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The *blp* Locus of *Streptococcus pneumoniae* Plays a Limited Role in the Selection of Strains That Can Cocolonize the Human Nasopharynx

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ABSTRACT

Nasopharyngeal colonization is important for *Streptococcus pneumoniae* evolution, providing the opportunity for horizontal gene transfer when multiple strains co-occur. Although colonization with more than one strain of pneumococcus is common, the factors that influence the ability of strains to coexist are not known. A highly variable *blp* (bacteriocin-like peptide) locus has been identified in all sequenced strains of *S. pneumoniae*. This locus controls the regulation and secretion of bacteriocins, small peptides that target other bacteria. In this study, we analyzed a series of cocolonizing isolates to evaluate the impact of the *blp* locus on human colonization to determine whether competitive phenotypes of bacteriocin secretion restrict cocolonization. We identified a collection of 135 nasopharyngeal samples cocolonized with two or more strains, totaling 285 isolates. The *blp* locus of all strains was characterized genetically with regard to pheromone type, bacteriocin/immunity content, and potential for locus functionality. Inhibitory phenotypes of bacteriocin secretion and locus activity were assessed through overlay assays. Isolates from single colonizations ($n = 298$) were characterized for comparison. Cocolonizing strains had a high diversity of *blp* cassettes; approximately one-third displayed an inhibitory phenotype *in vitro*. Despite *in vitro* evidence of competition, pneumococci cocolonized the subjects independently of *blp* pheromone type ($P = 0.577$), bacteriocin/immunity content, *blp* locus activity ($P = 0.798$), and inhibitory phenotype ($P = 0.716$). In addition, no significant differences were observed when single and cocolonizing strains were compared. Despite clear evidence of *blp*-mediated competition in experimental models, the results of our study suggest that the *blp* locus plays a limited role in restricting pneumococcal cocolonization in humans.

IMPORTANCE

Nasopharyngeal colonization with *Streptococcus pneumoniae* (pneumococcus) is important for pneumococcal evolution, as the nasopharynx represents the major site for horizontal gene transfer when multiple strains co-occur, a phenomenon known as cocolonization. Understanding how pneumococcal strains interact within the competitive environment of the nasopharynx is of chief importance in the context of pneumococcal ecology. In this study, we used an unbiased collection of naturally co-occurring pneumococcal strains and showed that a biological process frequently used by bacteria for competition—bacteriocin production—is not decisive in the coexistence of pneumococci in the host, in contrast to what has been shown in experimental models.

Streptococcus pneumoniae is an important bacterial pathogen associated with high morbidity and mortality worldwide (1–3). Notwithstanding, disease is a rare event compared with the frequency of asymptomatic nasopharyngeal colonization (4).

Nasopharyngeal colonization is particularly frequent among young children (5), and multiple strains can be simultaneously detected in the same sample (6–8). As the pneumococcus is naturally competent and known to evolve mainly through genetic recombination (9), cocolonization is fundamental for its evolution (10, 11). Donkor et al. (47) correlated the extensive recombination among pediatric colonization strains with a high prevalence of cocolonization in that population, highlighting the importance of having a better understanding of this phenomenon.

Little is known about how pneumococcal strains interact with each other within the nasopharynx. Data from longitudinal studies have shown that the pneumococcal population colonizing children is in constant turnover (5, 12). Although over 95 serotypes in the pneumococcal population have been described to date (13, 14), only a small subset are dominant in carriage, suggesting a higher competitive ability of these serotypes (15, 16). The fact that the natural niche of the pneumococcus is a dense polymicrobial

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environment with limited resources also suggests that competitive interactions are important in its lifestyle (17).

To date, two main molecular mechanisms have been implicated in pneumococcal intraspecies competition: competence-mediated fratricide and bacteriocin production (18–21). These systems were historically thought of as independent. However, recent work has demonstrated that they are coordinately regulated, suggesting that, under some circumstances, fratricide and bacteriocin production may work in concert to target neighboring cells (22, 23).

Fratricide is used by competent cells not only to eliminate direct competitors but also to obtain nutrients and DNA released by noncompetent lysed cells (19). Because pneumococcal populations are divided into two pherotypes that express and respond to distinct types of competence-stimulating peptides (CSP), it was originally hypothesized that CSP might influence cocolonization as a result of fratricide-mediated exclusion. Two studies that evaluated the impact of CSP pherotype on the coexistence of pneumococci in humans could not confirm this hypothesis (24, 25).

In *S. pneumoniae*, the *blp* locus (associated with bacteriocin production) has been shown to play a role in intraspecies competition in a murine model of cocolonization (20). The *blp* locus encodes the factors required for the regulation, production, and secretion of bacteriocins and their associated immunity proteins. The locus is controlled by a two-component regulatory system consisting of a histidine kinase (BlpH) and a response regulator (BlpR). The system is activated when the signaling peptide, BlpC, binds to its cognate BlpH receptor (20, 21), resulting in phosphorylation and activation of the regulator, BlpR. BlpR activation results in upregulation of the entire *blp* locus (26), including the bacteriocin/immunity region (BIR) that encodes the bacteriocins and their cognate cotranscribed immunity proteins (27, 28). Bacteriocins (and BlpC) are primarily secreted by the ABC transporter BlpAB (20–23, 26).

The *blp* loci are highly diverse. There is significant variability in the BIR; at least 16 bacteriocin peptides have been described, several with allelic variability, and strains can vary with respect to the number of encoded bacteriocins (27, 28). Also, at least five types of the BlpC peptide pheromone have been found (21, 26–28). In addition, a significant fraction of pneumococcal strains carry a disruption in the genes encoding the BlpAB transporter (27). These strains can respond to exogenous pheromone with the production of immunity proteins but cannot secrete bacteriocins. The prevalence of these strains suggests that strategies that limit locus stimulation and peptide secretion may provide an energetic advantage. It was recently shown that strains disrupted in *blpAB* can secrete a fraction of the *blp* pheromone, BlpC, through the activity of the homologous competence transporter, ComAB, allowing self-stimulation of the locus (22, 23). This secretion occurs only during the competent state when sufficient ComAB is produced to support BlpC secretion and is enhanced by cross-stimulation of some of the *blp* promoters by the ComE competence response regulator. The universal presence of the *blp* locus in pneumococci and the significant diversity of its content suggest that this locus provides a competitive advantage during either colonization or transmission (28). No studies published to date have used colonizing isolates from humans to examine whether *blp* locus activity is sufficiently widespread to restrict the range of strains that can coexist within the nasopharynx.

In this study, we explored the role of the *blp* locus and bacteriocin secretion in the coexistence of different *S. pneumoniae* strains in human nasopharyngeal samples.

MATERIALS AND METHODS

Sample selection and isolation of pneumococci. Nasopharyngeal samples collected from healthy children attending day care centers in Oeiras and Montemor-o-Novo, Portugal, were retrospectively selected from cross-sectional studies conducted in 2001, 2006, 2007, 2009, and 2010 (29–32). In those studies, samples were routinely plated in selective media for *S. pneumoniae* (5% blood–Trypticase soy agar containing 5 mg/liter gentamicin) and incubated overnight at 37°C under anaerobic conditions with an optochin disk. On the second day, pneumococcal colonies with different morphologies were picked and plated separately, one colony per morphology. In the majority (>90%) of the cases, only one morphology was detectable by the naked eye. The bacterial lawn of the primary selective plate, here called the "primary sample," was collected and frozen at −80°C in 1 ml Mueller-Hinton broth with 30% glycerol. On the third day, pure pneumococcal cultures were also frozen.

Pneumococcal strains were serotyped by multiplex PCR as described previously (33, 34) (http://www.cdc.gov/streplab/pcr.html) or by the Quellung reaction (35) (Statens Serum Institute, Copenhagen, Denmark), as appropriate.

To maximize the likelihood of detecting significant associations between serotypes in multiple carriage events, and given the high serotype diversity of pneumococci, we first identified all samples containing pneumococci. Then, based on our previous observation that the serotypes most frequently found in single and multiple carriage events are essentially the same (36), we identified the serotypes with the highest cumulative prevalence in the cross-sectional studies described above. These were serotypes 3, 6A, 6C, 11A, 15A, 15B/C, 19A, 19F, 21, 23A, and 23B and nonencapsulated pneumococci (NT). In total, 1,415 samples were identified and selected for molecular detection of cocolonization. Of these, 66.2% (936/1,415) were collected from children vaccinated with at least one dose of either the 7-valent or the 10-valent pneumococcal conjugate vaccine, 30.7% (435/1,415) were collected from nonvaccinated children, and 3.1% (44/1,415) were collected from children whose vaccination status was unknown.

DNA isolation. Total DNA was isolated from 200 μl of the primary sample frozen stock using a High Pure PCR template preparation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Detection of cocolonization and capsular typing of the cocolonizing strains. Detection of cocolonization was done using pneumolysin noncoding region restriction fragment length polymorphism (*ply*NCR-RFLP) (37) and the BuG@S SP-CPSv1.4.0 microarray for molecular serotyping (36) as previously described.

All serotypes identified by the microarray were confirmed by PCR using purified DNA of the primary sample as the template.

For the interpretation of the microarray results, a serotype was considered a major serotype whenever its relative abundance was ≥70%. Accordingly, a serotype was considered a minor serotype whenever its relative abundance was ≤30%. Serotypes with relative abundances of >30% and <70% were considered codominant.

Isolation of pneumococcal strains from the cocolonized samples. Aliquots of the primary sample frozen stock were prepared, serially diluted, and plated in 5% blood–Trypticase soy agar containing 5 mg/liter gentamicin plates to obtain isolated colonies. Individual colonies were picked for amplification of serotype-specific capsular genes. The number of colonies picked was calculated as described by Huebner et al. (38). One colony of each capsular type was subcultured and frozen. For isolation and correct identification of nonencapsulated pneumococci, the method described by Simões et al. was used (39).

MLST. Multilocus sequence typing (MLST) was done as described previously (40). Novel sequence types (STs) and the corresponding strain

TABLE 1 Reporter and control strains for characterization of the *blp* locus

Strain designation	Strain characteristics[a]	Reference
P537	Serotype 6A strain with deletion of *blpRHCBA*–BIR; susceptible to secretion of all bacteriocins; reporter of inhibitory activity	Son et al., 2011 (27)
PSD121	R6 background; insertion of a type P164 *blp* locus, with *blpC* deletion and *lacZ* reporter under the control of the BIR promoter; type P164 BlpC/BlpH reporter	Kochan and Dawid, 2013 (42)
PSD101	R6 background; insertion of a type R6 *blp* locus, with *blpC* deletion and *lacZ* reporter under the control of the BIR promoter; type R6 BlpC/BlpH reporter	Pinchas et al., 2015 (43)
PMP105	R6 background; insertion of a type 6A *blp* locus, with *blpC* deletion and *lacZ* reporter under the control of the BIR promoter; type 6A BlpC/BlpH reporter	Pinchas et al., 2015 (43)
PMP105	R6 background; insertion of a type T4 *blp* locus, with *blpC* deletion and *lacZ* reporter under the control of the BIR promoter; type T4 BlpC/BlpH reporter	Pinchas et al., 2015 (43)
P1	Serotype 6A, BIR with MNO, $BlpC_{6A}$; control for MNO BIR content	Kim and Weiser, 1998 (44)
P4	Serotype 6B, BIR with QMNO, $BlpC_{P164}$; control for QMNO BIR content	Kim and Weiser, 1998 (44)
P132	Serotype 29, BIR with K, $BlpC_{6A}$; control for K BIR content	Son et al., 2011 (27)
P133	Serotype 6A, BIR with IJK MNO, $BlpC_{R6}$; control for IJK MNO BIR content	Son et al., 2011 (27)
P140	Serotype 35B, BIR with IJK, $BlpC_{R6}$; control for IJK BIR content	Son et al., 2011 (27)

[a] IJK, BIR profiles with bacteriocin genes *blpI*, *blpJ*, and *blpK*; K, BIR profiles with bacteriocin gene *blpK*; MNO, BIR profiles with bacteriocin genes *blpM*, *blpN*, and *blpO*; QMNO, BIR profiles with bacteriocin genes *blpQ*, *blpM*, *blpN*, and *blpO*.

information were deposited in the public MLST database for *S. pneumoniae* (http://pubmlst.org/spneumoniae/).

Assignment of pherotype CSP1 or CSP2. Pherotype assignment was done by multiplex PCR amplification of specific *comC* gene fragments, as described previously (41).

PCR assignment of *blpC* type. Assignment of the five *blpC* types described to date was done by amplification of specific *blpC* gene fragments, using primers designed for this study (see Table S1 in the supplemental material). The PCRs varied according to primer pair, as described in Table S1.

Overlay assays. Inhibition and activity overlay assays were performed as described elsewhere (27), using the reporter strains listed in Table 1.

Assessment of *blpA* integrity. Detection of the 4-bp repeat insertion was done by PCR amplification of a *blpA* gene fragment containing the repeat insertion region using forward primer 21 (described in reference 27) and a reverse primer with the sequence AGCCGCTGATGAAATG GGC, followed by digestion with Cac8I (New England BioLabs, Ipswich, MA, USA), according to the manufacturer's instructions. Restriction occurs only when the 4-bp repeat is present.

Samples in which we failed to amplify the region containing the repeat insertion were amplified with primers 18 and 19 (described in reference 27) for amplification of the total *blpA* gene to confirm the presence of possible deletions by comparison of the size of the PCR product with that of a control strain with an intact *blpA* gene.

RFLP analysis of the bacteriocin/immunity region (BIR) and bacteriocin content prediction. RFLP profiles of all isolates were determined as previously described (27). Briefly, PCR products were obtained with primers 1 and 2, purified, and digested with AseI (New England BioLabs, Ipswich, MA, USA), according to the manufacturer's instructions. Digestion products were analyzed by capillary electrophoresis, and isolates with identical restriction patterns were assigned to the same group.

BIR content was predicted by comparison of the RFLP profiles with those of sequenced strains available at GenBank or ENA, using either VectorNTI or CLC Genomics software. Overlay assays were performed with reporter strains of known BIR content (Table 1) as a control for *in silico* predictions.

Data analysis. The frequencies of several trait types (serotype, genotype, CSP, BlpC, BlpA, and inhibitory activity) were compared between cocolonization and single-colonization samples (sample types). To detect statistically significant differences, 5,000 random data sets were generated by randomly permuting the strain's trait and sample types. Two-tailed *P* values were computed by comparing individual frequencies of each trait type in each sample type to permutation-derived frequencies. The deviation of the distribution of the complete trait/sample type(s) from the null hypothesis was evaluated using Fisher's exact test. A similar analysis was performed to compare strains classified as minor, codominant, or major, according to their relative abundances.

The frequencies with which pairs of serotypes (or of genotypes or CSP, BlpC, and BlpA types) appear together in cocolonizing samples were also compared using a null hypothesis where strains mix randomly. The null distribution was estimated through 5,000 random assignments of strains to the samples with cocolonization events. Each sample had to contain at least one strain from the set of serotypes used to select the samples included in this study (serotypes 3, 6A, 6C, 11A/D, 15A, 15B/C, 19A, 19F, 21, 23A, and 23B and NT). A similar analysis was performed to compare the frequencies of pair types among samples with major or minor versus codominant cocolonization events.

Frequency deviations were considered significant when *P* values were <0.05.

RESULTS

***S. pneumoniae* strains cocolonizing individuals are highly diverse and coexist independently of the capsular type and genotype.** Of the 1,415 samples included in the study to screen for pneumococcal cocolonization, 285 pneumococcal strains were isolated from 135 cocolonized samples (Fig. 1). Strains were found to be highly diverse: 36 capsular types were found, and MLST

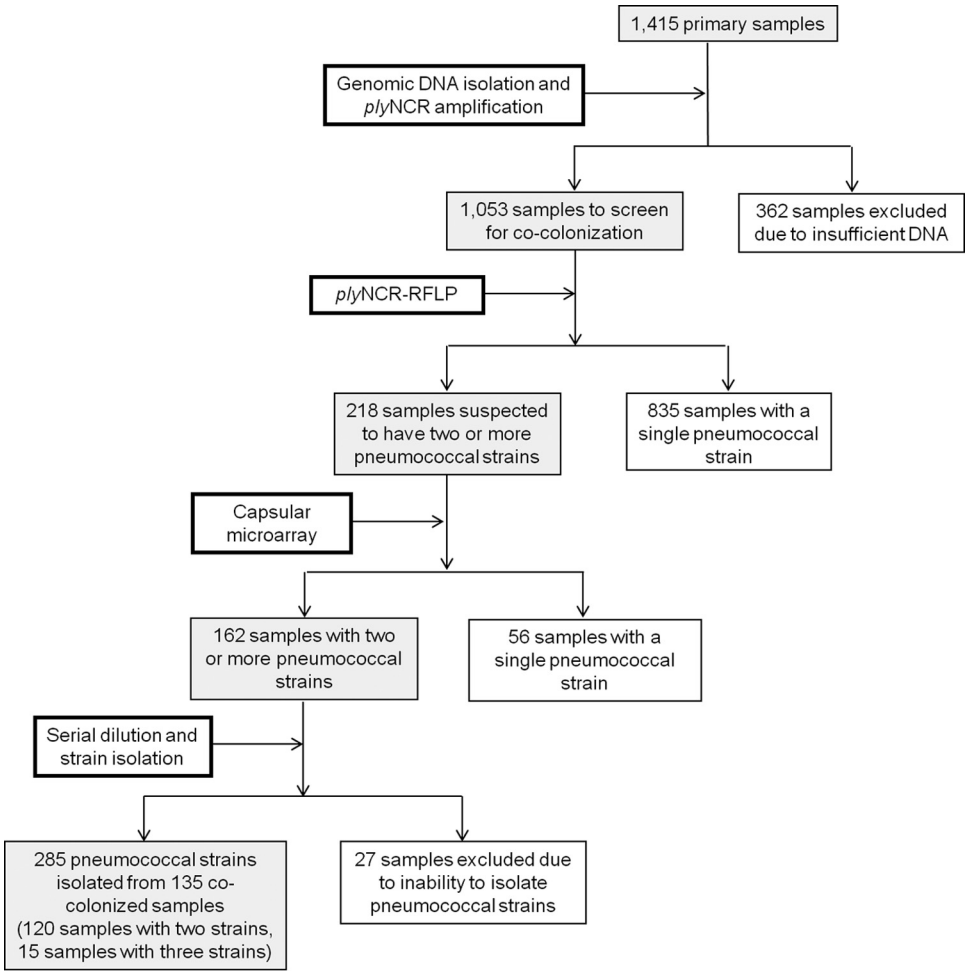

FIG 1 Strategy used for identification of cocolonized samples and isolation of cocolonizing pneumococcal strains.

genotyping clustered strains in 19 clonal complexes (CC; CC1 to CC19) and 36 singletons (S; S1 to S36) (Fig. 2). MLST analysis revealed 24 novel STs, 10 of which resulted from the presence of new alleles (ST9146, ST9147, ST9148, ST9149, ST9150, ST9151, ST9722, ST9724, ST9725, and ST9726) and 14 of which resulted from novel allele combinations (ST9152, ST9153, ST9154, ST9155, ST9156, ST9157, ST9158, ST9159, ST9160, ST9161, ST9162, ST9163, ST9164, and ST9720). Among the latter, for three STs (ST9160, ST9162, and ST9164), the allele separating the novel ST from its single-locus variant (SLV) was shared with the cocolonizing strain, suggesting that within-host recombination might have occurred with the cocolonizing strain (Table 2).

Positive associations between some serotypes and sequence types were found in this collection, although these correlations are of unclear significance. Statistically significant correlations are listed in Table S2 in the supplemental material.

CSP assignment of all strains showed that 35.2% of the interactions occurring in the 135 cocolonized samples were between two strains of CSP1, 10.9% were between two strains of CSP2, and 51.5% were between strains of different pherotypes (CSP1, CSP2, and other types present in strains of serotype 38). When the frequency with which CSP types appeared together in cocolonization was compared with the frequency estimated under the hypothesis that strains mix randomly, the results suggested a tendency for cocolonization with strains of different CSP types, although with low robustness of the results ($P = 0.048$).

Despite some positive associations found at the serotype and genotype levels, the large diversity of our collection seems to suggest that pneumococcal strains cocolonize the human nasopharynx independently of their capsule type, genetic background, and CSP type.

Genetic characterization of the *blp* locus of cocolonizing pneumococci reveals high diversity of *blp* cassettes. The genetic characterization of the *blp* locus of the cocolonizing strains was focused on the RFLP analysis of the BIR, on the assignment of the *blpC* type, and on the assessment of *blpA* integrity. The results are summarized in Fig. 2.

Thirty-nine BIR RFLP profiles were identified; among those profiles, 16 could be assigned to known BIR sequences available in GenBank and were found to account for 70.9% ($n = 202/285$) of the strains. The remaining 23 profiles corresponded to not-yet-described BIRs and accounted for 25.6% ($n = 73/285$) of the strains. In 10 strains (3.5%; 10/285), we were not able to amplify the BIR, despite several attempts. Of note, some BIR profiles, such as profiles 7 and 11, were detected in several clonal complexes (CC). Other profiles, highly prevalent as well, were restricted to a single CC each; examples included profiles 10 and 8, associated

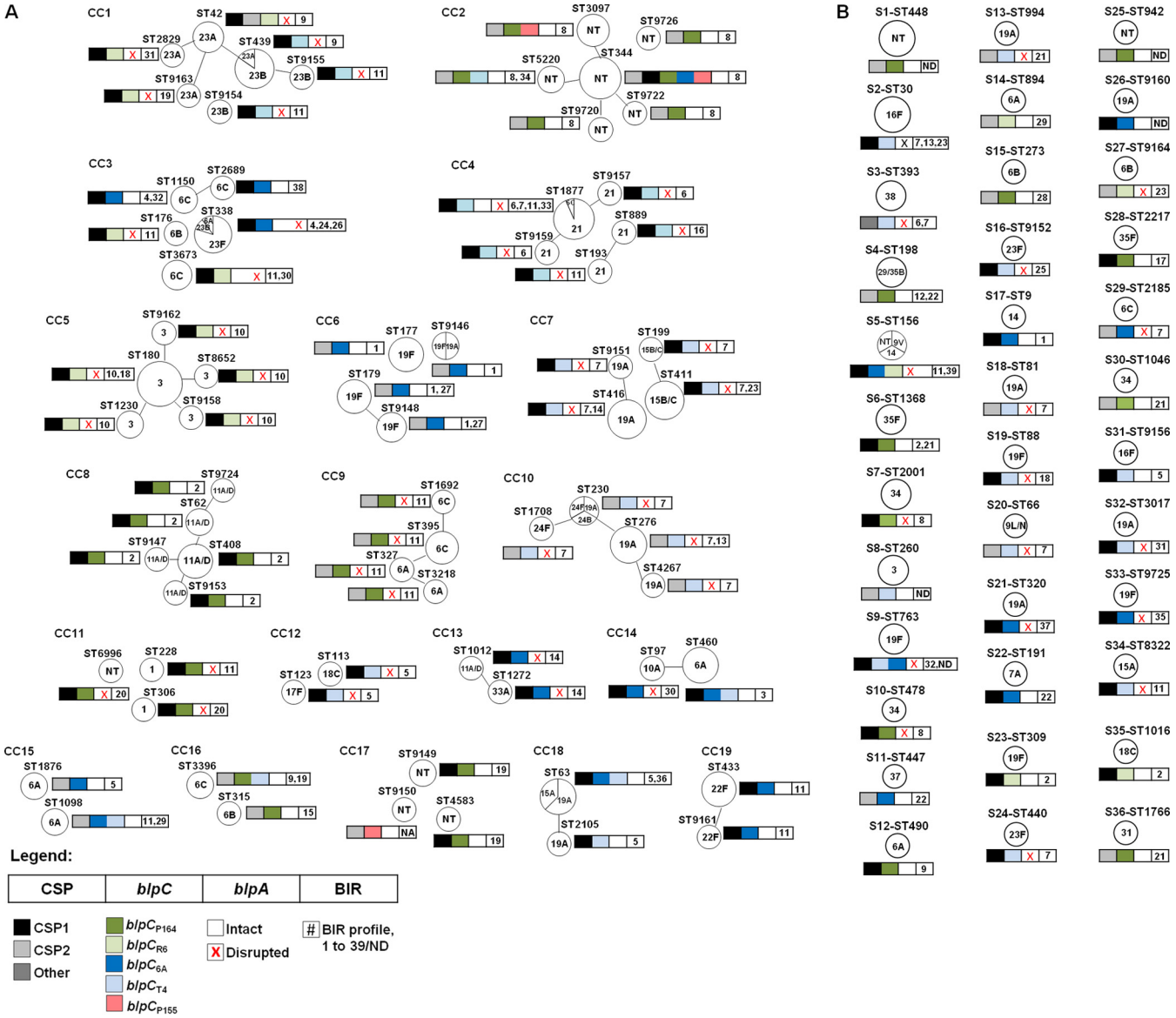

FIG 2 Genetic diversity and characteristics of the *blp* locus of cocolonizing pneumococci. Interpretation of results was performed using the goeBURST algorithm to determine possible evolutionary relationships between isolates: strains sharing 5 of 7 alleles were considered genetically related. Clonal complexes (CC) were assigned considering only the collection of cocolonizing pneumococci. Each circle represents a sequence type (ST). The size of the circle is proportional to the number of isolates of that ST. The serotype(s) of the isolates belonging to a given ST is indicated inside each circle. Related STs are grouped in clonal complexes (CC) (A), and unrelated STs are represented as singletons (S) (B). For each ST, information on CSP type and on genetic characterization of *blp* locus is represented by the bars; the first section represents CSP type, the second section represents *blpC* type, the third section represents the presence or absence of disruption in *blpA*, and the fourth section represents the BIR profiles, numbered 1 to 39. BIR RFLP profiles were matched to sequenced genome strains as follows: profile 1, GenBank/ENA accession number AILM01000012.1 (GA13723); profile 2, NC_014494.1 (AP200); profile 3, NZ_AGPB01000003.1 (GA13856); profile 4, NZ_ALCN01000004.1 (GA54354); profile 5, ALBB01000005.1 (2070335); profile 7, NZ_AGPV01000004.1 (GA47439); profile 9, NC_014498.1 (670-6B); profile 10, NC_017592.1 (OXC141); profile 11, AGPJ01000004.1 (GA17971); profile 12, NZ_ABFT01000002.1 (CDC1087); profile 14, ERR163218 (46518); profile 18, AP010935.1 (SP14-BS69); profile 19, AGNV01000005.1 (GA47502); profile 20, NC_012468.1 (70585); profile 21, ERR163199 (8a-SA64); and profile 22, ERR026719 (5a-14-3). ND, BIR profiles not determined due to failure to amplify the BIR.

with CC5 and CC2, respectively. Our BIR predictions indicated that the number of bacteriocin peptides present in the cocolonizing strains ranged between 2 and 6. Interestingly, the profiles predicted to contain the highest number of bacteriocin peptides (profiles 7 and 11) were associated mostly with strains with a disrupted *blpA* gene (described below).

High prevalence of strains with a disrupted *blpA* gene cocolonizing in nature. Disruption of the *blpA* transporter gene has been previously associated with a "cheater" phenotype, i.e., a non-inhibitory immune-only phenotype (27). Analysis of the *blpA* gene showed a disruption in the gene in 155 (54.4%) of 285 strains. Of these, 151 strains contained the 4-bp repeat insertion described by Son et al. (27) and four strains had a larger deletion of approximately 840 bp.

In the sets of cocolonized samples, 27.9% of dual interactions occurred between two cheater strains, 20.6% occurred between

TABLE 2 Evidence for putative *in vivo* horizontal gene transfer among cocolonizing strains in nasopharyngeal samples characterized in this study[a]

NP sample or strain	Serotype	ST	Allele no. according to *S. pneumoniae* MLST database						
			aroE	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*
Sample 8043									
Strain 1	19A	**ST9160**	**1**	60	9	8	6	3	29
Strain 2	6C	ST395	**1**	5	7	12	17	1	14
Strain 3	31	ST1766	**1**	5	29	1	46	14	18
Closest ST to strain 1	19A	ST1151	7	60	9	8	6	3	29
Sample 8058									
Strain 1	3	**ST9162**	**2**	15	2	10	6	1	22
Strain 2	23A	**ST9163**	**2**	8	9	9	6	4	6
Closest ST to strain 1	3	ST180	7	15	2	10	6	1	22
Closest ST to strain 2	23A/23F	ST190	8	8	9	9	6	4	6
Sample 8098									
Strain 1	6B	**ST9164**	**7**	8	4	18	15	4	31
Strain 2	3	ST180	**7**	15	2	27	2	11	71
Closest ST to strain 1	6A/6B	ST5516	2	8	4	18	15	4	31

[a] Closest ST to strain 1 (or strain 2), closest ST found in the MLST database (http://spneumoniae.mlst.net/). NP, nasopharyngeal; ST, sequence type. Novel STs and alleles shared with the cocolonizing strain that generated novel STs are represented in bold. In three samples, at least one of the strains generated a novel MLST profile not previously described in the MLST database. In all cases, the closest match was a single-locus variant. The novel allele was shared with the cocolonizing strain, suggesting within-host recombination.

strains with intact *blpA* genes (non-cheater strains), and 51.5% occurred between a cheater strain and a non-cheater strain. Comparison of the estimated and observed proportions of events involving strains with the same status or different *blpA* statuses (intact or disrupted) did not show significant differences, suggesting that the cheater phenotype alone does not restrict cocolonization of pneumococci ($P = 0.713$).

BlpC pheromone peptides are not equally distributed in the population and do not restrict cocolonization. The coexistence of strains secreting different BlpC peptides implies that the strain at the higher cell density would activate its *blp* locus earlier and be at a competitive advantage. To assess whether the type of BlpC could prevent or facilitate coexistence of pneumococci in the host, we determined the *blpC* allele of the 285 cocolonizing strains. The results showed that 36.8% of the strains were of type $blpC_{T4}$, 22.5% of type $blpC_{P164}$, 22.1% of type $blpC_{6A}$, 16.5% of type $blpC_{R6}$, and 2.1% of type $blpC_{P155}$. Of note, the lattermost *blpC* type was associated exclusively with nonencapsulated pneumococci.

To evaluate the ability of the strains to activate their own loci, signaling overlay assays were performed for all strains using BlpC-specific reporter strains for each BlpC of the four major types (Table 1). The BlpC type was confirmed in all strains in which it was possible to observe locus activity, as indicated by the β-galactosidase activity in the assays ($n = 59$). However, we were not able to confirm phenotypically the secretion of the encoded BlpC type for most strains (79%; $n = 226/285$). This could be largely explained by the fact that 69% ($n = 155/226$) of the noninducible strains were in fact cheater strains. *blpA* disruption was mainly associated with strains of type $blpC_{R6}$ and type $blpC_{T4}$ ($P = 0.0001$ for both [Fisher's exact test]) (Fig. 3). In the remaining 71 strains, a *blpA* disruption was not detected. A likely explanation for the lack of BlpC secretion in these strains would be the presence of nonrepeat mutations in *blpA* or of mutations in *blpB*, *blpH*, or *blpR*, a *blpC-blpH* mismatch, or mutations in the promoter regions of *blpABC* or *blpRH*, all of which have been described in other collections (22, 27, 45).

Looking at the sets of cocolonization samples, 40.6% of the dual interactions were between strains of the same BlpC type, while 59.4% were between strains of different BlpC types. When the frequency with which BlpC types appeared together in cocolonization was compared with the frequency estimated under the hypothesis that strains mix randomly, no significant difference was found ($P = 0.577$). The same analysis was performed by considering only pairs of strains in which secretion of BlpC was confirmed phenotypically through overlay assays in one of the strains; no significant difference was found in the results of this analysis as well ($P = 0.798$).

Phenotypes of bacteriocin secretion do not restrict cocolonization. To determine the phenotype of bacteriocin secretion, i.e., the inhibitory activity of the pneumococcal strains, overlay assays with a susceptible reporter pneumococcal strain P537 (Δ*blp*) were performed.

A total of 84 of 285 (29.5%) cocolonizing strains displayed

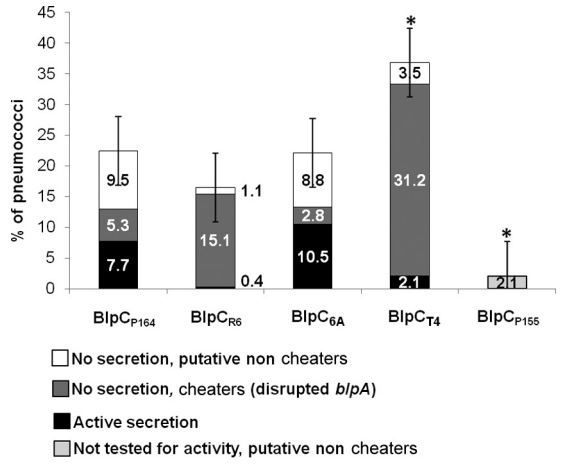

FIG 3 Distribution of *blpC* types in the cocolonization strains. Asterisks indicate statistically significant differences. Error bars represent standard errors.

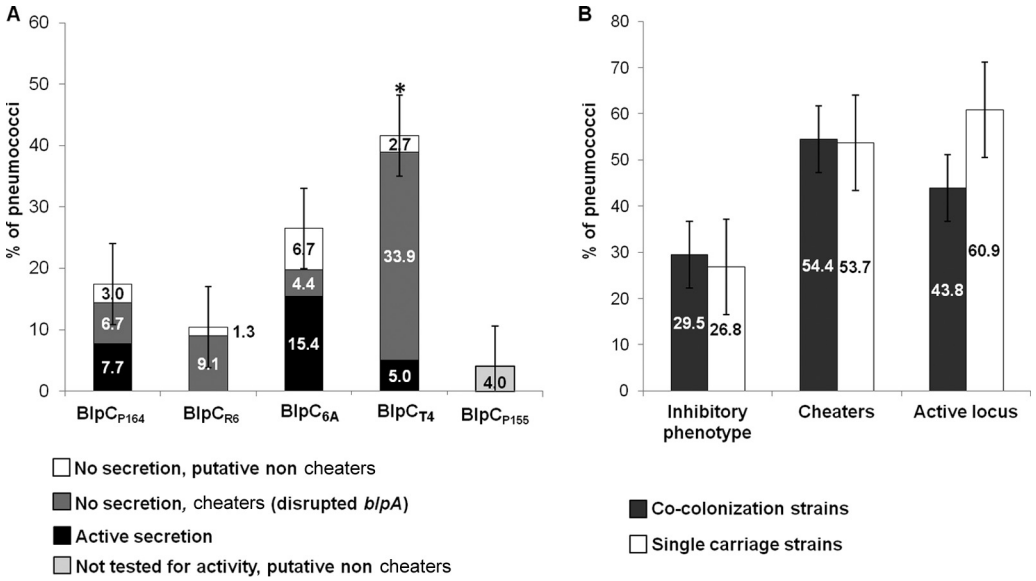

FIG 4 Characterization of the *blp* locus of the strains isolated from single carriage events. (A) Distribution of *blpC* types in the cocolonization strains. The asterisk indicates statistically significant differences. Error bars represent standard errors. (B) Comparison of the collections of single and cocolonized strains regarding the proportions of strains with an inhibitory phenotype, a cheater genotype, and locus inducibility. Error bars represent standard errors.

inhibitory activity against P537. Of these strains, 21 (25%) had a disrupted *blpA* gene and no evidence of pheromone secretion as analyzed using reporter strains (suggesting non-blp-mediated inhibition) and 63 had an intact *blpA* gene. Among the latter, 53 strains were in the group in which BlpC pheromone secretion was observed in the signaling overlays. For the other 10, the signaling overlay assay results were negative.

Among the strains that did not display inhibitory activity ($n = 201$), 67% were cheater strains and 30% did not secrete pheromone in the signaling overlays, despite an apparently intact *blpA* gene.

In the sets of cocolonized samples, the rate of coexistence of strains that displayed an inhibitory phenotype was not different from what would be expected by chance, suggesting that the presence of an inhibitory phenotype alone does not prevent cocolonization ($P = 0.715$).

Considering this result, we performed *in silico* prediction of the outcome of the interaction of each pair of strains in every sample, taking into account the genetic content of the BIR, the integrity of the *blpA* transporter gene, and the type of signaling peptide BlpC secreted. Our prediction resulted in 50 outcomes of inhibition (30.3%) and 115 outcomes of no inhibition (69.7%). Comparison of the estimated and predicted proportions of the outcomes of inhibition and no inhibition did not show a significant difference ($P = 0.274$).

The results obtained with the genotypic and phenotypic approaches suggest that the *blp* locus alone does not seem to prevent the coexistence of pneumococcal strains in the host.

Phenotypes of bacteriocin secretion are the same in strains isolated from single and cocolonization events. In order to assess whether strains found in single and cocolonization events would display different genotypic and/or phenotypic *blp* characteristics, we selected a subset ($n = 298$) of the initial 1,053 nasopharyngeal samples in which only one strain was detected for comparison with the collection of cocolonized samples. This selection was performed randomly while maintaining matched numbers of samples from the two collections from each year. The characteristics of this collection are summarized in Table S3 in the supplemental material.

Overall, the prevalences of each *blpC* type, as well as the distributions of inducible, noninducible, and cheater strains within each *blpC* type, were similar in the two collections (Fig. 3 and 4A). Similarly, no significant differences were observed in the two collections regarding the proportions of strains with an inhibitory phenotype, cheater strains, and strains with an active locus (Fig. 4B).

DISCUSSION

Bacteriocin production is a common trait in organisms that reside in polymicrobial communities. Bacteriocin-mediated competition has been shown to alter the composition of the microbiota in environmental communities and on human surfaces (46).

Animal models of pneumococcal colonization have demonstrated that bacteriocin production provides a competitive advantage during establishment on the mucosal surface (20, 27). These experiments were performed by simultaneously inoculating mixtures of competing strains at a 1:1 ratio into the nasopharynx of a mouse. Colonization dynamics is likely to be far more complex in humans than under the experimental conditions used in these studies. Although cocolonization by distinct pneumococcal strains is quite common, the relative proximity of these strains within the human host is not known. What is clear, based on extensive genome studies, is that such organisms are in close enough proximity to support genetic exchange (9, 47). In this study, we used a collection of cocolonizing and singly colonizing isolates that reflected true colonization patterns in the human host to better understand the contribution of bacteriocin production to global colonization dynamics.

Characterization of the cocolonization strains at the level of serotype and genotype enabled us to investigate positive associa-

tions between specific capsular types and genotypes, although no straightforward observations could be made to explain those associations. At the capsular level, we explored properties previously shown to be dependent on the capsular type, such as the polysaccharide structure and, by association, the fitness cost of capsule production (48, 49) and the surface charge of the capsular type (50). All these properties have been shown to predict the prevalence of the serotypes (48, 49). Interestingly, Trzciński et al. (51) revealed a reproducible hierarchy of capsular types in a mouse model of multiple serotype carriage which correlated with the metabolic cost and the surface charge measured *in vitro*. Our prevalence results are in agreement with the proposed hypothesis, but none of the explored properties could explain the serotype associations identified in this study. This may have been due to the fact that interactions are likely to be far more complex in the human host than in experimental systems.

The genetic characterization of the *blp* locus of the cocolonizing strains allowed us to show very great diversity in this locus that originated from the diversity in the *blpC* and *blpA* genes and the BIR, supporting observations from other studies (20, 26–28, 52). Notwithstanding, MLST genotyping of the strains enabled us to show a fairly high level of conservation of the *blp* locus among closely related strains, suggesting that this extensive genetic diversification is occurring at a rate sufficiently low for some clonality to be sustained.

Overall, the results of our genotypic and phenotypic characterization of the *blp* locus of cocolonizing pneumococcal strains seem to suggest that the phenotypes of bacteriocin secretion do not have an impact on the coexistence of pneumococci in the nasopharynx, an observation that was supported by the lack of differences in the results obtained in the characterization of the single carriage isolates. Given the large diversity in this locus, it is not surprising that the effect of *blp*-mediated competition on cocolonization is not as straightforward as might be expected, and several aspects must be taken into account.

First, the high diversity of BlpH receptors due to the existence of naturally occurring chimeras for the $blpH_{6A}$ gene, as described by Pinchas et al. (43), may affect the likelihood of cross-stimulation between cocolonizing pairs. Those authors have shown that these BlpH variants have different specificities for cognate and noncognate BlpC peptides, which can affect the outcome of activation of neighboring *blp* loci.

Second, the large array of bacteriocins and immunity proteins that can be present in a strain, and the fact that the natural pneumococcal niche is a polymicrobial environment, raises the hypothesis that this competition mechanism might be used for mediating interactions not only with bacteria of the same species but also with other inhabitants of the nasopharynx. In fact, Lux et al. (52) have demonstrated the inhibitory activity of pneumococci against bacteria of different species.

Finally, the natural and highly frequent occurrence of strains that display a cheater phenotype also contributes to the complexity of this competition mechanism. The reason why these cheater strains are so highly prevalent remains to be addressed, and the advantage of displaying this phenotype is not completely clear. On the one hand, these strains avoid the fitness cost of bacteriocin secretion while they are still able to express immunity proteins. On the other hand, they become at risk of elimination upon encountering a strain secreting a different BlpC type. Two recent publications (22, 23) have demonstrated a regulatory connection between the *com* system, controlling competence, and the *blp* system. Those studies demonstrated that BlpC could be secreted by the ComAB competence-regulated transporter under competence-inducing conditions. ComAB-secreted BlpC could then stimulate the *blp* locus through the BlpHR regulatory mechanism. None of these studies had, however, shown the ability of these strains to use ComAB as a bacteriocin secretion system, suggesting that the previously named "cheater" strains may be able to self-activate the locus during competence but have the capability to display an immune phenotype only. We have performed signaling overlays in close to 600 strains, among which over half had a disrupted *blpA* gene, and we were never able to see locus activation in a strain with *blpA* disruption. Nevertheless, as the conditions of our assays did not control for competence activation, we have repeated the analysis performed in this study, considering that all strains would be able to activate the *blp* locus and express immunity, and the conclusion that bacteriocin secretion does not restrict cocolonization was maintained: predictions resulted in 95 outcomes of inhibition, 51 outcomes of no inhibition, and 19 events in which it was not possible to predict an outcome due to unknown bacteriocin/immunity content in the strains ($P = 0.628$ [Fisher's exact test]).

Our study had some limitations. First, we did not sequence the entire BIR, which might have helped in explaining the lack of BlpC secretion in the 71 strains with an apparently intact *blpA*. Lack of BlpC secretion might have been due to mutations in *blpA*, *blpB*, *blpH*, or *blpR* (22, 27, 45). In fact, looking at over 4,000 pneumococcal genomes, Kjos et al. (22) showed that only 23% of the genomes had intact *blpAB* genes, which is in agreement with the 21% proportion of secretor strains that we obtained with the signaling overlays. Additional alternative explanations could be the presence of a *blpC-blpH* mismatch in these strains (43), lack of sensitivity of the assay, or the requirement of particular conditions for locus activation in those strains. In addition, the few noninhibitory strains with intact pheromone secretion may have harbored mutations in their bacteriocin genes that would not be detected with the RFLP analysis. Second, we did not determine the opaque/transparent phenotype of our strains, which might have helped to explain the lack of inhibitory activity in the 69 intact strains that failed to inhibit the susceptible strain. Dawid et al. (53) showed that the opaque and transparent variants of a strain with an intact *blpA* gene had different inhibitory profiles, despite the fact that the amounts of *blpMNPO* transcripts were the same. These differences were attributed to different expression levels of the HtrA protease. In particular, expression of HtrA in opaque variants was higher, resulting in degradation of BlpC and hence in decreased bacteriocin secretion (42). Still, this link between opacity variants and HtrA expression was shown for a single 6A strain only and it is unknown whether this is a general property of pneumococcal strains. Third, the cross-sectional nature of our study prevented us from measuring the effect of *blp*-mediated competition on the duration of carriage. Also, we did not measure the pneumococcal load in the samples and it is possible that the effects of competition might be reflected at the level of carriage density. Even so, we measured the relative proportion of each strain in the samples and could not establish a correlation between outcomes of bacteriocin secretion and strain density (see Fig. S1 in the supplemental material).

Our study also had significant strengths. We used a very-well-characterized, natural collection of co- and singly colonized sam-

ples identified through the use of highly sensitive molecular methods. This enabled us to obtain an unbiased and highly diverse collection of both single and cocolonizing pneumococcal strains to measure the impact of *blp* bacteriocin production on competition in the nasopharynx.

Our results demonstrate the importance of using human samples to support conclusions drawn using idealized animal models and are likely to have implications for other bacteriocin systems that have been exclusively studied in animal models. For example, similarly to the case with the *blp* bacteriocins, studies performed in animal models have shown that bacteriocin secretion is important in *Streptococcus mutans* competition in early dental biofilms (54) and in the ability of *Enterococcus faecalis* to colonize the mammalian gut (55). In light of the results of our study, it would be of interest to determine if the expected correlations hold true when natural samples from the human host are studied.

Although we have not seen an effect of *blp*-mediated competition on cocolonization, the fact that this locus is present in all pneumococci and has been maintained by evolution (28) suggests an important function, a conclusion supported by the results obtained in competition experiments showing that the locus is active *in vivo* (20). The link between the *blp* and *com* systems suggests that the *blp* locus could be important in increasing the DNA pool for transformation (22, 23). Also, it could be important under specific conditions (e.g., nutrient limitation) or during the establishment of a strain during colonization by creating an isolated niche. Moreover, studies with a longitudinal design would help to determine whether *blp*-mediated competition might be acting at the level of carriage duration or density.

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