased further (60). Planktonic transformations of Rx1 performed at 34°C to mimic the temperature of the nasopharynx (62) showed increased, although not significantly so ($P = 0.056$), natural transformation efficiencies of 4.38×10^{-4} compared with 1.11×10^{-4} at 37°C, somewhat supporting those studies.

To test if the lower culture temperature of our *in vitro* biofilms (grown at 34°C) contributed to their increased transformation efficiency, we repeated our single-strain biofilm experiment with addition of exogenous DNA and our dual-strain biofilm transformations also at 37°C. Addition of D39-C08P2 DNA to SP670 biofilms resulted in high transformation efficiency at both 34°C and 37°C. This correlated with similar biofilm biomasses at the two temperatures (mean biomass at 37°C of 4.7 $\log_{10}$ CFU/biofilm compared with 5.3 $\log_{10}$ CFU/biofilm at 34°C; $P = 0.82$). Addition of D39-C08P2 DNA to Rx1 biofilms resulted in high transformation efficiency at 34°C, whereas no transformation was observed at 37°C. However, Rx1 failed to form effective biofilms at 37°C (average biomass of 1.9 $\log_{10}$ CFU/biofilm compared with 6.7 $\log_{10}$ CFU/biofilm at 34°C), which may explain the differences. Thus, when comparable biomasses are obtained, exogenous DNA will be taken up equally well.

To make it more physiologically relevant, dual-strain biofilms were made at each temperature. Although a dual-strain biofilm was formed by D39-C08P2 and SP670 at 37°C (average biomass of 4 biofilms was 5.1 $\log_{10}$ CFU/biofilm), it was somewhat impaired compared with biofilms at 34°C (total biomass of 8 biofilms, 6.3 $\log_{10}$ CFU/biofilm; $P < 0.05$), and no transformants were recovered after 72 h of biofilm growth at 37°C. Dual-strain biofilms between HT6 and SP456 could not be formed at 37°C, and therefore, no transformants could be recovered, whereas at 34°C an average biomass of 5.5 $\log_{10}$ CFU/biofilm was obtained with high transformation efficiency (Fig. 4B). These data suggest that the nasopharyngeal temperatures play an important role both for the ability of the bacteria to organize into an optimal biofilm structure and for their ability to exchange genetic material and are more important in cocultures than when exogenous DNA is added. Combined, the results with host factors suggest that the biofilm model used in our assays represents a more closely associated *in vitro* surrogate assay to investigate transformation during *in vivo* colonization.

Population dynamics of mixed-species biofilms. When analyzing the results of our *in vitro* and *in vivo* transformation experiments, we found that even though multistrain biofilms were seeded with equal total inocula, they showed a trend of increased biomass compared to single-species biofilms. While this phenomenon was not statistically significant, it prompted us to explore the population dynamics within our dual-strain biofilms. More-detailed examination revealed that strains that were poor biofilm formers *in vitro* and weak colonizers in our *in vivo* model became strong biofilm formers *in vitro* and colonized more effectively *in vivo* in the presence of another strain (Fig. 5A to 5C). Similar results were not seen during planktonic growth or sepsis, where D39 outcompeted SP670 rather than stabilizing the strain as seen in the biofilms formed during colonization (Fig. 5D and 5E).

In a striking example of the protective effect of multistrain collaboration, we found that cocolonization of mice with the effective colonizing strain EF3030 and the unencapsulated D39 derivative (AM1000) allowed for the unencapsulated strain to persist at higher density in the nasopharynx (Fig. 5F). Of importance, however, was the fact that this increased fitness during cocolonization was not directly associated with the acquisition of colonization factors, as the strains after cocolonization did not colonize the murine nasopharynx better than they did prior to the cocolonization experiments. This suggests that part of fitness may be associated with a protective effect from efficiently colonizing strains.

***In vivo* biofilms induce transformation that increases fitness.** Evolutionary theory suggests that in addition to generating genetic diversity and acquiring novel functions, natural genetic transformation may also serve to capture genetic material from other cells to facilitate repair of damaged genes to increase fitness. To test this hypothesis, we inoculated 6-week-old BALB/cByJ mice intranasally for 48 h with a mixture of 2.5×10^6 CFU of D39 wild type and 2.5×10^6 CFU of the *pspA*/*pspC* null D39 strain (TRE121; Erm^r Tet^r) and another control group with 5×10^6 CFU of TRE121 alone. After 48 h, bacteria were plated on selective medium to identify the resulting populations. Consistent with previous findings demonstrating that PspC is required for colonization (63), we found that all mice inoculated with the *pspA*/*pspC* null strain alone rapidly cleared the bacteria. In contrast, mice inoculated with the combined wild-type and mutant population showed a population of repaired mutants that had reacquired the *pspC* gene (Erm^r Tet^s) (Fig. 6). These results were confirmed by PCR for the repair of *pspC*, and the functionality of the *pspC* gene was shown by rechallenge of mice with clones from the repaired *pspC* population. Mice inoculated with the TRE121 repaired-*pspC* strain maintained stable nasopharyngeal colonization after 48 h (Fig. 6).

More unexpectedly, we also identified a surviving population of the *pspA*/*pspC* null strain as well as mutants that had repaired the *pspA* locus but remained *pspC* negative (Fig. 6). These data support the conclusions that mixed strains cocolonizing in the nasopharynx expand the bacterium's supragenome, increasing its adaptive potential.

DISCUSSION

The implications of horizontal gene transfer for bacterial evolution and adaptation are far reaching (64). The rapid emergence and spread of antibiotic resistance and capsular switching are the most commonly recognized manifestations of this process (7). Lacking systems for conjugation or transduction, pneumococci incorporate foreign DNA by transformation. Spontaneous gene uptake and transformation of *S. pneumoniae* strains have previously been reported to occur at extremely low frequencies *in vivo* (9, 10, 65, 66). Similarly, natural competence is extremely poor in clinical isolates *in vitro* and has been observed only in a limited collection of rough laboratory strains. However, most previous work investigating this phenomenon has done so in the context of sepsis, lung infection, subcutaneous infection, intraperitoneal infection, or other disease states and stands in stark contrast to the epidemiological evidence that suggests a high resistance selection occurring mainly in pneumococci colonizing children, where

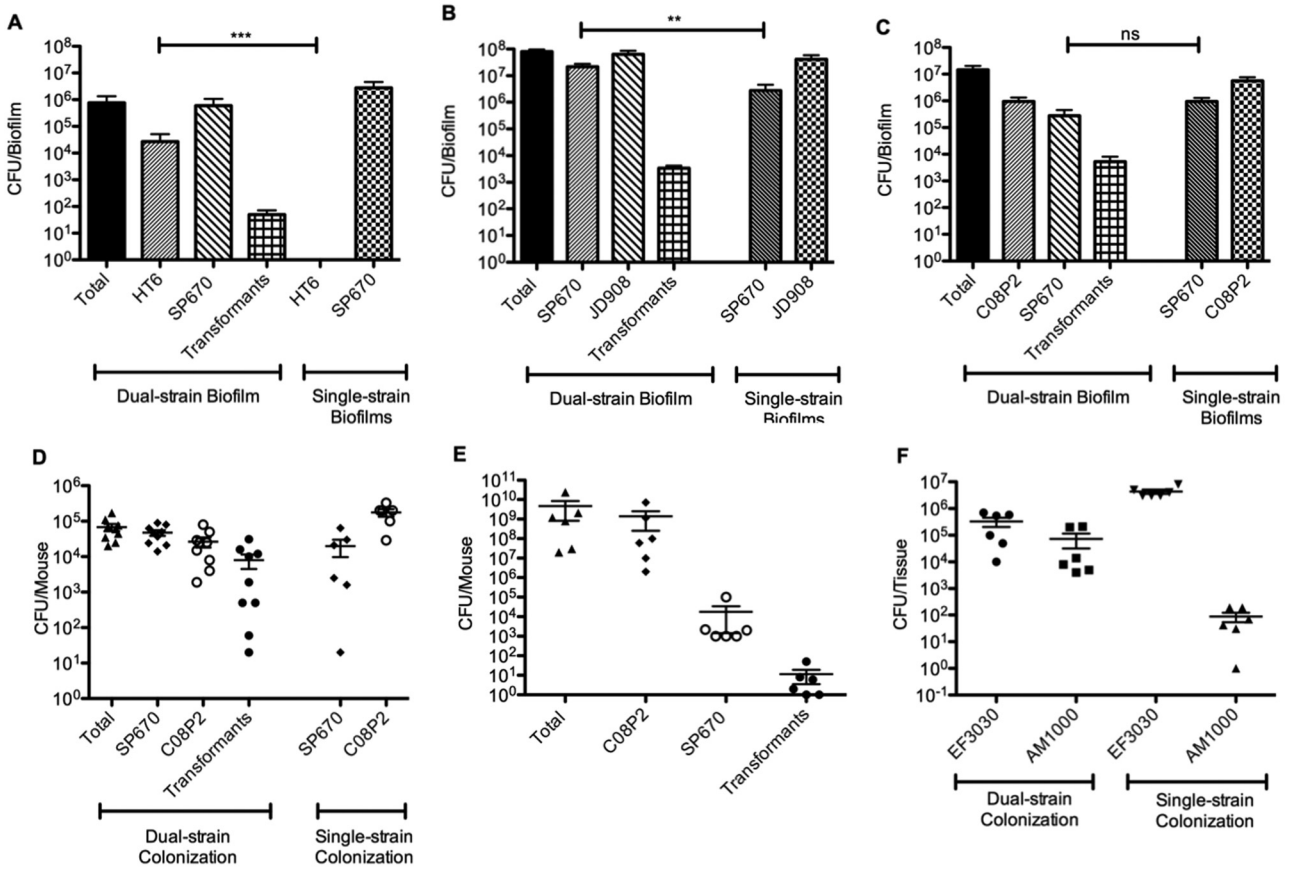

FIG 5 (A to C) Total population and subpopulations from dual-strain biofilms of HT6/SP670 (A), SP670/JD908 (B), and P2A1/SP670 (C), compared to the biomass of single-strain biofilms. (D) Total nasopharyngeal colonization and subpopulations after 48-h dual-strain colonization by SP670 and D39-C08P2 compared to colonization by each single strain. (E) Total bacterial burden and isolated subpopulations during sepsis induced by SP670 and D39-C08P2. (F) Isolated encapsulated (EF3030) and unencapsulated (AM1000) populations recovered after 48 h of nasopharyngeal carriage from dual-strain challenge (lanes 1 and 2, coculture of AM1000 and EF3030) or single-strain nasopharyngeal carriage (lanes 3 and 4).

high carriage rates and exposure to antibiotics favor the selection of drug-resistant strains (29, 67, 68). Therefore, while these papers demonstrate that pneumococci are transformable in a variety of infection models, thus far surprisingly little is known about the efficiency and function of this system during pneumococcal colonization of its main niche, the nasopharynx.

The focus of this paper was therefore to comprehensively address the exchange of genetic material in the nasopharynx, something that has not been done previously, and characterize the bacterial and host factors required for this to occur optimally. We found that cocolonization and sequential colonization in the nasopharynx of mice of two strains of pneumococci carrying various genetic markers resulted in a highly elevated level of natural transformation that was ~10^7-fold higher than the transformation efficiency seen during planktonic growth of the strains in a sepsis model. Interestingly, the transformation efficiency was high even though no antibiotic pressure was induced in the model system, suggesting a very high basic exchange of genetic material in the host. Also, the sequential colonization experiments suggested that the nasopharyngeal bacterial environment is dynamic enough to allow integration of new strains in already-established communities to facilitate genetic exchange. These studies are the first to address the transformation efficiency of cocolonization *in vivo*. The high frequency of natural transformation during colonization may explain current epidemiological typing data indicating frequent recombination within the pneumococcal population (69).

As we have recently shown that colonizing bacteria grow in biofilms rather than planktonic growth associated with sepsis (32), we investigated what factors and environmental conditions promoted biofilm formation and genetic exchange in the nasopharynx and compared those to standard techniques involved in transforming planktonic bacteria. We first investigated regulation of the competence system in biofilms and planktonic bacteria. We show here, as others have shown earlier, that in contrast to the transient induction of competence occurring during planktonic growth, competence is permanently upregulated during biofilm formation *in vitro* (35) and during tissue-associated growth *in vivo* (34). This upregulation is most likely associated with a shifting subpopulation of genetically competent organisms within the dynamic biofilm, making the biofilm as a whole constantly competent. As our model system is different and our studies were more prolonged than those reported earlier, it is important to point out that constitutive upregulation does not occur during a phase of rapidly changing cell density and is not limited in time, instead providing increased opportunities over a prolonged period for genetic recombination. This factor combined with the increased

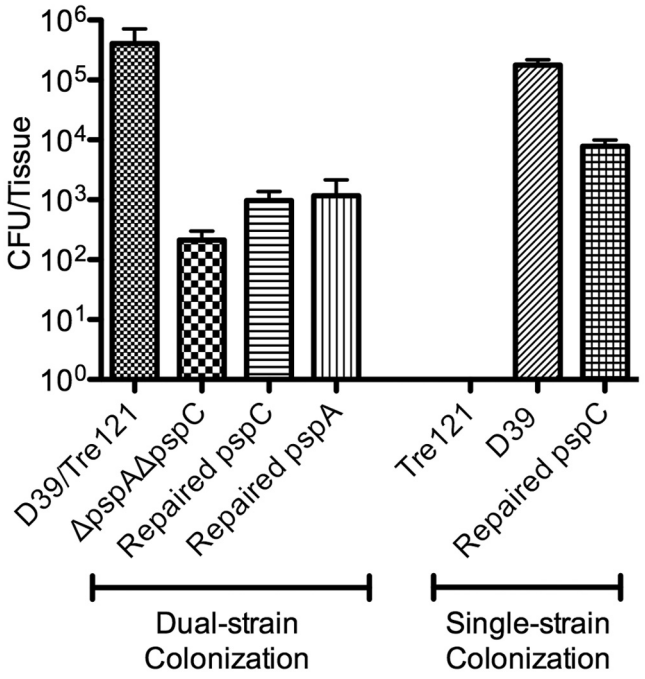

FIG 6 Colonization levels of each subpopulation 48 h after dual-strain colonization between D39 and TRE121 (lanes 1 to 4) and single-strain colonizations (lanes 5 to 7). Lane 1, total recovered pneumococci; lane 2, *pspA/pspC* null pneumococcus TRE121; lane 3, TRE121 pneumococci with repaired *pspC* gene; lane 4, TRE121 pneumococci with repaired *pspA* gene; lane 5, single-strain colonizing ability of TRE121 alone; lane 6, single-strain colonizing ability of D39 alone; lane 7, rechallenge of mice with isolate from dual colonization with strain with repaired *pspC* gene (from lane 3).

time of exposure to DNA compared to the shorter period of competence of planktonic cells and shorter exposure to DNA likely contributes significantly to the increased genetic recombination seen during biofilm growth.

Another factor associated with decreased transformation efficiency is capsule expression, which is upregulated during planktonic growth and during septic infection in mice (34). In our biofilm model, we showed that the major capsular gene *cps2* was significantly downregulated compared with planktonically growing bacteria. This phenotypic variation of capsular expression (51) may further facilitate the binding of DNA and contribute to increased transformation efficiency during colonization. Although capsule has been shown before to be downregulated during biofilm formation on abiotic surfaces (59), we showed here that the downregulation of capsule was even greater than has been described earlier, which may be related to the interaction with epithelial cells that has also been shown to promote capsule downregulation (70). Our epithelium substratum model may therefore promote an optimal environment and signaling situation to allow effective exchange of genetic material.

Although competence induction and capsule downregulation have been associated with biofilm formation *in vitro* and both have been associated with increased transformation efficiency of planktonic bacteria, this study provided evidence that the competence induction and capsule downregulation in biofilms were also associated with increased transformation efficiency, which is a novel finding. However, these two bacterial factors are most certainly not the only ones involved in this phenomenon. The quorum sensing molecule LuxS has been implicated in both early biofilm formation *in vitro* and regulation of competence induction but has not been directly studied for transformation efficiency (71, 72); CbpD, involved in release of target cell DNA during fratricide, has been shown to have some impact on transformation in early-stage biofilms (16); and a multitude of virulence determinants have been shown to be differentially expressed in biofilms and planktonic bacteria (34, 50, 73), although different biofilm models show different results that may or may not correlate well with the *in vivo* situation (35). Dissection of these factors in relevant models will be of major importance for colonization, transformation, and horizontal spread in the future.

Less attention has been focused on the role of the host environment in transformation efficiency. Until recently, a variety of model systems, all on abiotic surfaces, have been used to examine biofilm formation. However, none fully mimic the *in vivo* environment, with little association between the ability of clinical isolates to form biofilms in these model systems and their ability to colonize or infect the host (33, 74–78). As stated above, the biofilm model used in this study includes environmental factors associated with the nasopharyngeal environment (32), and we have shown a strong correlation between the ability of clinical strains and colonization-deficient mutants to form biofilms in this model and their ability to colonize the murine nasopharynx, which was not the case when biofilms were grown on glass and other abiotic surfaces.

In this study, the epithelial cell substratum for biofilm formation was shown to provide increased transformation efficiency. Biofilms formed over a prefixed epithelial substratum were naturally transformed with pure DNA or lysate from dead cells at a high frequency, and dual-strain biofilms, with strains carrying two different antibiotic markers, showed similarly high levels of genetic recombination and antibiotic resistance transfer as what we detected *in vivo*. Although biofilms formed on abiotic surfaces also supported genetic transformation, the density of the biofilms was markedly lower and the efficiency was significantly less pronounced. This suggests that the density and organization of the biofilms are highly important for effective transformation to occur and suggests that our model of biofilm growth could potentially be used as a surrogate model for *in vivo* colonization to better understand the mechanistic aspects of transformation in the context of biofilm growth *in vivo*. An important side note is that the high efficiency of exchange of genetic material seen in this study between clinical strains, which are unable to undergo natural transformation *in vitro*, some even in the presence of CSP, suggests that this biofilm model facilitates common laboratory transformations of more challenging *S. pneumoniae* strains with the added benefit of a more streamlined technique not requiring additional reagents, competence buffers, or synthetic CSP.

However, epithelial cell interactions were not the only environmental factor required for optimal transformation efficiency. It has been shown elsewhere that ion and nutrient concentrations are important variables during competence development in planktonic pneumococcal cultures (79–81). Likewise, we showed that while planktonic transformation efficiency did not differ between the synthetic medium CDM and the more complex medium THY, CDM supported biofilm formation and therefore natural transformation within biofilms much better than did the complex medium THY. This specific nutrient environment therefore appears to support an increase in genetic exchange that is

largely due to its supportive effect on biofilm formation. In addition, competence development during planktonic growth can also be modulated by changes in growth conditions such as temperature (61, 80), pH, or oxygen concentration (82, 83). Interestingly, growth environments associated with sepsis and meningitis, such as anaerobic growth conditions and a temperature of 37°C, are optimal for planktonic growth of the bacteria but induce competence repression. In contrast, colonization of the upper respiratory tract, where the pneumococcus resides as a commensal, exposes the bacteria to lower temperatures (closer to 34°C) and a pressure of oxygen closer to that of the atmosphere, both conditions that favor biofilm formation and natural transformation. We showed that transformation efficiency was increased 10^7-fold during nasopharyngeal colonization, where the temperature is lower (~34°C), over that with sepsis, where the temperature is 37°C. Similarly, biofilm formation and transformation efficiency *in vitro* on epithelial cells at 34°C were significantly improved over that with the same parameters at 37°C, so much so that some strains were unable to produce biofilms at 37°C. As the growth rate of pneumococci at 34°C is half of that of pneumococci grown at 37°C with a parallel decrease in the metabolic rate, it is likely that the higher temperature causes increased cell turnover with more H_2O_2 production and autolysis occurring in biofilms that may be responsible for the difficulty in organizing stable biofilms with high biomass and transformation efficiency. Competence induction from the increased oxygen concentration in the nasopharynx may likewise contribute to the increased transformation efficiency of colonizing pneumococci.

While no single environmental parameter appears to account for the entire difference observed between planktonic and biofilm bacteria, these observations suggest that the combined heterogeneous environmental conditions found during colonization of the nasopharynx and associated with a developing biofilm generate distinct physiological signals that together are optimal for biofilm formation and that optimal biofilm formation is a prerequisite for competence expression and increased horizontal gene transfer.

An unexpected finding in our studies was the observation that dual-strain biofilms *in vitro* formed between poor biofilm formers such as SP670 or HT6 (type 3 capsular isolate unable to form biofilms) and strains with a higher biofilm-forming ability increased the persistence of the poor-biofilm-forming strain *in vitro*. The same cooperative population dynamics between poor- and high-colonizing strains were also detected during cocolonization experiments *in vivo* and were seen even during cocolonization with encapsulated and unencapsulated strains. Alone, *S. pneumoniae* strains require at least some level of capsule expression for murine colonization (84) and are incapable of causing pneumonia or sepsis (85, 86). Yet, unencapsulated *S. pneumoniae* strains are frequently identified as the causative agents of bacterial conjunctivitis outbreaks. Our findings suggest that cocolonization with a second strain may enable rare serotypes and nonencapsulated isolates to persist within biofilms in the host, protecting them from host clearance and providing a different type of cooperative fitness, not dependent on acquisition of genetic factors. This phenomenon has recently been recognized in epidemiological surveys where rare serotypes (generally considered poor colonizers) and nontypeable or unencapsulated isolates are detected nearly 10-fold more often in the nasopharynx of multiply colonized individuals than in that of singly colonized individuals (28).

Finally, we also showed that dual-strain biofilms formed *in vitro* or cocolonizing in the nasopharynx may further expand the genetic reservoir—supragenome—for this bacterium, increasing the repertoire of genes available for adaptation to environmental stresses and improving colonization fitness (87). Pneumococci lacking a gene essential for colonization (*pspC*) could repair its critical defect in the presence of a wild-type strain. However, consistent with our findings that colonization-deficient strains persist better within multistrain biofilms than in single-strain biofilms, a substantial population of the colonization-deficient *pspA-pspC*- mutant strain remained even after 72 h, when alone this strain was completely cleared within 48 h. We also observed repair of the nonessential *pspA* gene (which retained the *pspC*-negative genotype) in these experiments, suggesting that fitness provided by the biofilm environment as well as direct fitness obtained by uptake of genetic material exists in the context of the nasopharyngeal community. As strains were identified based on resistance, it is also possible that these isolates could be D39 that acquired the *pspA*:: Erm^r DNA by transformation (with no fitness defect associated with loss of PspA) and then became Tet^r through an atypical recombination event that retained wild-type PspC. It is also possible that repair of both genes occurred in our experiments, although we have no way of detecting these strains, as they are genotypically similar to the wild-type strain used in the cocolonization. However, despite these limitations, taken together these observations suggest that cocolonization may increase the potential of strains to adapt in response to the host environment and increase fitness either by incorporation of genes providing improved fitness and/or by being incorporated in a protective bacterial community. Simultaneous colonization and the formation of multistrain biofilms, therefore, most certainly represent an important adaptive strategy for survival in the host.

In conclusion, in this study we have provided novel evidence that genetic exchange and natural transformation of *S. pneumoniae* occur primarily in the context of nasopharyngeal colonization and that growth conditions associated with this niche, such as temperature, nutrient availability, and epithelial cell interaction and its ensuing downregulation of bacterial capsule expression and upregulation of competence genes, all help in facilitating this process. This work integrates our understanding of the role of competence and the supragenome within biofilm communities, suggesting that the continuous recombination between colonizing strains may serve as a supravirulence/fitness factor for pneumococci. Understanding these interactions within the colonizing biofilm communities may provide critical information in the face of the global increase in antibiotic resistance and serotype replacement postvaccination.

MATERIALS AND METHODS

Ethics statement. This study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at the University at Buffalo, Buffalo, NY (protocol number MIC36048Y). All bacterial inoculations and treatments were performed under conditions to minimize any potential suffering of the animals.

Reagents. Cell culture reagents were from Invitrogen, Carlsbad, CA. Bacterial and cell culture media and reagents were from VWR Inc., Radnor, PA. Chemically defined bacterial growth medium (CDM) was obtained from JRH Biosciences, Lenexa, KS. Sheep blood was purchased from BioLink, Inc., Liverpool, NY. All antibiotics and remaining reagents were purchased from Sigma-Aldrich, St. Louis, MO.

Cells and bacterial strains. NCI-H292 cells (ATCC CCL-1848) were grown on various surfaces as described previously (88). Pneumococcal strains were grown in a synthetic medium (CDM) containing 2% choline chloride or in Todd-Hewitt medium containing 0.5% yeast extract (THY) as described previously (89). The *S. pneumoniae* strains used in this study are listed in Table 1. MICs of bacterial strains were determined in 96-well microtiter plates by the microdilution method according to approved standards of the CLSI, except that Todd-Hewitt medium supplemented with 0.5% yeast extract, which yields reproducible MIC results, was used as the test medium for *S. pneumoniae* (90, 91).

Static biofilm model. Pneumococci were grown in CDM to mid-logarithmic phase (OD_{600}, 0.5), washed, and resuspended in fresh pre-warmed medium to a density of 2×10^4 CFU in a 500-μl volume, and suspensions were used to seed sterile round glass coverslips in the bottom of polystyrene 24-well plates with or without a substratum of confluent H292 epithelial cells that were prefixed in 4% paraformaldehyde as described previously. Biofilms were cultured at 34°C in 5% CO_2 for indicated times with a change of culture medium every 12 hours. Before plating, biofilms were washed with phosphate-buffered saline (PBS), sealed, floated on a sonicator water bath, and sonicated for 2 s to disperse the bacteria (verified by light microscopy). Cells were then collected and vortexed three times for 20 s at high speed to ensure a homogenous solution, and the dispersed biofilm cells were used to determine viable CFU per ml by viable plate counts as described previously (32). Results are reported as the total CFU per biofilm.

(i) Mouse sepsis and nasopharyngeal colonization model. Six-week-old female BALB/cByJ mice from Jackson Laboratories (Bar Harbor, ME) were maintained in filter-top cages on standard laboratory chow and water *ad libitum* until use. The animal experiments were performed essentially as described earlier (89). Frozen stocks of bacteria were thawed, washed once, and resuspended in 1.5 ml sterile, cold PBS. Bacteria were plated on tryptic soy agar (TSA)-blood agar plates to verify quantities in stocks. For nasopharyngeal colonization experiments, mice were colonized with a total of 5×10^6 CFU, either all from a single strain or as 2.5×10^6 CFU from each strain, resuspended in a 20-μl volume pipetted into the nares of nonanesthetized mice. Mice were monitored and sacrificed at 48 h postinoculation. Nasopharyngeal tissue was dissected as described previously (89) by removing the upper skull bone and harvesting the tissue present in the nasal conchae with forceps. Harvested tissue was homogenized, and the homogenate was serially diluted on TSA-5% blood agar plates. To assess transformation frequencies during sepsis, we infected BALB/c mice intraperitoneally with 1×10^5 CFU of SP670 and 1×10^5 CFU of D39-C08P2 resuspended in 50 μl of PBS.

For all transformation experiments, equal volumes were plated on TSA-5% blood agar plates containing 1 μg/ml penicillin G to detect SP670 bacteria, 3 μg/ml erythromycin to detect D39-C08P2 bacteria, and 1 μg/ml penicillin G plus 3 μg/ml erythromycin to detect transformants. Double mutants were verified by replating a limited number of cells from double-resistant colonies in each experiment and performing MIC assays (described below) for penicillin G and erythromycin on these isolates.

For cocolonization experiments between encapsulated and unencapsulated strains EF3030 and AM1000, 5×10^6 CFU (either of a single strain or as 2.5×10^6 CFU of each strain combined) was resuspended in a 20-μl volume pipetted into the nares of nonanesthetized mice. Mice were monitored and sacrificed at 48 h postinoculation, and nasopharyngeal tissue was dissected as described above. Unencapsulated colonies were identified based on their rough (nonmucoid) appearance on blood agar.

(ii) Planktonic pneumococcal transformations. *S. pneumoniae* was transformed using exogenous addition of 100 ng/ml CSP as described previously (63). Natural transformations (no exogenous addition of CSP) were performed as described previously (58). In brief, glycerol stocks of cells grown at 37°C to a density of ~1×10^8 CFU/ml in THY were diluted 100-fold into competence medium (THY plus 0.2% bovine serum albumin and 0.01% $CaCl_2$) and incubated at 37°C. At 10-minute intervals, cells were removed and competence was tested by adding 1 μg/ml DNA (final concentration) and incubating cells at 37°C for 2 h before plating them on selective medium. Antibiotic susceptibility was determined on plates containing various combinations of indicated antibiotics. Plates were incubated for 24 h at 37°C. The following antibiotic concentrations were used for selection: 1 μg/ml penicillin G (Pen), 3 μg/ml erythromycin (Erm), 500 μg/ml kanamycin (Kan), 4 μg/ml chloramphenicol (Cm), and 4 μg/ml tetracycline (Tet). Antibiotics were obtained from Sigma-Aldrich, St. Louis, MO.

Calculation of transformation efficiency. For all transformation experiments, transformation efficiency was calculated as the number of transformants obtained relative to the total number of pneumococcal cells recovered.

RNA isolation and qRT-PCR. Briefly, frozen stocks of bacteria were used to inoculate a 20-ml culture in THY for planktonic growth. When the bacteria reached an OD_{600} of ~0.1 and an OD_{600} of ~0.6, 10 and 1 ml, respectively, were removed and bacteria were pelleted by centrifugation at 9,000 $\times$ *g* for 2 min at 4°C. For isolation of RNA from biofilms, biofilms were seeded and maintained as described above. At indicated time points (24, 48, and 72 h), biofilms were sonicated and resuspended in 1 ml of PBS and pelleted by centrifugation at 9,000 $\times$ *g* for 2 min at 4°C. Pellets were resuspended in 0.5 ml of 0.9% NaCl, 1 ml of RNAprotect (Qiagen, Valencia, CA) was added, and the mixture was incubated at room temperature for 5 min. Cells were then pelleted at 9,000 $\times$ *g* for 2 min at room temperature, and RNA was purified using Qiashreeder columns and the RNeasy minikit as described previously (89).

Statistical analysis. The data were analyzed for statistical significance by a two-tailed Student *t* test for paired or unpaired data as indicated. A *P* value of <0.05 was considered significant.

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