The *blp* locus of *Streptococcus pneumoniae* plays a limited role in the selection of which strains can co-colonize the human nasopharynx

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Running title: Impact of the *blp* locus on pneumococcal co-colonization

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KEY WORDS

Streptococcus pneumoniae; multiple serotype carriage; co-colonization; competition; bacteriocins; *blp* locus

LIST OF ABBREVIATIONS

CSP (competence stimulating peptide); BIR (bacteriocin/immuntity region); *ply*NCR-RFLP (pneumolysin non-coding region restriction fragment length polymorphism); MLST (multilocus sequence typing); CC (clonal complex); S (singleton).

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 co-exist 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 co-colonizing isolates to evaluate the impact of the *blp* locus on human colonization to determine whether competitive phenotypes of bacteriocin secretion restrict co-colonization.

We identified a collection of 135 nasopharyngeal samples with two or more strains totaling 285 isolates. The *blp* locus of all strains was characterized genetically with regards 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 colonization (n=298) were characterized for comparison.

Co-colonizing strains had a high diversity of *blp* cassettes; approximately one third displayed an inhibitory phenotype *in vitro*. Despite in vitro evidence of competition, pneumococci co-colonized individuals independently of their *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 co-colonizing strains were compared.

Despite clear evidence of *blp*-mediated competition in experimental models, our study suggests that the *blp* locus plays a limited role in restricting pneumococcal co-colonization in humans.

IMPORTANCE

Nasopharyngeal colonization with *Streptococcus pneumoniae* (pneumococcus) is important for pneumococcal evolution as it represents the major site for horizontal gene transfer when multiple strains co-occur, a phenomenon known as co-colonization. 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 co-existence of pneumococci in the host, contrary to what has been shown in experimental models.

INTRODUCTION

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).

84

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

91

92 Little is known about how pneumococcal strains interact with each other within the
93 nasopharynx. Data from longitudinal studies have shown that the pneumococcal population
94 colonizing children is in constant turnover (5, 12). Although over 95 serotypes have been
95 described to date in the pneumococcal population (13, 14), only a small subset are dominant in
96 carriage suggesting a higher competitive ability of these serotypes (15, 16). The fact that the
97 pneumococcus' natural niche is a dense polymicrobial environment with limited resources also
98 suggests that competitive interactions are important in its lifestyle (17).

99

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

105

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

113

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

124

125 The *blp* locus is highly diverse. There is significant variability in the BIR, at least sixteen
126 bacteriocin peptides have been described, several with allelic variability and strains can vary
127 with respect to the number of encoded bacteriocins (27, 28). Also, at least five types of the
128 peptide pheromone BlpC have been found (21, 26-28). In addition, a significant fraction of
129 pneumococcal strains carry a disruption in the genes encoding the BlpAB transporter (27). These
130 strains can respond to exogenous pheromone with the production of immunity proteins but
131 cannot secrete bacteriocins. The prevalence of these strains suggests that strategies that limit
132 locus stimulation and peptide secretion may provide an energetic advantage. It was recently
133 shown that *blpAB* disrupted strains can secrete a fraction of the *blp* pheromone, BlpC, through
134 the homologous competence transporter, ComAB, allowing for self-stimulation of the locus (22,
135 23). This secretion only occurs during the competent state when sufficient ComAB is produced
136 to support BlpC secretion and is enhanced by cross stimulation of some of the *blp* promoters by
137 the competence response regulator, ComE. The universal presence of the *blp* locus in
138 pneumococci and the significant diversity of its content suggest that this locus provides a
139 competitive advantage either during colonization or transmission (28). No studies to date have
140 used colonizing isolates from humans to examine whether *blp* locus activity is sufficiently
141 widespread to restrict the strains that can co-exist within the nasopharynx.
142
143 In this study we explored the role of the *blp* locus and bacteriocin secretion on the co-existence
144 of *S. pneumoniae* in human nasopharyngeal samples.
145

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 5mg/L gentamicin) and incubated overnight at 37^{o}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 of the cases (>90%) only one morphology was detected with naked eye. The bacterial lawn of the primary selective plate, herein called "primary sample", was collected and frozen at -80^{o}C in 1mL Mueller-Hinton broth with 30% glycerol. On the third day, pneumococcal pure 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, as appropriate (35)(Statens Serum Institute, Copenhagen, Denmark).

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 more frequently found in single and multiple carriage events are essentially the same (36), we

identified the serotypes with highest cumulative prevalence in the cross-sectional studies described above. These were: 3, 6A, 6C, 11A, 15A, 15B/C, 19A, 19F, 21, 23A, 23B and non-encapsulated pneumococci (NT). In total, 1,415 samples were identified and selected for molecular detection of co-colonization. Of these, 66.2% (936/1,415) were collected from children vaccinated with at least one dose of either the 7- or the 10-valent pneumococcal conjugate vaccine, 30.7% (435/1,415) were collected from non-vaccinated children, and 3.1% (44/1,415) were collected from children from whom the vaccination status was unknown.

DNA isolation

Total DNA was isolated from 200µL of the primary sample frozen stock using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Detection of co-colonization and capsular typing of the co-colonizing strains

Detection of co-colonization was done using the plyNCR-RFLP (37) and the BuG@S SP-CPSv1.4.0 microarray for molecular serotyping as previously described (36).

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

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 > 30% and <70% were considered co-dominant.

Isolation of pneumococcal strains from the co-colonized samples

Aliquots of the primary sample frozen stock were prepared, serially diluted and plated in 5% blood trypticase soy agar containing 5mg/L 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 non-encapsulated pneumococci the method described by Simões *et al.* was used (39).

MLST

Multilocus sequence typing was done as described previously (40). Novel STs and the corresponding strain information were deposited at 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).

208

209 **PCR assignment of *blpC* type**

210 Assignment of the five *blpC* types described up to now was done by amplification of specific
211 *blpC* gene fragments, using primers designed for this study (Table S1). The PCR reactions varied
212 according to primer pair, as described in Table S1.

213

214 **Overlay assays**

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

217

218 **Assessment of *blpA* integrity**

219 Detection of the 4 bp repeat insertion was done by PCR amplification of a *blpA* gene fragment
220 containing the region of repeat insertion using forward primer 21 described in (27) and reverse
221 primer with sequence AGCCGCTGATGAAATGGGC, followed by digestion with Cac8I (New
222 England Biolabs, Ipswich, MA, USA), according to the manufacturer's instructions. Restriction
223 occurs only when the 4 bp repeat is present.

224

225 Samples in which we failed to amplify the region containing the repeat insertion were amplified
226 with primers 18 and 19 described in (27) for amplification of the total *blpA* gene to confirm
227 presence of possible deletions by comparison of the size of the PCR product with that of a
228 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 RFLP profiles with those of sequenced strains available at GenBank, using either VectorNTI or CLC Genomics softwares. Overlay assays with reporter strains of known BIR contents (Table 1) were performed as a control for *in silico* predictions.

Data analysis

The frequencies of several trait types (serotypes, genotype, CSP, BlpC, BlpA and inhibitory activity) were compared between co-colonization and single colonization samples (sample types). To detect statistically significant differences, 5000 random datasets 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 complete trait/sample types distribution from the null hypothesis was evaluated through a Fisher's Exact Test. A similar analysis was performed to

compare strains classified as minor, co-dominant or major, according to their relative abundance.

The frequency with which pairs of serotypes (or of genotypes, CSP, BlpC and BlpA types) appear together in co-colonizing samples was also compared with a null hypothesis where strains mix randomly. The null distribution was estimated through 5000 random assignments of strains to the samples with co-colonization events. Each sample had to contain at least one strain from the set of serotypes used to select the samples included in this study (3, 6A, 6C, 11A/D, 15A, 15B/C, 19A, 19F, 21, 23A, 23B, NT). A similar analysis was performed to compare the pair type frequency among samples with major/minor versus co-dominant co-colonization events.

Frequency deviations were considered significant when p-values<0.05.

RESULTS

S. pneumoniae strains co-colonizing individuals are highly diverse and co-exist independently of the capsular type and genotype

Out of the 1,415 samples included in the study to screen for pneumococcal co-colonization, 285 pneumococcal strains were isolated from 135 co-colonized samples (Figure 1). Strains were found to be highly diverse: 36 capsular types were found and MLST genotyping clustered strains in 19 clonal complexes (CC, CC1-CC19) and 36 singletons (S, S1-S36) (Figure 2). MLST analysis revealed 24 novel STs, 10 of which were originated by the presence of new alleles (STs 9146,

9147, 9148, 9149, 9150, 9151, 9722, 9724, 9725, and 9726) and 14 resulted from novel allele combinations (STs 9152, 9153, 9154, 9155, 9156, 9157, 9158, 9159, 9160, 9161, 9162, 9163, 9164, and 9720). Among the latter, for three STs (ST9160, ST9162 and ST9164) the allele separating the novel ST from its SLV was shared with its co-colonizing strain, suggesting that within host recombination might have occurred with the co-colonizing strain (Table 2).

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

CSP assignment of all strains showed that 35.2% of the interactions occurring in the 135 co-colonized 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 type present in strains from serotype 38). When the frequency in which CSP types appear together in co-colonization was compared with the frequency estimated under the hypothesis that strains mix randomly, the results suggested a tendency for co-colonization with strains of different CSP types, although with low robustness (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 co-colonize the human nasopharynx independently of their capsule, genetic background and CSP type.

Genetic characterization of the *blp* locus of co-colonizing pneumococci reveals high diversity of *blp* cassettes

The genetic characterization of the *blp* locus of the co-colonizing 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 Figure 2.

Thirty-nine BIR RFLP profiles were identified, from which 16 could be assigned to known BIR sequences available in GenBank and accounted for 70.9% (n=202/285) of the strains. The remaining 23 profiles corresponded to not yet described BIR regions and accounted for 25.6% (n=73/285) of the strains. In ten strains (3.5%, 10/285) we were not able to amplify the BIR region, 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, such as profiles 10 and 8, associated with CC5 and CC2, respectively. Our BIR predictions indicated that the number of bacteriocin peptides present in the co-colonizing strains varied between 2 and 6. Interestingly, profiles predicted to contain the highest number of bacteriocin peptides (profiles 7 and 11) were associated mostly with strains with a disrupted *blpA* (described below).

High prevalence of strains with disrupted *blpA* gene co-colonizing in nature

312 Disruption of the *blpA* transporter gene has been previously associated with a *cheater*
313 phenotype, i.e., non-inhibitory immune-only (27). Analysis of the *blpA* gene showed a disruption
314 in the gene in 155 out of 285 strains (54.4%). Of these, 151 strains contained the 4 bp repeat
315 insertion described by Son *et al.* (27) and four strains had a larger deletion of approximately 840
316 bp.
317
318 In the sets of co-colonized samples, 27.9% of dual interactions occurred between two *cheater*
319 strains, 20.6% between strains with intact *blpA* genes (non-*cheaters*), and 51.5% occurred
320 between a *cheater* and a non-*cheater* strain. Comparison of the estimated and observed
321 proportions of events involving strains with same or different *blpA* status (intact or disrupted)
322 did not show a significant difference, suggesting that the *cheater* phenotype alone does not
323 restrict co-colonization of pneumococci (p=0.713).
324
325 **Pheromone peptides BlpC are not equally distributed in the population and do not restrict co-**
326 **colonization**
327 Co-existence of strains secreting different BlpC peptides implies that the strain at higher cell
328 density would activate its *blp* locus earlier and be at a competitive advantage. To assess if the
329 type of BlpC could prevent or facilitate co-existence of pneumococci in the host we determined
330 the *blpC* allelle of the 285 co-colonizing strains. The results showed that 36.8% of the strains
331 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, this latter *blpC* type was associated exclusively with non-encapsulated pneumococci.

To evaluate the ability of the strains to activate their own locus, 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, for most strains (79%, n=226/285) we were not able to confirm phenotypically the secretion of the encoded BlpC type. This could be largely explained by the fact that 69% (n=155/226) of the non-inducible strains were in fact *cheaters*. *blpA* disruption was mainly associated with strains of type *blpC*$_{R6}$ and *blpC*$_{T4}$ (p=0.0001 for both, Fisher's exact test) (Figure 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 non-repeat mutations in *blpA*, or mutations in *blpB, blpH* or *blpR*, a *blpC/blpH* mismatch, or mutations in the promotor regions of *blpABC* or *blpRH*, all of which have been described in other collections (22, 27, 45).

Looking at the sets of co-colonization 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 in which BlpC types appear together in co-colonization 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 considering only pairs of strains in

353 which secretion of BlpC was confirmed phenotypically through overlay assays in one of the
354 strains; no significant difference was found as well (p=0.798).
355
356 **Phenotypes of bacteriocin secretion do not restrict co-colonization**
357 To determine the phenotype of bacteriocin secretion, *i.e.*, the inhibitory activity of the
358 pneumococcal strains, overlay assays with a susceptible reporter pneumococcal strain P537
359 (Δ*blp*) were performed.
360
361 Eighty-four out of 285 (29.5%) co-colonizing strains displayed inhibitory activity against P537. Of
362 these, 21 strains (25%) had a disrupted *blpA* gene and no evidence of pheromone secretion
363 using reporter strains (suggesting a non-*blp* mediated inhibition) and 63 strains had an intact
364 *blpA* gene. Among the latter, 53 strains were in the group in which BlpC pheromone secretion
365 was observed in the signaling overlays. For the other 10, the signaling overlay assay had been
366 negative.
367
368 Among the strains that did not display inhibitory activity (n=201), 67% were *cheaters* and 30%
369 did not secrete pheromone in the signaling overlays, despite an apparently intact *blpA* gene.
370
371 In the sets of co-colonized samples, co-existence of strains that displayed an inhibitory
372 phenotype was not different from what would be expected by chance, suggesting that an
373 inhibitory phenotype alone does not prevent co-colonization (p=0.715).

374

375 Considering this result, we performed an *in silico* prediction of the outcome of the interaction of
376 each pair of strains in every sample, taking into account the genetic content of the BIR, the
377 integrity of the *blpA* transporter gene and the type of signaling peptide BlpC secreted. Our
378 prediction resulted in 50 outcomes of inhibition (30.3%) and 115 outcomes of no inhibition
379 (69.7%). Comparison of the estimated and predicted proportions of outcomes of inhibition and
380 no inhibition did not show a significant difference (p=0.274).

381

382 The results obtained with the genotypic and phenotypic approaches suggest that the *blp* locus
383 alone does not seem to prevent the co-existence of pneumococci in the host.

384

385 **Phenotypes of bacteriocin secretion are the same in strains isolated from single and co-**
386 **colonization events**

387 In order to assess whether strains found in single and co-colonization events would display
388 different genotypic and/or phenotypic *blp* characteristics, we selected a subset (n=298) of the
389 initial 1,053 nasopharyngeal samples in which only one strain was detected, for comparison
390 with the collection of co-colonized samples. This selection was performed randomly but
391 maintaining a matched number of samples from each year between both collections. The
392 characteristics of this collection are summarized in Table S3.

393

394 Overall, the prevalence of each *blpC* type was similar in the two collections, as well as the
395 distribution of inducible, non-inducible and *cheater* strains within each *blpC* type (Figures 3 and
396 4A). Similarly, no significant differences were observed in the two collections regarding the
397 proportion of strains with an inhibitory phenotype, *cheaters*, and an active locus (Figure 4B).
398
399 **DISCUSSION**
400 Bacteriocin production is a common trait in organisms that reside in polymicrobial communities.
401 Bacteriocin mediated competition has been shown to alter the composition of the microbiota in
402 environmental communities and on human surfaces (46).
403
404 Animal models of pneumococcal colonization have demonstrated that bacteriocin production
405 provides a competitive advantage during establishment on the mucosal surface (20, 27). These
406 experiments were performed by inoculating mixtures of competing strains at a 1:1 ratio
407 simultaneously into the nasopharynx of a mouse. Colonization dynamics in humans is likely to
408 be far more complex than the experimental conditions used in these studies. Although co-
409 colonization with distinct pneumococcal strains is quite common, the relative proximity of these
410 strains within the human host is not known. What is clear, based on extensive genome studies,
411 is that organisms are in close enough proximity to support genetic exchange (9, 47). In this
412 study, we used a collection of co-colonizing and single colonizing isolates that reflect true
413 colonization patterns in the human host to better understand the contribution of bacteriocin
414 production to global colonization dynamics.

415

416 Characterization of the co-colonization strains at the level of serotype and genotype enabled us
417 to investigate positive associations between specific capsular types and genotypes, although no
418 straightforward observations could be found to explain those associations. At the capsular level,
419 we explored properties shown to be dependent on the capsular type, such as the
420 polysaccharide structure, and, by association, the fitness cost of capsule production (48, 49),
421 and the surface charge of the capsular type (50). All these properties have been shown to
422 predict the prevalence of the serotypes (48, 49). Interestingly, Trzciński *et al.* (51) have shown a
423 reproducible hierarchy of capsular types in a mouse model of multiple serotype carriage, which
424 correlated with the metabolic cost and the surface charge measured *in vitro.* Our prevalence
425 results are in agreement with the proposed hypothesis but none of the explored properties
426 could explain the serotype associations identified in this study. This may have been due to the
427 fact that interactions in the human host are likely to be far more complex than in experimental
428 systems.

429

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

436

437 Overall our genotypic and phenotypic characterization of the *blp locus* of co-colonizing
438 pneumococci seems to suggest that the phenotypes of bacteriocin secretion do not have an
439 impact in the co-existence of pneumococci in the nasopharynx, an observation that was
440 supported by the lack of differences in the results obtained in the characterization of the single
441 carriage isolates. Given the large diversity in this locus, it is not surprising that the effect of *blp*-
442 mediated competition on co-colonization is not as straightforward as might be expected and
443 several aspects must be taken into account.

444

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

450

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

457

458 Finally, the natural and highly frequent occurrence of strains that display a *cheater* phenotype
459 also contributes to the complexity of this competition mechanism. The reason why these
460 *cheater* strains are so highly prevalent remains to be addressed and the advantage of displaying
461 this phenotype is not completely clear. On one hand, these strains avoid the fitness-cost of
462 bacteriocin secretion while they are still able to express immunity proteins. On the other hand,
463 they become at risk of elimination upon the encounter of a strain secreting a different BlpC
464 type. Two recent publications (22, 23) have demonstrated a regulatory connection between the
465 *com* system, controlling competence and the *blp* system. These studies demonstrated that BlpC
466 could be secreted by the competence regulated transporter, ComAB under competence
467 inducing conditions. ComAB secreted BlpC could then stimulate the *blp* locus through the BlpHR
468 regulatory mechanism. None of these studies has, however, shown the ability of these strains to
469 use ComAB as a bacteriocin secretion system, suggesting that the previously named "*cheater*"
470 strains may be able to self-activate the locus during competence but have the capability to
471 display only an immune phenotype. We have performed signalling overlays in close to 600
472 strains, from which over half had a disrupted *blpA* gene, and we were never able to see locus
473 activation in a strain with *blpA* disruption. Nevertheless, as the conditions of our assays do not
474 control for competence activation, we have repeated the analysis performed in this study
475 considering that all strains would be able to activate the *blp* locus and express immunity and the
476 conclusion that bacteriocin secretion does not restrict co-colonization was maintained:
477 predictions resulted in 95 outcomes of inhibition, 51 outcomes of no inhibition and 19 events in

478 which it was not possible to predict an outcome due to unknown bacteriocin/immunity contents
479 in the strains (p=0.628, Fisher's exact test).
480
481 Our study has some limitations. First, we did not sequence the entire BIR locus, which might
482 have helped in explaining the lack of BlpC secretion in the 71 strains with an apparently intact
483 *blpA.* Lack of BlpC secretion may have been due to mutations in *blpA, blpB, blpH* or *blpR* (22, 27,
484 45). In fact, looking at over 4,000 pneumococcal genomes, Kjos *et al.* (22) have shown that only
485 23% of the genomes had intact *blpAB* genes, which is in agreement with the 21% proportion of
486 secretor strains that we have obtained with the signaling overlays. Yet other alternative
487 explanations could be a *blpC/blpH* mismatch in these strains (43), lack of sensitivity of the assay,
488 or the requirement of particular conditions for locus activation in those strains. In addition, the
489 few non-inhibitory strains with intact pheromone secretion may have harbored mutations in
490 their bacteriocin genes that would not be detected with the RFLP analysis. Second, we did not
491 determine the opaque/transparent phenotype of our strains, which might have helped to
492 explain the lack of inhibitory activity in the 69 intact strains that failed to inhibit the susceptible
493 strain. Dawid *et al* (53) have shown that opaque and transparent variants of a strain with an
494 intact *blpA* gene had different inhibitory profiles, despite the fact that the amount of *blpMNPO*
495 transcripts was the same. These differences were attributed to different expression levels of the
496 HtrA protease. In particular, in opaque variants expression of HtrA was higher resulting in
497 degradation of BlpC and hence in decreased bacteriocin secretion (42). Still, this link between
498 opacity variants and HtrA expression was shown for a single 6A strain only and it is unknown

499 whether this is a general property of pneumococcal strains. Third, the cross-sectional nature of
500 our study prevented us from measuring the effect of *blp*-mediated competition on the duration
501 of carriage. Also, we did not measure the pneumococcal load in the samples and it is possible
502 that the effects of competition might be reflected at the level of carriage density. Even so, we
503 measured the relative proportion of each strain in the samples and could not establish a
504 correlation between outcomes of bacteriocin secretion and strain density (Figure S1).
505
506 Our study has also significant strengths. We have used a very well characterized, natural
507 collection of co- and singly colonized samples identified through the use of highly sensitive
508 molecular methods. This enabled us to obtain an unbiased and highly diverse collection of both
509 single and co-colonizing pneumococcal strains to measure the impact of *blp*-bacteriocin
510 production on competition in the nasopharynx.
511
512 Our results demonstrate the importance of using human samples to support conclusions drawn
513 using idealized animal models and are likely to have implications for other bacteriocin systems
514 that have been exclusively studied in animal models. For example, similar to the case with the
515 *blp* bacteriocins, studies performed in animal models have shown that bacteriocin secretion is
516 important in *Streptococcus mutans* competition in early dental biofilms (54) and in the ability of
517 *Enterococcus faecalis* to colonize of the mammalian gut (55). In light of our study, it would be of
518 interest to determine if the expected correlations hold true when natural samples from the
519 human host are studied.

520

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

530

531 **AUTHOR CONTRIBUTIONS**

532 The study was designed by RSL, CV and SD. Data acquisition, analysis and interpretation were
533 performed by CV, SD, FP, JH, ASS, KG, LAM, and RSL. RSL, SD, JH and HML contributed reagents
534 or materials. The manuscript was drafted by CV and RSL and critically revised by all authors. All
535 authors read and approved the final version of the manuscript.

536

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FIGURE LEGENDS

Figure 1. Strategy used for identification of co-colonized samples and isolation of co-colonizing pneumococcal strains.

Figure 2. Genetic diversity and characteristics of the blp locus of co-colonizing pneumococci. Interpretation of results was performed using the goeBURST algorithm to determine possible evolutionary relationships between isolates: strains sharing 5 out of 7 alleles were considered genetically related. Clonal complexes (CC) were assigned considering only the collection of co-colonizing 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 ST are grouped in clonal complexes (CC, Panel A) and unrelated ST are represented as singletons (S, Panel B). For each ST information on CSP type and genetic characterization of *blp* locus is represented by the bars; the first section represents CSP type (CSP1 in black, CSP2 in light gray, other type in dark gray); the second section represents *blpC* type (dark green for $blpC_{P164}$, light green for $blpC_{R6}$, dark blue for $blpC_{6A}$, light blue for $blpC_{T4,}$ and pink for $blpC_{P155}$); the third section represents presence (red cross) or absence (white) of disruption in *blpA*; the fourth section represents the BIR profile, numbered 1 to 39. BIR RFLP profiles matched to genome sequenced strains (GenBank designations): 1 (GA13723), 2 (AP200), 3 (GA13856), 4 (GA54354), 5 (20703335), 7 (GA47439), 9 (670-6B), 10 (OXO141), 11 (GA17971), 12 (CDC1087), 14 (46518), 18 (SP14-BS69), 19 (GA47502), 20 (70585),

722 21 (8a-SA64), and 22 (5a-14-3). ND, BIR profiles not determined due to failure to amplify the BIR
723 region.

724

725 **Figure 3. Distribution of *blpC* types in the co-colonization strains.** Asterisks indicate statistically
726 significant differences. Black, active secretion; dark gray, no secretion, *cheaters*; white, no
727 secretion, putative non *cheaters*; light gray, not tested for active secretion, putative non
728 *cheaters*. Error bars represent standard error.

729

730 **Figure 4. Characterization of the *blp* locus of the strains isolated from single carriage events.**
731 (A) Distribution of *blpC* types in the co-colonization strains. Asterisk indicates statistically
732 significant differences. Black, active secretion; dark gray, no secretion, *cheaters*; white, no
733 secretion, putative non *cheaters*; light gray, not tested for active secretion, putative non
734 *cheaters*. Error bars represent standard error. (B) Comparison of the single and co-colonized
735 collections regarding proportions of strains with inhibitory phenotype, cheater genotype and
736 locus inducibility. Dark gray, co-colonization strains; white, single carriage strains. Error bars
737 represent standard error.

738

739
740
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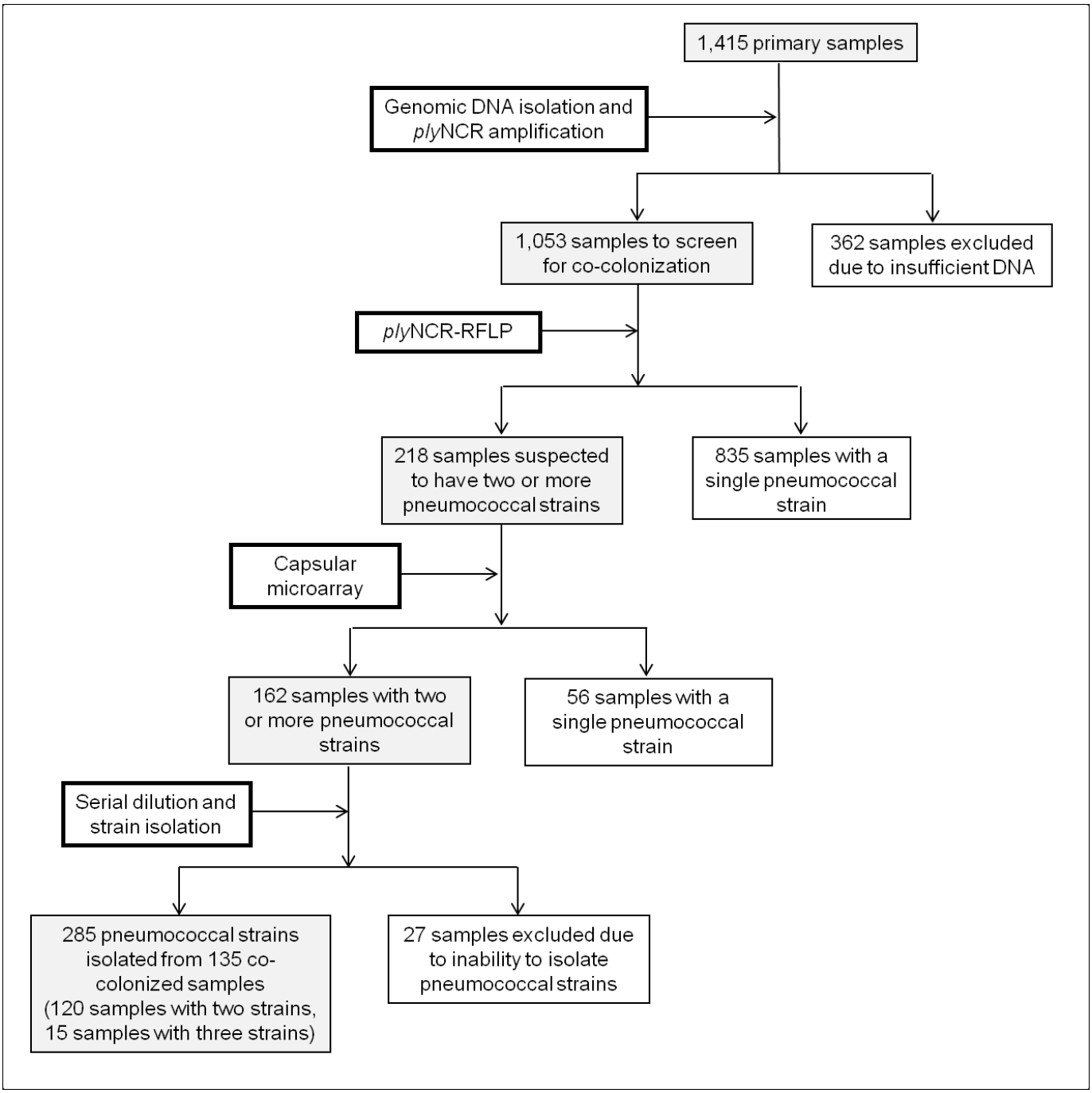

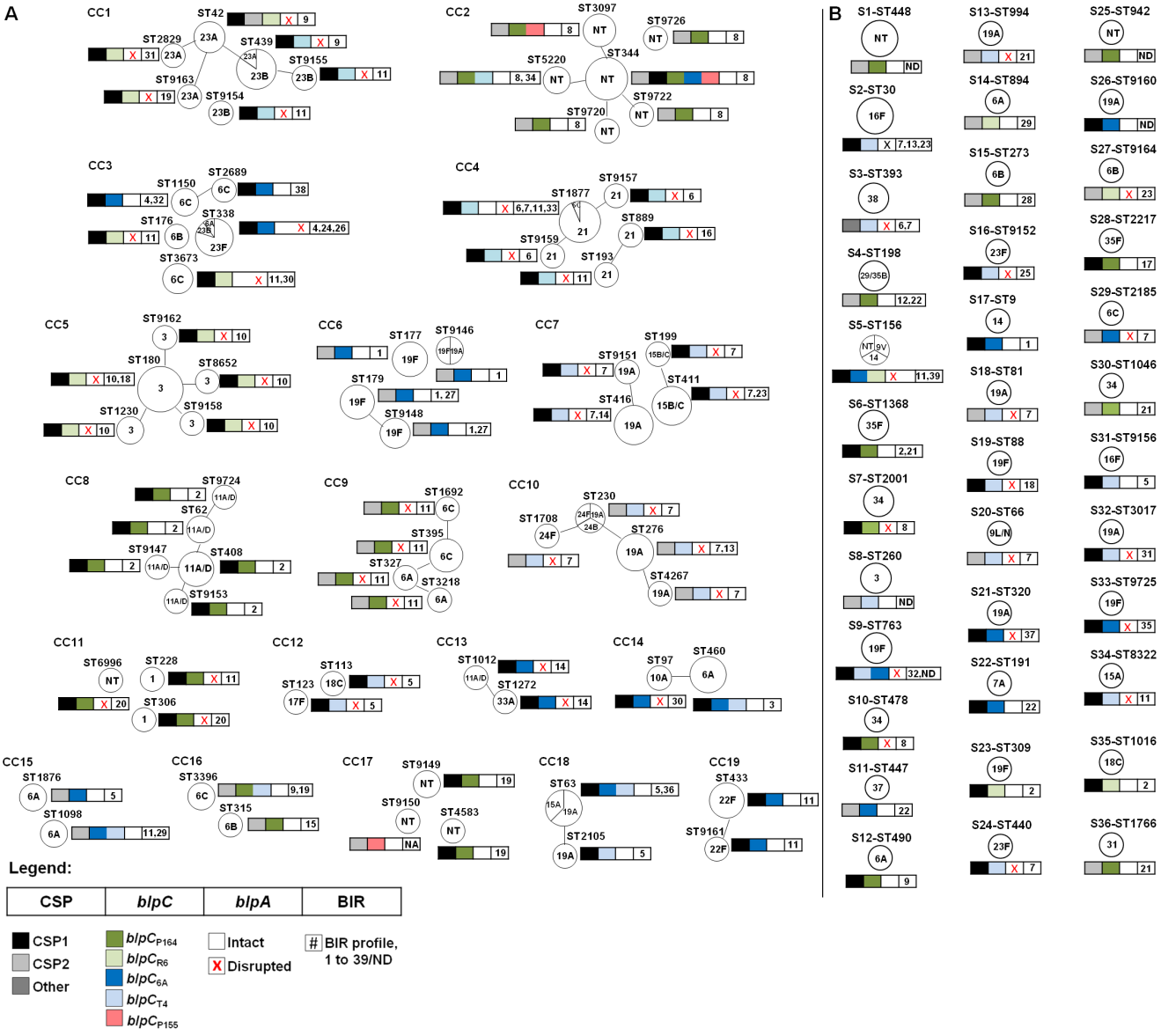

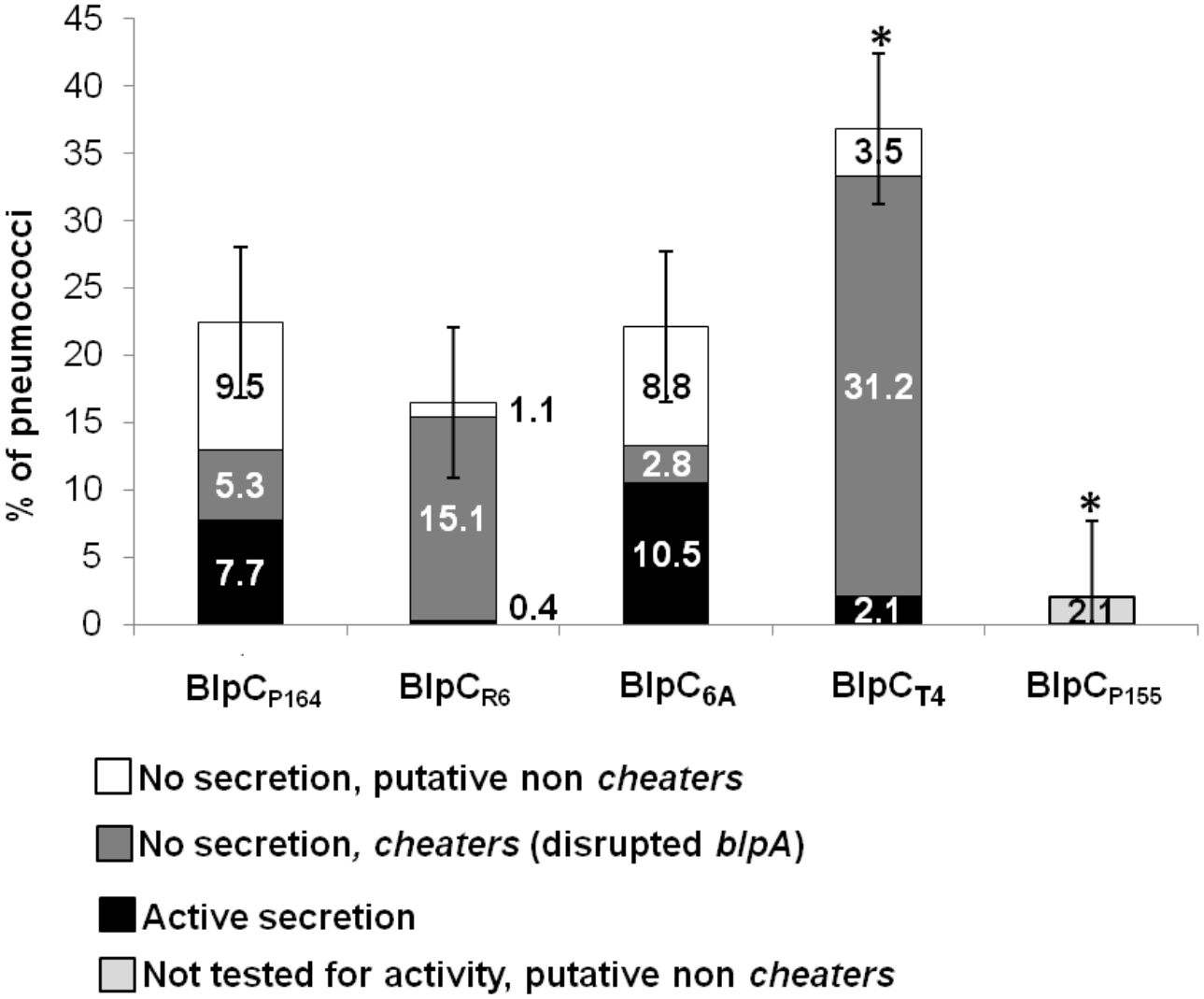

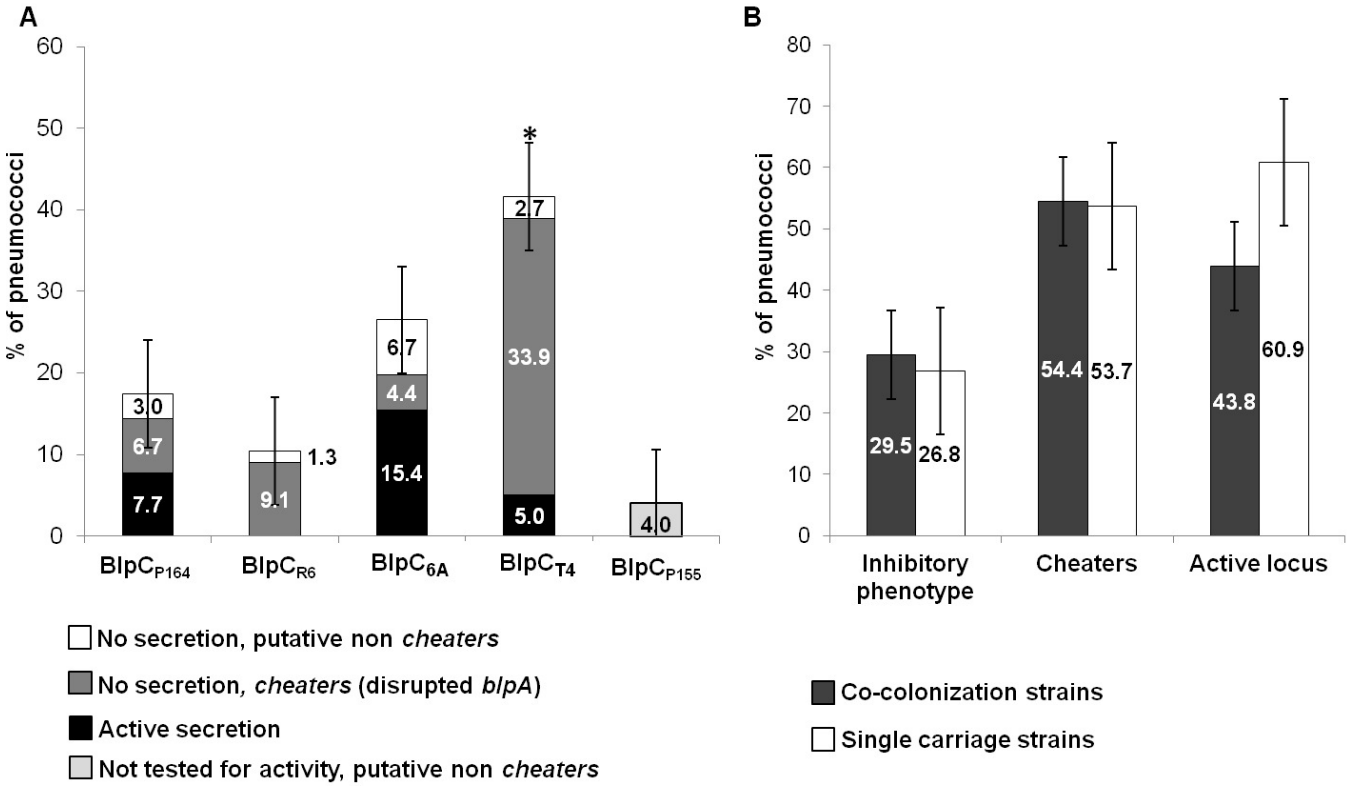

Table 1. Reporter and control strains for characterization of the *blp* locus

Strain designation	Strain characteristics	Reference
P537	Serotype 6A strain with deletion of *blpRHCBA-BIR*. Susceptible to all bacteriocins secretion. Reporter of inhibitory activity	Son *et al*., 2011 (27)
PSD121	R6 background; insertion of a type P164 *blp* locus, with *blpC* deletion and *lac-Z* reporter under control of the BIR promoter. Type P164 BlpC/BlpH reporter	Kochan *et al.*, 2013 (42)
PSD101	R6 background; insertion of a type R6 *blp* locus, with *blpC* deletion and *lac-Z* reporter under 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 *lac-Z* reporter under 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 *lac-Z* reporter under control of the BIR promoter.	Pinchas *et al.*, 2015 (43)

	Type T4 BlpC/BlpH reporter	
P1	Serotype 6A, BIR with MNO, $BlpC_{6A}$. Control for MNO BIR content	Kim *et al*., 1998 (44)
P4	Serotype 6B, BIR with QMNO, $BlpC_{P164}$. Control for QMNO BIR content	Kim *et al*., 1998 (44)
P132	Serotype 29, BIR with K, type 3 $BlpC_{6A}$. 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)

Table 2. Evidence for putative *in vivo* horizontal gene transfer among co-colonizing strains in nasopharyngeal samples characterized in this study.

NP sample	Serotype	ST	aroE	gdh	gki	recP	spi	xpt	ddl
Sample 8043									
Strain 1	19A	**9160**	**1**	60	9	8	6	3	29
Strain 2	6C	395	**1**	5	7	12	17	1	14
Strain 3	31	1766	**1**	5	29	1	46	14	18
Closest ST to strain 1[a]	19A	1151	7	60	9	8	6	3	29
Sample 8058									
Strain 1	3	**9162**	**2**	15	2	10	6	1	22
Strain 2	23A	**9163**	**2**	8	9	9	6	4	6
Closest ST to strain 1[a]	3	180	7	15	2	10	6	1	22
Closest ST to strain 2[a]	23A/23F	190	8	8	9	9	6	4	6
Sample 8098									
Strain 1	6B	**9164**	**7**	8	4	18	15	4	31
Strain 2	3	180	**7**	15	2	27	2	11	71
Closest ST to strain 1[a]	6A/6B	5516	2	8	4	18	15	4	31

[a] Closest ST found in the MLST database (http://spneumoniae.mlst.net/). NP, nasopharyngeal; ST, sequence type. Novel STs and alleles shared with co-colonizing strain that generated novel STs 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 its co-colonizing strain, suggesting within host recombination.