



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Recombination in *Streptococcus pneumoniae* Lineages Increase with Carriage Duration and Size of the Polysaccharide Capsule

Citation for published version:

Chaguza, C, Andam, CP, Harris, SR, Cornick, JE, Yang, M, Bricio-Moreno, L, Kamng'ona, AW, Parkhill, J, French, N, Heyderman, RS, Kadioglu, A, Everett, DB, Bentley, SD & Hanage, WP 2016, 'Recombination in *Streptococcus pneumoniae* Lineages Increase with Carriage Duration and Size of the Polysaccharide Capsule' *mBio*, vol 7, no. 5. DOI: 10.1128/mBio.01053-16

Digital Object Identifier (DOI):

[10.1128/mBio.01053-16](https://doi.org/10.1128/mBio.01053-16)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

mBio

Publisher Rights Statement:

2016 Chaguza et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



Recombination in *Streptococcus pneumoniae* Lineages Increase with Carriage Duration and Size of the Polysaccharide Capsule

Chrispin Chaguza,^{a,b} Cheryl P. Andam,^{a,c} Simon R. Harris,^d Jennifer E. Cornick,^{a,b} Marie Yang,^a Laura Bricio-Moreno,^a Arox W. Kamng'ona,^{a,b,e} Julian Parkhill,^d Neil French,^{a,b} Robert S. Heyderman,^{b,f} Aras Kadioglu,^a Dean B. Everett,^{a,b} Stephen D. Bentley,^{a,d} William P. Hanage^c

Department of Clinical Infection, Microbiology and Immunology, Institute of Infection and Global Health, University of Liverpool, Liverpool, United Kingdom^a; Microbial Ecology, Malawi-Liverpool-Wellcome Trust Clinical Research Programme, University of Malawi, College of Medicine, Blantyre, Malawi^b; Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, Massachusetts, USA^c; Pathogen Genomics, Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, United Kingdom^d; Department of Biomedical Sciences, University of Malawi, College of Medicine, Blantyre, Malawi^e; Division of Infection and Immunity, University College London, London, United Kingdom^f

D.B.E., S.D.B., and W.P.H. contributed equally to this work.

ABSTRACT *Streptococcus pneumoniae* causes a high burden of invasive pneumococcal disease (IPD) globally, especially in children from resource-poor settings. Like many bacteria, the pneumococcus can import DNA from other strains or even species by transformation and homologous recombination, which has allowed the pneumococcus to evade clinical interventions such as antibiotics and pneumococcal conjugate vaccines (PCVs). Pneumococci are enclosed in a complex polysaccharide capsule that determines the serotype; the capsule varies in size and is associated with properties including carriage prevalence and virulence. We determined and quantified the association between capsule and recombination events using genomic data from a diverse collection of serotypes sampled in Malawi. We determined both the amount of variation introduced by recombination relative to mutation (the relative rate) and how many individual recombination events occur per isolate (the frequency). Using univariate analyses, we found an association between both recombination measures and multiple factors associated with the capsule, including duration and prevalence of carriage. Because many capsular factors are correlated, we used multivariate analysis to correct for collinearity. Capsule size and carriage duration remained positively associated with recombination, although with a reduced *P* value, and this effect may be mediated through some unassayed additional property associated with larger capsules. This work describes an important impact of serotype on recombination that has been previously overlooked. While the details of how this effect is achieved remain to be determined, it may have important consequences for the serotype-specific response to vaccines and other interventions.

IMPORTANCE The capsule determines >90 different pneumococcal serotypes, which vary in capsule size, virulence, duration, and prevalence of carriage. Current serotype-specific vaccines elicit anticapsule antibodies. Pneumococcus can take up exogenous DNA by transformation and insert it into its chromosome by homologous recombination. This mechanism has disseminated drug resistance and generated vaccine escape variants. It is hence crucial to pneumococcal evolutionary response to interventions, but there has been no systematic study quantifying whether serotypes vary in recombination and whether this is associated with serotype-specific properties such as capsule size or carriage duration. Larger capsules could physically inhibit DNA uptake, or given the longer carriage duration for larger capsules, this may promote recombination. We find that recombination varies among capsules and is associated with capsule size, carriage duration, and carriage prevalence and negatively associated with invasiveness. The consequence of this work is that serotypes with different capsules may respond differently to selective pressures like vaccines.

Received 10 June 2016 Accepted 2 September 2016 Published 27 September 2016

Citation Chaguza C, Andam CP, Harris SR, Cornick JE, Yang M, Bricio-Moreno L, Kamng'ona AW, Parkhill J, French N, Heyderman RS, Kadioglu A, Everett DB, Bentley SD, Hanage WP. 2016. Recombination in *Streptococcus pneumoniae* lineages increase with carriage duration and size of the polysaccharide capsule. *mBio* 7(5):e01053-16. doi:10.1128/mBio.01053-16.

Editor Paul Stephen Keim, Northern Arizona University

Copyright © 2016 Chaguza et al. This is an open-access article distributed under the terms of the [Creative Commons Attribution 4.0 International license](https://creativecommons.org/licenses/by/4.0/).

Address correspondence to Chrispin Chaguza, Chrispin.Chaguza@liverpool.ac.uk, or William P. Hanage, whanage@hsph.harvard.edu.

The global mortality due to invasive pneumococcal disease (IPD) has been estimated at more than 1 million per annum, with the majority of deaths occurring in children less than 5 years old (1). Use of a 7-valent pneumococcal conjugate vaccine (PCV7) and subsequent 13-valent (PCV13) formulations has been followed by a precipitate reduction in IPD due to vaccine

serotypes (VTs) in the United States, including a substantial herd effect in unvaccinated individuals (2, 3). PCVs are plainly an effective selective pressure on the pneumococcal population. The response to that pressure has been notable for the increase in prevalence of nonvaccine serotypes (NVTs). More than 90 distinct serotypes have been described (4–10), so even PCV13 protects

against a small minority of antigenic diversity in this organism. Following vaccination, the prevalence of NVTs in carriage, and to a lesser extent in IPD, has increased as the NVTs took advantage of the removal of their competitors in a process termed serotype replacement (11–13). An important component of serotype replacement has been the success of “capsule switch” vaccine escape variants (14). These variants are lineages that were previously associated with a vaccine serotype but have persisted in the postvaccine environment by the introduction of genes for an NVT capsule through homologous recombination (15). The known ability of the pneumococcus to undergo homologous recombination has also been associated with the acquisition of drug resistance (16, 17) and numerous examples of capsule switch variants (15, 18–20). As a result of this recombination-mediated shuffling of serotype and genotype, when PCVs were introduced, the population already contained potential vaccine escape variants, some of which have subsequently become common and important causes of disease.

The polysaccharide capsule is known to influence multiple aspects of pneumococcal biology. Serotypes vary in their nasopharyngeal carriage rates (21), and their propensity to cause IPD was usually expressed as the “invasive potential” in order to account for the various exposures to different serotypes (22–24). Moreover, these properties have been linked to basic biochemical features of the capsule: those capsules with fewer carbons in the repeat unit of the polysaccharide tend to be thicker and exhibit a more negative surface charge (25) and are associated with a longer duration of carriage and a higher carriage prevalence—an outcome that may be linked to enhanced resistance to opsonophagocytic killing (21). Those serotypes more associated with virulence meanwhile, tend to have smaller capsules and shorter durations of carriage (21, 22, 24).

Despite the importance of both recombination and capsule to pneumococcal biology, whether serotypes vary in their recombination rates in nature and what drives it have not been extensively studied. Genomic studies have shown that different pneumococcal lineages plainly vary in the amount that recombination has contributed to their diversification (20, 26) but have not linked this to the different serotypes associated with each lineage. These studies have also suggested that lineages that completely lack capsule may have a higher relative rate of recombination, suggesting that the capsule can impede the uptake of DNA (26). Support for this comes from the observation that mechanisms such as biofilm formation and downregulation of capsule biosynthesis have been reported to facilitate DNA uptake and chromosomal integration *in vitro* (27). This finding suggested that the polysaccharide capsule may inhibit genetic exchange in encapsulated isolates either physically or structurally (28).

There are hence potentially antagonistic roles for serotype in recombination. A larger capsule is associated with a higher prevalence and longer duration of carriage, therefore offering more opportunities to undergo recombination, but conversely, experimental and observational data suggest that the absence of capsule increases transformation and recombination (26, 27). As yet, there has been no attempt to quantify the association between serotype properties, such as capsule size and carriage duration, and the amount of recombination that those serotypes experience. Understanding this is important because as noted, recombination is crucial to the response of pneumococci to medical interventions (19), and if serotypes vary in recombination in a predictable fashion,

we might be able to predict those that are a higher risk of developing drug resistance or vaccine escape.

In the present study, we used whole genomes of 439 pneumococcal isolates collected in Malawi, a low-income country in sub-Saharan Africa with high pneumococcal carriage rates (29) and which contains a wide range of serotypes, including serotypes with very low prevalence or that are absent in industrialized countries such as serotype 1 and 5 (18). We quantified the extent to which recombination has contributed to the history of serotype-specific lineages and combined this with data on carriage duration, invasive potential, carriage prevalence, and capsule size to quantitatively investigate the association. Our results demonstrate significant associations between the recombination rate and frequency with the carriage duration, invasive potential, and size of the outer cell wall polysaccharide capsule in pneumococcal isolates.

RESULTS

We sequenced the genomes of 439 *Streptococcus pneumoniae* clinical isolates collected from the Queen Elizabeth Central Hospital (QECH) in Blantyre, Malawi (southeastern Africa), between 2002 and 2010. The set of samples comprised 364 invasive isolates and 75 carriage isolates and representatives of 48 distinct serotypes. The most common serotypes were serotype 1 with 83 isolates (52 isolates from blood, 28 isolates from cerebral spinal fluid [CSF], and 3 isolates from carriers), serogroup 6 with 46 isolates (25 isolates from blood, 11 isolates from CSF, and 10 isolates from carriers), serotype 5 with 25 isolates (17 isolates from blood, 6 isolates from CSF, and 2 isolates from carriers), and other serotypes (285 isolates; 126 isolates from blood, 99 isolates from CSF, and 60 isolates from carriers) (see Table S1 in the supplemental material). Whole-genome sequencing was done using the HiSeq platform (Illumina, CA, USA) as described in Materials and Methods. The raw sequence reads had an average length of 72.23 bp (95% confidence interval [95% CI], 70.58 to 73.87 bp), average read quality of 33.48 (95% CI, 33.22 to 33.75), and average insert size of 263.30 kb (95% CI, 263.3 to 270.10 kb) per genome (Fig. S1). Consensus *de novo* sequence assemblies were generated (30, 31) with an average of 66.43 contigs (95% CI, 63.65 to 69.22) and an average chromosome size of 2.09 Mb (95% CI, 2.09 to 2.10 Mb) and average contig size of 37.31 kb (95% CI, 35.91 to 38.72 kb) per genome. The phylogeny of all the isolates was constructed using a 0.79-Mb multiple-sequence alignment of 852 concatenated core genes containing 51,389 single nucleotide polymorphisms (SNPs) (32). For the analysis of serotype-specific evolution of lineages identified as described below, SNPs were called against reference genomes (Table S2).

Population structure. Analysis of the population using BAPS (33, 34) identified 14 primary sequence clusters (SCs) (Fig. 1). Of these 14 SCs, SC1 to SC13 were monophyletic clades. The remaining SC, SC14, contained sequences that could not be assigned to a monophyletic SC, and the streptococci in this polyphyletic SC are a diverse group of rare lineages, the grouping of which does not necessarily reflect common evolutionary history, and hence, we undertake no further analysis of this group. Most SCs contained isolates with a single serotype and multiple closely related sequence types (STs) as determined by multilocus sequence typing (MLST) (Fig. 1; see Table S1 in the supplemental material). In some cases, SCs contained multiple serotypes separated by long branches. A notable example is SC9, which consisted of clearly distinct subclades of serotype 6A and 35B (Fig. 1), which we split

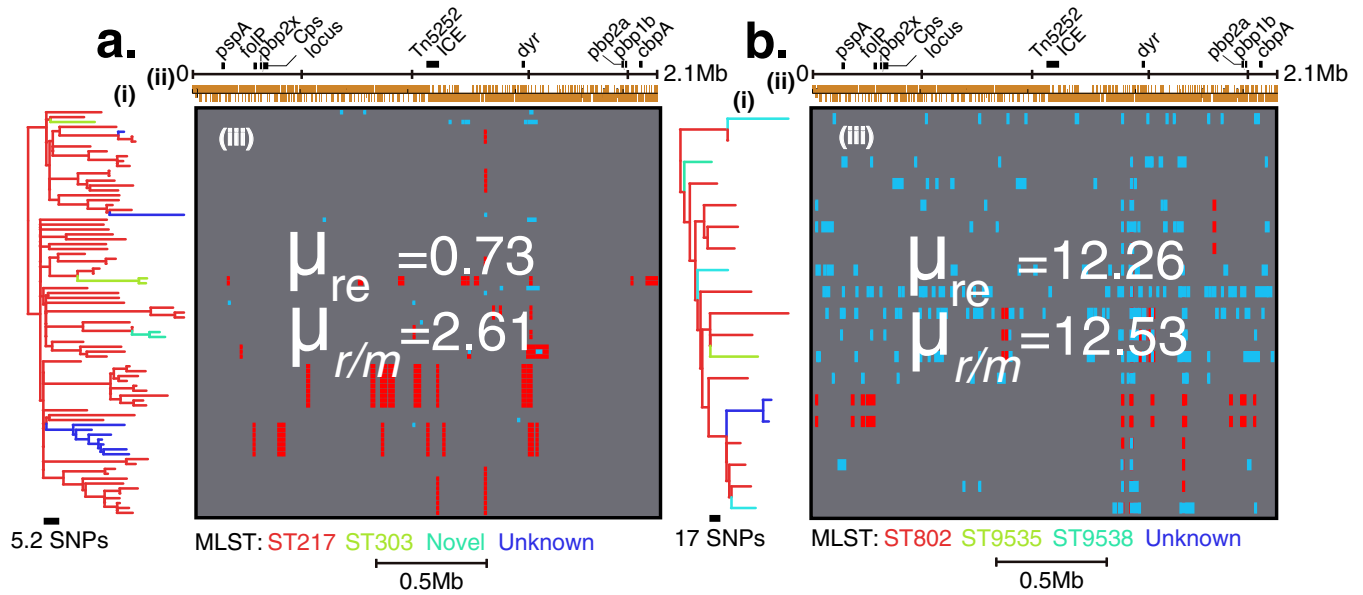


FIG 2 Recombination inferred by Gubbins. Recombination events mapped onto internal nodes (red blocks) and terminal branches (blue blocks) are illustrated for a typical low-recombination serotype (serotype 1 [SC1]) (a) and a high-recombination serotype (serotype 6A [SC3]) (b). (i) Maximum likelihood phylogenetic trees of the different serotypes. Branches and tips of the phylogeny are colored according to the MLST sequence type (ST). (ii) Schematic representation of the reference *S. pneumoniae* genome showing all the genetic annotations and locations of some well-known genes. (iii) Matrix showing the tracks representing each genome. The locations and distribution of regions, which have acquired exogenous DNA through recombination, are colored depending on the number of strains that contain them. Recombination events in internal branches (red) were present in multiple isolates and were shared through clonal descent rather than independent acquisitions, while those in the terminal branches (blue) were isolate specific and represent independent recent acquisitions. The recombination rate ($\mu_{r/m}$), i.e., mean number of the inferred distinct recombination events per isolate (each shared ancestral recombination event that occurred once and spread in the clone via clonal descent was counted once). The recombination frequency (μ_{re}), i.e., the mean number of SNPs introduced through recombination to those introduced through mutation, is shown. A high presence of recent rather than shared recombination events implies high μ_{re} .

which identifies regions with an atypically high density of SNPs using a sliding window approach (Fig. 2a and b; see Fig. S3a to S3o in the supplemental material). We distinguish between two separate recombination parameters. The relative recombination rate ($\mu_{r/m}$) is the ratio of the numbers of SNPs introduced by recombination to the number introduced by mutation. It hence measures the total amount of diversity accumulated by a lineage. In contrast, the recombination frequency (μ_{re}) is defined as the average number of recombination events inferred per isolate, regardless of how much variation they have introduced. Hence, a large amount of recombination with closely related strains could lead to a high μ_{re} but a low $\mu_{r/m}$. Furthermore, higher presence of shared ancestral recombination events acquired through clonal descent than strain-specific events introduced independently may lead to a high μ_{re} . The averages of both these parameters were calculated for each SC. While SC1 and SC2 both contained isolates with a single serotype (serotype 1 and 5, respectively), in the case of SCs containing more than one serotype, the statistics for each were calculated independently (Table 1). Separate estimates were obtained for the two distinct 6A clades in SC3 and SC9, which we refer to as 6A-SC3 and 6A-SC9, respectively, on the basis of the predominant STs in each.

Estimates of $\mu_{r/m}$ ranged from 0.44 to 23.45 with a mean of 8.89 (95% CI, 5.243 to 12.43) (Table 1), and the only estimate less than 1 was found in the 16F lineage SC5. The highest values of $\mu_{r/m}$ were 12.53 for serotypes 23F-SC8, 23.45 for 6A-SC3, 12.85 for 6A-SC9, and 17.02 for 35B-SC9, while serotypes 1-SC2 ($\mu_{r/m} = 2.61$), 16F-SC5 ($\mu_{r/m} = 0.44$), 4-SC13 ($\mu_{r/m} = 1.67$), and 5-SC1 ($\mu_{r/m} = 2.82$) showed the lowest values (Table 1). The distribution of $\mu_{r/m}$ values

deviated from a normal distribution ($P < 0.0001$) on the basis of three normality tests (see Materials and Methods). In serotypes of the same SC, such as 35B, 6A, and 18B/C in SC4, SC9, and SC13, respectively, the distribution of r/m ratios per branch were not different with P values of 0.7431 and 0.9350, respectively. The average estimated μ_{re} was 6.316 (95% CI, 3.316 to 9.316) and ranged from 0.11 to 20.08. The highest μ_{re} estimates were 12.26 for 23F-SC8, 20.08 for 35B-SC9, 8.91 for 6A-SC3, and 11.92 for 6A-SC9, while serotypes 1-SC2 ($\mu_{re} = 0.73$), 16F-SC5 ($\mu_{re} = 0.11$), 4-SC13 ($\mu_{re} = 0.75$), and 5-SC1 ($\mu_{re} = 2$) showed the lowest μ_{re} values (Table 1). Significant differences ($P = 0.0005$) in the average numbers of recombination events in each serotype were also observed by using the same test (see Fig. S2b in the supplemental material). The Kruskal-Wallis test was used because of significant deviations of the distributions of both $\mu_{r/m}$ per branch in each serotype and the average number of recombination events for each serotype from the normal distribution. Interestingly, both distinct lineages with 6A capsule type consistently showed high $\mu_{r/m}$ (23.45 in SC3 and 12.85 in SC9) and μ_{re} (8.91 in SC3 and 11.92 in SC9) despite having a dissimilar genetic backbone. In addition, $\mu_{r/m}$ positively correlated with μ_{re} ($R^2 = 0.5168$; $P = 0.0025$) (Fig. 3). The pneumococcal phenotypes or competence-stimulating peptides (CSP) encoded by the *comC* gene were diverse and variably distributed in the SCs but showed no obvious association with the recombination parameters (Table 1).

Serotype-specific carriage duration, prevalence, and invasive potential correlate with recombination. Different pneumococcal lineages vary in the extent of recombination that has influenced their genomes (20, 26). Serotypes are known to vary in their du-

TABLE 1 Summary statistics for the genetic recombination events identified in each serotype

Serotype	Sequence cluster (SC)	No. of isolates (n)	Mean no. of recombination events/isolate (μ_{re})	Mean recombination to mutation ($\mu_{r/m}$)		Recombination size (bp)		Phenotype ^a		
				Mean	95% CI ^b	Mean	95% CI	CSP1	CSP2	Other
5	SC1	25	2	2.82	0.29–5.35	7,642	3,877–11,407	100	0	0
1	SC2	83	0.73	2.61	0.74–4.48	8,727	5,753–11,702	98.78	0	1.22
6A	SC3	11	8.91	23.45	1.54–45.44	11,579	8,872–14,286	0	100	0
19A	SC4	12	5.58	11.26	4.64–17.89	9,143	6,308–11,979	0	0	100
16F	SC5	9	0.11	0.44	0–1.39	36,616	0	0	0	100
3	SC6	10	8.1	9.45	0–23.53	8,020	6,201–9,839	0	0	100
10B	SC7	14	2.07	4.15	0–8.70	9,855	4,805–14,905	76.47	17.65	5.88
23F	SC8	19	12.26	12.53	7.33–17.72	8,094	6,737–9,451	94.74	0	5.26
35B	SC9	13	20.08	17.02	5.31–28.74	9,022	7,987–10,056	100	0	0
6A	SC9	12	11.92	12.85	4.55–21.15	7,115	5,697–8,532	100	0	0
12F	SC10	19	5.16	8.17	2.38–13.97	7,709	6,016–9,401	94.74	0	5.26
7A/F	SC11	20	6.4	8.74	3.09–14.38	6,924	5,547–8,302	95	0	5
14	SC12	15	4	13.93	4.70–23.16	6,745	4,926–8,563	100	0	0
18B/C	SC13	8	6.67	3.49	0–11.15	9,469	6,215–12,724	83.78	16.22	0
4	SC13	6	0.75	1.67	0–3.67	9,208	0–20,175	38.75	32.56	28.29

^a Proportion of the pneumococcal phenotypes or competence-stimulating peptide (CSP) encoded by the *comC* gene and its variants in each SC. Detailed information about the CSPs is shown in Fig. S2c and S2d in the supplemental material.

^b 95% CI, 95% confidence interval.

ration of carriage and propensity to cause invasive disease per exposure. A longer duration of carriage could offer more opportunities for cocolonization with different strains and possible recombination. We hence tested the association for each serotype between $\mu_{r/m}$ and μ_{re} and previously published serotype-specific estimates of carriage duration (36), carriage prevalence data from this study, capsule size (21), and invasive potential (defined as the odds ratio for causing invasive disease to carriage [IC_{OR}] [22, 37]) using univariate linear regression. We observed a positive association between both $\mu_{r/m}$ ($R^2 = 0.4664$; $P = 0.0071$) and μ_{re} ($R^2 = 0.3393$; $P = 0.0367$) with carriage duration (Fig. 4a and Table 2; see Fig. S4a in the supplemental material), but only $\mu_{r/m}$ showed a

significant association ($R^2 = 0.4173$; $P = 0.0093$) with carriage prevalence of the serotypes (Table 2; see Fig. S6a and S6b in the supplemental material). When modeling the relationship between carriage duration and μ_{re} , serotype 35B-SC9 was excluded as an outlier, but its inclusion decreased the P value ($R^2 = 0.2406$; $P = 0.0749$). Both $\mu_{r/m}$ ($R^2 = 0.4549$; $P = 0.0228$) and μ_{re} ($R^2 = 0.7155$; $P = 0.0010$) showed negative association with the invasive potential of the studied serotypes (Fig. 4b and Table 2; Fig. S4b and S4c). In multivariable regression, the association of carriage duration, prevalence, and invasive potential disappeared (Table 2). Repeated univariate and multivariate analyses using only those serotypes for which all response variables were available found similar results (Table S3). A multivariate regression to account for this removed the significant association with serotype invasive potential, while the association with carriage duration and capsule size remained (Table 2).

Recombination correlates with larger polysaccharide capsules. Capsule size has been associated with the factors described above—the serotype-specific duration of carriage and prevalence. The polysaccharide capsule size data for different pneumococcal serotypes, determined by the fluorescein isothiocyanate-labeled dextran (FITC-dextran) assay, were obtained from Weinberger and colleagues (21). By using univariate regression, we observed positive association between the capsule size and μ_{re} ($R^2 = 0.5892$; $P = 0.0095$), but not $\mu_{r/m}$ ($R^2 = 0.1186$; $P = 0.300$) (Fig. 5; Table 2). Excluding serotype 35-SC9, which was deemed to be an outlier observation, the linear association between capsule size and $\mu_{r/m}$ increased ($R^2 = 0.3519$; $P = 0.0544$). This was robust to multivariate analysis ($P = 0.0296$). We also did a multivariable regression to account for collinearity among capsule size, duration of carriage, carriage prevalence, and invasive potential and identify the independent contribution of each, which showed no association between $\mu_{r/m}$ and μ_{re} (Table 2; see Table S3 in the supplemental material).

DISCUSSION

Recombination is a fundamental process in the evolution and adaptation of many pathogens. It is capable of shuffling existing

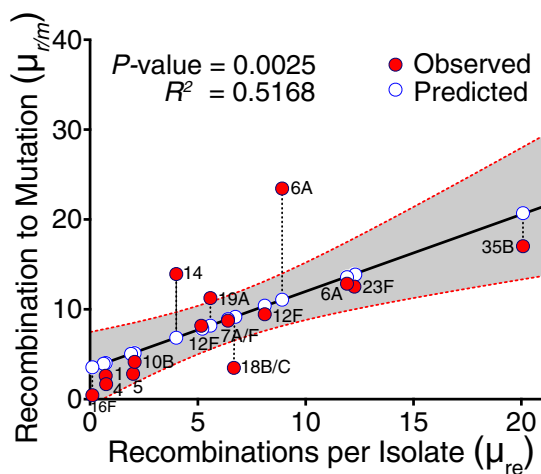


FIG 3 Relationship between rate and frequency of genetic recombination estimates in different *S. pneumoniae* serotypes. The solid red circles show the observed values, while the solid white circles show the estimated values by the univariate linear regression model. The dashed lines connecting the red circles to the white circles represent the residual, the difference between the observed and predicted values by the model. The gray band surrounding the regression line shows the 95% confidence interval (95% CI) for prediction of each data point in the horizontal axis. The two serotype 6A symbols in the figure originated from distinct clades, SC3 and SC9, as shown in Fig. 1 and Table 1.

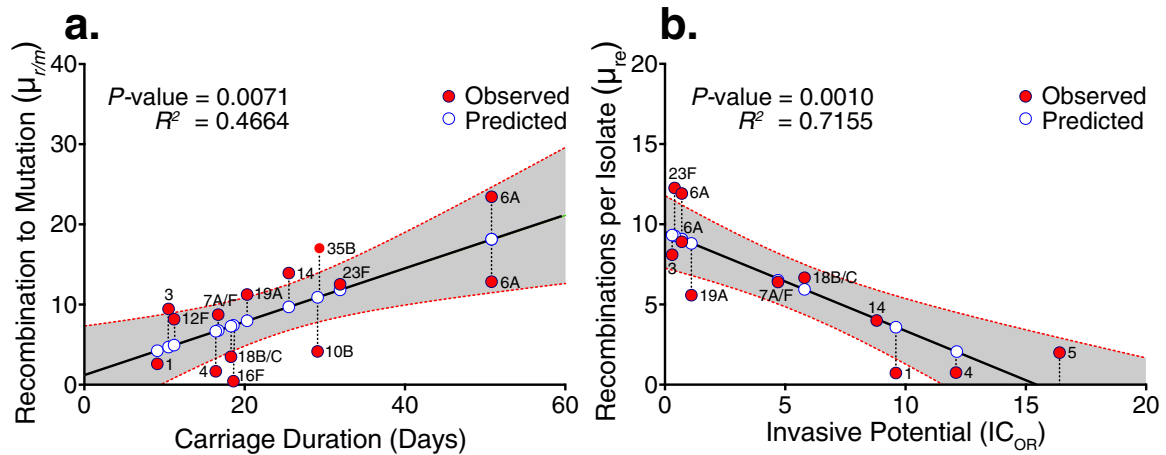


FIG 4 Statistical relationships between recombination rate ($\mu_{r/m}$) and frequency (μ_{re}) with carriage duration and serotype invasive potential (IC_{OR}). Univariate regression between $\mu_{r/m}$ and carriage duration (a) and μ_{re} and IC_{OR} (invasive to carriage odds ratio) (b). The solid red circles show the observed values, while the solid white circles show the estimated values by the univariate linear regression model. The dashed lines connecting the red circles to the white circles represent the residual, the difference between the observed and predicted values by the model. The gray band surrounding the regression line shows the 95% confidence interval (CI) for prediction of each data point in the horizontal axis. The two serotype 6A symbols in the figure originated from distinct clades, SC3 and SC9, as shown in Fig. 1 and Table 1.

variation into more fit combinations, and in the case of pneumococcus, it has been an important force in its response to medical interventions like vaccines and antibiotics (19). Despite the obvious benefits to the pathogen that recombination offers in this regard, the rates with which it occurs vary greatly among pneumococcal lineages and serotypes (26) for reasons that are obscure. Understanding the factors that contribute to this variation is important as we attempt to limit the potential of pneumococcal evolution to erode the benefits of clinical innovation.

Previous studies have commented on the low presence of recombination in certain serotypes, such as the “highly invasive” serotypes 1 and 7F (20, 38, 39). A systematic comparison of capsular types is challenging because some serotypes are comparatively rare or absent from samples taken in industrialized countries, and while it might be possible to combine studies from different sites, it would be important to account for any bias arising from variation in recombination in different regions—for instance, previous work with MLST data found more evidence of recombination in samples from African carriers and speculated that this might be the consequence of a higher carriage rate (40).

We have used a set of pneumococcal samples that includes an unusually wide range of serotypes with diverse properties, and we found a clear association between serotype and $\mu_{r/m}$ and μ_{re} . Because of lack of data in resource-poor settings, we used previously published data on the properties of serotypes, such as capsule size, carriage duration, prevalence, and invasive capacity (all of which vary relatively little among sample sites); we found that all were associated with one recombination parameter or the other recombination parameter or both. In univariate analysis, a negative association with invasive capacity mirrored a positive association of $\mu_{r/m}$ with carriage duration and prevalence—likely reflecting the fact that the majority of highly invasive serotypes are also infrequently carried. However, it should be noted that we did not have specific invasiveness data from pneumococci infecting the Malawi population, and there are few estimates for this quantity from resource-poor settings, so this is an important focus for future work.

We have also found more frequent recombination in serotypes with larger outer cell wall polysaccharide capsules, which shows that despite the potential physical barrier offered by the capsule,

TABLE 2 Summarized estimates from the univariate, multivariable, and multivariate multiple regression

Response variable	Predictor variable	Univariate			Multivariable			Multivariate		
		Estimate	SE	<i>P</i> value ^a	Estimate	SE	<i>P</i> value	Estimate ^b	Approx. <i>F</i>	<i>P</i> value ^a
Recombination rate ($\mu_{r/m}$)	Capsule size	0.0205	0.0186	0.3000	-0.0185	0.0242	0.487	0.9044	14.183	0.0296*
	Carriage duration	0.3336	0.1030	0.0071*	0.2149	0.3301	0.550	0.9085	14.884	0.0277*
	Invasive potential	-0.7930	0.2894	0.0228*	-0.8847	0.7927	0.327	0.4032	1.0134	0.4611
	Carriage prevalence	1.0030	0.3287	0.0093*	0.1081	0.9468	0.915	0.5457	1.8021	0.3062
Recombination frequency (μ_{re})	Capsule size	0.0263	0.0078	0.0095*	0.0057	0.0081	0.516	0.9044	14.183	0.0296*
	Carriage duration	0.1707	0.0718	0.0367*	0.2595	0.1101	0.078	0.9085	14.884	0.0277*
	Invasive potential	-0.6142	0.1291	0.0010*	-0.4127	0.2645	0.194	0.4032	1.0134	0.4612
	Carriage prevalence	0.5443	0.3260	0.1189	-0.4967	0.3159	0.191	0.5457	1.8021	0.3062

^a The asterisks in the *P* value columns indicate significant values.

^b All test statistics, i.e., Pillai-Bartlett trace criterion, Wilks' lambda, Hotelling-Lawley trace, and Roy's greatest root, were used to summarize the results from the multivariate analysis of variance (MANOVA) of the multivariate multiple regression model. All the tests showed identical *P* values. For illustration, only estimates using the Pillai-Bartlett trace are shown.

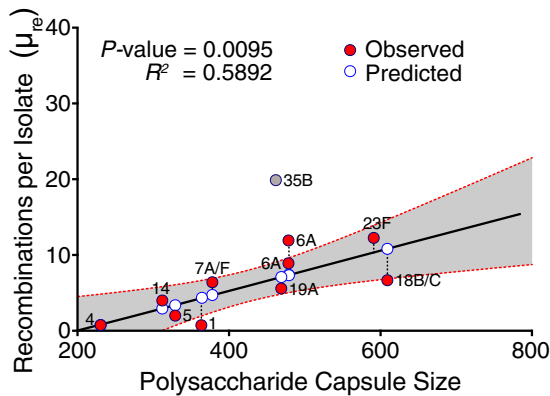


FIG 5 Relationship between frequency of recombination (μ_{re}) and polysaccharide capsule size in pneumococcal isolates. Serotype 35-SC9 was excluded from the regression, as it was deemed an outlier observation. The solid red circles show the observed values, while the solid white circles show the estimated values by the univariate linear regression model. The dashed lines connecting the red circles to the white circles represent the residual, the difference between the observed and predicted values by the model. The gray band surrounding the regression line shows the 95% confidence interval (CI) for prediction of each data point in the horizontal axis. The two serotype 6A symbols in the figure originated from distinct clades, SC3 and SC9, as shown in Fig. 1 and Table 1.

the presence of a larger capsule itself does not simply reduce recombination. While this could be considered surprising, it is important to note that serotypes with enlarged capsules are typically carried for longer duration and resist neutrophil phagocytosis (21) and complement-mediated immune responses (41, 42) than serotypes with smaller capsules. In addition, this may be because some of the serotypes with larger capsules have lower metabolic costs (21) associated with capsule expression and may have a higher ability to form biofilms which may prevent elimination (27). This suggests that longer duration of carriage and lower clearance rates by the host's immune system in serotypes with enlarged capsules leads to sustained exposure to cocolonizers (potential donors of exogenous DNA), which has been previously described to facilitate recombination (40). This in turn results in higher recombination than in serotypes with thinner capsules, which are presented with limited opportunities for recombination due to rapid clearance. The intrinsic recombination rates *in vitro* in the absence of capsule-induced immune clearance may be different than observed in the natural population where various factors such as strain competition and immune responses can directly affect the recombination rate. Hence, the results of *in vitro* studies may be misleading when applied to the nasopharynx. In addition, there was no obvious association between the pneumococcal phenotypes such as CSP1, CSP2, and other variants encoded by the *comC* gene and its allelic variants, which suggests that the observed differences in recombination may not be due to the differences in competence. The evidence presented here certainly strongly suggests that capsule does not prevent recombination in any straightforward fashion. However, while capsule size was associated with a higher frequency of recombination (μ_{re}), the association with $\mu_{r/m}$ was not significant in the univariate regression. Nevertheless, the effect of capsule size and carriage duration in a multivariate analysis accounts for the high correlation between the two recombination parameters, i.e., $\mu_{r/m}$ and μ_{re} , indicating that these two factors are key contributors to the observed

variation. The capsule data used in this study were obtained *in vitro*, and the expressed capsule sizes during carriage and transmission remain unclear and should be investigated in follow-up studies.

The properties of the capsule that we have studied here are also highly correlated (Pearson correlation coefficients are shown in Fig. S7 in the supplemental material), and accounting for this removed the association (Table 2). As a result, we have not been able to precisely define the underlying causes. A plausible mechanism is that serotypes with larger capsules are more exposed to other cocolonizing strains because they are carried for a longer period and at a higher prevalence. One way to test this would be to use serotype switches as natural experiments and compare $\mu_{r/m}$ and μ_{re} between switched lineages where the two serotypes involved vary in their size. However, this is limited by the fact that serotype switches identified in Fig. 1 and other studies typically involve serotypes that are effective colonizers such as serogroups 6, 15, 18, 19, and 23 (15). While this means we cannot use the present data set to do the above analysis, we should note that this observation is consistent with the suggestion that more commonly carried types are more likely to undergo recombination with each other.

In conclusion, we have shown that in a set of samples from Malawi containing many different serotypes, there is a linear relationship between μ_{re} and $\mu_{r/m}$ and properties of the capsule. In general, there is evidence that recombination increases with carriage duration and polysaccharide capsule size. We have not been able to pick out the causal roots of this relationship due to the complexity of the relationships between other important properties also associated with capsule, but the observation shows conclusively that all serotypes are not alike and that serotypes differ in how recombination has affected their evolution history, which has been associated with many different responses from drug resistance to vaccine escape. The serotypes observed in settings with a high burden of carriage and disease, such as sub-Saharan Africa, are often different from those found in industrialized nations, and there has been concern that lineages with serotypes targeted by vaccine such as serotypes 1, 5, and 7F that are relatively invasive, with a short duration of carriage and small capsules, might survive a serotype switch (21, 22, 36). The results of this study suggest that such concerns may be misplaced.

MATERIALS AND METHODS

Isolate collection and genome sequencing. We collected 439 *S. pneumoniae* isolates from the Malawi-Liverpool-Wellcome Trust Clinical Research Programme (MLW) pneumococcal isolate archive in Blantyre, Malawi. The isolates were routinely collected from patients at the Queen Elizabeth Central Hospital, a 1,250-bed referral hospital and the largest hospital located in the Blantyre District in southern Malawi from 2002 to 2011. All isolates were collected before the introduction of the 13-valent pneumococcal conjugate vaccine (PCV13) in routine childhood immunization programs in Malawi in 2011. Isolates were collected from carriers ($n = 75$) and patients with invasive disease ($n = 364$) from 2002 to 2011 (see Table S1 in the supplemental material). Isolates were cultured in Todd-Hewitt broth, and genomic DNA was extracted using the Promega Wizard DNA genomic DNA purification kit (Promega, USA). DNA sequencing was done using the Illumina genome analyzer II (Illumina, CA, USA) platform at the Wellcome Trust Sanger Institute. A robust bespoke pipeline (43) that was composed of the Velvet v1.2.09 De Bruijn graph-based DNA sequence assembler (30), Velvet Optimizer (31), SSPACE Basic v2.0 contig-scaffolding software (44), GapFiller (45), and SMALT v0.7.4 short-read aligner (<http://sourceforge.net/projects/smalt/>) was

used to generate sequence assemblies from the paired-end Illumina sequence reads for the study isolates. Serotypes were identified from the whole-genome sequence data using an *in silico* approach implemented with Perl and BioPerl (46) as described by Croucher et al. (47).

Population structure and phylogenetic analysis. Phylogenetic trees were constructed using a 0.79-Mb sequence alignment of 51,389 single nucleotide polymorphisms (SNPs) in 852 concatenated core genes present in single copies (no paralogs). The conserved (core) and nonconserved (accessory) genes were identified using the Roary bacterial genome analysis pipeline (32). The population structure of the isolates was inferred using the hierBAPS module implemented in BAPS v6.0 (33, 34). Maximum likelihood phylogenies of the concatenated core gene alignments for all the study isolates and serotype-specific isolates in the inferred sequence clusters (SCs) from BAPS were constructed using RAXML v7.0.4 (48). We used a generalized time reversible (GTR) (49) model with gamma (γ) heterogeneity across nucleotide sites and 100 bootstrap replicates. The tree was rooted with an outgroup clade of nontypeable (NT) isolates, which form a distinct clade from other pneumococci (50). Phylogenetic trees and associated metadata, namely, sequence types (STs), serotypes, and inclusion of the sample's serotype in the PCV13 vaccine formulation, were overlaid on the phylogenies and visualized in iTOL (51) and BioPython scripts (52).

Recombination detection and phylogeny construction. Phylogenies for specific serotypes were generated for downstream phylogenetic analysis through mapping paired-end short sequence reads against different published reference whole-genome sequences (see Table S2 in the supplemental material) for different pneumococcal serotypes using SMALT v0.7.4. The output binary alignment map (BAM) files were realigned using the Genome Analysis Tool Kit (GATK) v3.3.0 (53). The locations of recombination events in each chromosome in the alignments were detected using Gubbins v1.1.1 (35). The serotype-specific phylogenies with recombination removed were constructed using RAXML v7.0.4 (48) as above and rooted in the middle of the branch, separating the two most divergent isolates. Visualization of the phylogenies was done as described in "Population structure and phylogenetic analysis" above.

Carriage duration, serotype invasive potential, and capsule size. We used previously published data from Kilifi in Kenya, data on carriage duration of pneumococcal serotypes in the human nasopharynx from Abdullahi and colleagues (36) and data on serotype invasive potential (odds ratio for IPD to carriage [IC_{OR}]) from Brueggemann and colleagues (22). Additional data for serotype 5 invasive potential were obtained from Smith and colleagues (37). The polysaccharide capsule sizes for the serotypes were obtained from Weinberger and colleagues (21) where the degree of encapsulation was determined by measuring the zone of exclusion using the fluorescein isothiocyanate-labeled dextran (FITC-dextran) assay. The specific methods used to obtain the data sets are described in the respective publications. We excluded serotypes with estimated IC_{OR} values of 0 (22) to prevent overfitting as a consequence of the smaller number of isolates used from invasive disease sample collection. Genomic data on the relative recombination rate and frequency of pneumococcal lineages and prevalence of serotypes in carriage were obtained from Malawi (this study).

Statistical analysis. We used univariate, multivariable, and multivariate multiple regression to examine the association between known properties of serotypes and the relative recombination rate ($\mu_{r/m}$) and frequency (μ_{re}). The univariate regression model were separately formulated as $Y_i = \beta_0 + \beta_1 x_i + \varepsilon_i$ for each dependent variable Y_i ($\mu_{r/m}$ and μ_{re}) against each independent variable x_i where i designates the polysaccharide capsule size, carriage duration, serotype invasive potential, and carriage prevalence. The intercept and regression coefficient for the independent variables are represented by β_0 and β_1 , respectively, while ε_i represents the residuals. We formulated the multivariable model similarly but included multiple independent variables to account for collinearity between them as $Y_i = \beta_0 + \beta_1 x_i + \beta_2 x_i + \beta_3 x_i + \beta_4 x_i + \varepsilon_i$, where β_0 represents the intercept and the terms β_1 , β_2 , β_3 , and β_4 are the regression coefficients for

each independent variable (x_i) where i is polysaccharide capsule size, carriage duration, serotype invasive potential and carriage prevalence. We also modeled the relationship between both dependent variables ($\mu_{r/m}$ and μ_{re}) and the independent variables in the multivariable regression this time with a multivariate multiple regression model. The multivariate model was formulated as $Y_{n \times m} = X_{n \times (p+1)} \beta_{(p+1) \times m} + \varepsilon_{n \times m}$, where n designates each serotype and lineage combination as follows 1-SC2, 5-SC1, 6A-SC3, . . . , and 4-SC13 (Table 1). The m in the matrix $Y_{n \times m}$ takes values 1 and 2 that designate dependent variables $\mu_{r/m}$ and μ_{re} , respectively. The model matrix is the term $X_{n \times (p+1)}$ and consists of $p+1$ regressors where p represents polysaccharide capsule size, carriage duration, serotype invasive potential, and carriage prevalence of serotypes. The first column in the model matrix $X_{n \times (p+1)}$ consists of 1s, which represents the regression constants. The matrix of regression coefficients for all the independent variables in $X_{n \times (p+1)}$ is represented by parameter β . The residual term in the model is represented by the matrix $\varepsilon_{n \times m}$. For comparison with the multivariable and multivariate analyses, we repeated the univariate analyses with the same sample size.

We calculated the pairwise Pearson correlation coefficients for independent and response variables and plotted them using the ggplot2 package. The regression analysis, normality tests, namely, D'Agostino and Pearson, Shapiro-Wilk, and Kolmogorov-Smirnov tests, Kruskal-Wallis and analysis of variance (ANOVA) tests and summary statistics, such as means and ranges, were calculated using R v3.1.2 (R Core Team, 2014) and GraphPad Prism v6.0 (GraphPad Software, Inc., CA, USA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01053-16/-/DCSupplemental>.

Figure S1, EPS file, 2.1 MB.

Figure S2, TIF file, 1 MB.

Figure S3, TIF file, 1.6 MB.

Figure S4, EPS file, 1.9 MB.

Figure S5, EPS file, 1 MB.

Figure S6, EPS file, 1.9 MB.

Figure S7, EPS file, 1.2 MB.

Table S1, DOCX file, 0.2 MB.

Table S2, DOCX file, 0.1 MB.

Table S3, DOCX file, 0.1 MB.

ACKNOWLEDGMENTS

We thank Daniel Weinberger for kindly providing data on the capsule sizes of different pneumococcal serotypes and Helen Jenkins for helpful discussions. We acknowledge the support of the laboratory staff at the Malawi-Liverpool-Wellcome Trust Clinical Research Programme (MLW) and the library preparation, sequencing, and core informatic teams at the Wellcome Trust Sanger Institute.

C.C. and W.P.H. conceived and designed the study and analysis protocols. D.B.E., S.D.B., and W.P.H. supervised the study. N.F. and D.B.E. collected the samples. C.C. with advice from W.P.H. carried out the bioinformatics and statistical analyses and prepared tables and figures. W.P.H. advised on the regression analysis. C.C. and W.P.H. interpreted the results and drafted the manuscript. All authors contributed to the discussions and interpretation of the results and commented on the manuscript. All authors have read and approved the final manuscript.

This work was supported by funding from the Bill & Melinda Gates Foundation grant OPP1023440 (to D.B.E. [<http://www.pagegenome.org>]) and the Wellcome Trust Major Overseas Programme (MLW) core award 084679/Z/08/Z. We also acknowledge funding from the Commonwealth Scholarship Commission (Ph.D. studentship to C.C.).

The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding agencies. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

FUNDING INFORMATION

This work, including the efforts of Robert Heyderman and Dean Everett, was funded by Wellcome Trust (084679/Z/08/Z). This work, including the efforts of Dean Everett, was funded by Bill and Melinda Gates Foundation (Bill & Melinda Gates Foundation) (OPP1023440).

REFERENCES

- O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, Lee E, Mulholland K, Levine OS, Cherian T, Hib and Pneumococcal Global Burden of Disease Study Team. 2009. Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. *Lancet* 374:893–902. [http://dx.doi.org/10.1016/S0140-6736\(09\)61204-6](http://dx.doi.org/10.1016/S0140-6736(09)61204-6).
- Moore MR, Link-Gelles R, Schaffner W, Lynfield R, Lexau C, Bennett NM, Petit S, Zansky SM, Harrison LH, Reingold A, Miller L, Scherzinger K, Thomas A, Farley MM, Zell ER, Taylor TH, Jr, Pondo T, Rodgers L, McGee L, Beall B, Jorgensen JH, Whitney CG. 2015. Effect of use of 13-valent pneumococcal conjugate vaccine in children on invasive pneumococcal disease in children and adults in the USA: analysis of multistate, population-based surveillance. *Lancet Infect Dis* 15:301–309. [http://dx.doi.org/10.1016/S1473-3099\(14\)71081-3](http://dx.doi.org/10.1016/S1473-3099(14)71081-3).
- Whitney CG, Farley MM, Hadler J, Harrison LH, Bennett NM, Lynfield R, Reingold A, Cieslak PR, Plishvili T, Jackson D, Facklam RR, Jorgensen JH, Schuchat A, Active Bacterial Core Surveillance of the Emerging Infections Program Network. 2003. Decline in invasive pneumococcal disease after the introduction of protein–polysaccharide conjugate vaccine. *N Engl J Med* 348:1737–1746. <http://dx.doi.org/10.1056/NEJMoa022823>.
- Bentley SD, Aanensen DM, Mavroidi A, Saunders D, Rabbinowitsch E, Collins M, Donohoe K, Harris D, Murphy L, Quail MA, Samuel G, Skovsted IC, Kalltoft MS, Barrell B, Reeves PR, Parkhill J, Spratt BG. 2006. Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. *PLoS Genet* 2:e31. <http://dx.doi.org/10.1371/journal.pgen.0020031>.
- Calix JJ, Nahm MH. 2010. A new pneumococcal serotype, 11E, has a variably inactivated wcjE gene. *J Infect Dis* 202:29–38. <http://dx.doi.org/10.1086/6053123>.
- Jin P, Kong F, Xiao M, Oftadeh S, Zhou F, Liu C, Russell F, Gilbert GL. 2009. First report of putative *Streptococcus pneumoniae* serotype 6D among nasopharyngeal isolates from Fijian children. *J Infect Dis* 200:1375–1380. <http://dx.doi.org/10.1086/606118>.
- Park IH, Pritchard DG, Cartee R, Brandao A, Brandileone MC, Nahm MH. 2007. Discovery of a new capsular serotype (6C) within serogroup 6 of *Streptococcus pneumoniae*. *J Clin Microbiol* 45:1225–1233. <http://dx.doi.org/10.1128/JCM.02199-06>.
- Oliver MB, van der Linden MP, Kuntzel SA, Saad JS, Nahm MH. 2013. Discovery of *Streptococcus pneumoniae* serotype 6 variants with glycosyltransferases synthesizing two differing repeating units. *J Biol Chem* 288:25976–25985. <http://dx.doi.org/10.1074/jbc.M113.480152>.
- Calix JJ, Porambo RJ, Brady AM, Larson TR, Yother J, Abeygunwardana C, Nahm MH. 2012. Biochemical, genetic, and serological characterization of two capsule subtypes among *Streptococcus pneumoniae* serotype 20 strains: discovery of a new pneumococcal serotype. *J Biol Chem* 287:27885–27894. <http://dx.doi.org/10.1074/jbc.M112.380451>.
- Park IH, Geno KA, Yu J, Oliver MB, Kim KH, Nahm MH. 2015. Genetic, biochemical, and serological characterization of a new pneumococcal serotype, 6H, and generation of a pneumococcal strain producing three different capsular repeat units. *Clin Vaccine Immunol* 22:313–318. <http://dx.doi.org/10.1128/CI.00647-14>.
- Hanage WP, Bishop CJ, Huang SS, Stevenson AE, Pelton SI, Lipsitch M, Finkelstein JA. 2011. Carried pneumococci in Massachusetts children: the contribution of clonal expansion and serotype switching. *Pediatr Infect Dis J* 30:302–308. <http://dx.doi.org/10.1097/INF.0b013e318201a154>.
- Hanage WP, Bishop CJ, Lee GM, Lipsitch M, Stevenson A, Rifas-Shiman SL, Pelton SI, Huang SS, Finkelstein JA. 2011. Clonal replacement among 19A *Streptococcus pneumoniae* in Massachusetts, prior to 13 valent conjugate vaccination. *Vaccine* 29:8877–8881. <http://dx.doi.org/10.1016/j.vaccine.2011.09.075>.
- Hanage WP, Finkelstein JA, Huang SS, Pelton SI, Stevenson AE, Kleinman K, Hinrichsen VL, Fraser C. 2010. Evidence that pneumococcal serotype replacement in Massachusetts following conjugate vaccination is now complete. *Epidemics* 2:80–84. <http://dx.doi.org/10.1016/j.epidem.2010.03.005>.
- Brueggemann AB, Pai R, Crook DW, Beall B. 2007. Vaccine escape recombinants emerge after pneumococcal vaccination in the United States. *PLoS Pathog* 3:e168.
- Croucher NJ, Kagedan L, Thompson CM, Parkhill J, Bentley SD, Finkelstein JA, Lipsitch M, Hanage WP. 2015. Selective and genetic constraints on pneumococcal serotype switching. *PLoS Genet* 11:e1005095. <http://dx.doi.org/10.1371/journal.pgen.1005095>.
- Hanage WP, Fraser C, Tang J, Connor TR, Corander J. 2009. Hyper-recombination, diversity, and antibiotic resistance in pneumococcus. *Science* 324:1454–1457. <http://dx.doi.org/10.1126/science.1171908>.
- Chaguza C, Cornick JE, Everett DB. 2015. Mechanisms and impact of genetic recombination in the evolution of *Streptococcus pneumoniae*. *Comput Struct Biotechnol J* 13:241–247. <http://dx.doi.org/10.1016/j.csbj.2015.03.007>.
- Everett DB, Cornick J, Denis B, Chewapreecha C, Croucher N, Harris S, Parkhill J, Gordon S, Carrol ED, French N, Heyderman RS, Bentley SD. 2012. Genetic characterisation of Malawian pneumococci prior to the roll-out of the PCV13 vaccine using a high-throughput whole genome sequencing approach. *PLoS One* 7:e44250. <http://dx.doi.org/10.1371/journal.pone.0044250>.
- Croucher NJ, Harris SR, Fraser C, Quail MA, Burton J, van der Linden M, McGee L, von Gottberg A, Song JH, Ko KS, Pichon B, Baker S, Parry CM, Lambertsen LM, Shahinas D, Pillai DR, Mitchell TJ, Dougan G, Tomasz A, Klugman KP, Parkhill J, Hanage WP, Bentley SD. 2011. Rapid pneumococcal evolution in response to clinical interventions. *Science* 331:430–434. <http://dx.doi.org/10.1126/science.1198545>.
- Croucher NJ, Finkelstein JA, Pelton SI, Mitchell PK, Lee GM, Parkhill J, Bentley SD, Hanage WP, Lipsitch M. 2013. Population genomics of post-vaccine changes in pneumococcal epidemiology. *Nat Genet* 45:656–663. <http://dx.doi.org/10.1038/ng.2625>.
- Weinberger DM, Trzciński K, Lu YJ, Bogaert D, Brandes A, Galagan J, Anderson PW, Malley R, Lipsitch M. 2009. Pneumococcal capsular polysaccharide structure predicts serotype prevalence. *PLoS Pathog* 5:e1000476. <http://dx.doi.org/10.1371/journal.ppat.1000476>.
- Brueggemann AB, Griffiths DT, Meats E, Peto T, Crook DW, Spratt BG. 2003. Clonal relationships between invasive and carriage *Streptococcus pneumoniae* and serotype- and clone-specific differences in invasive disease potential. *J Infect Dis* 187:1424–1432. <http://dx.doi.org/10.1086/374624>.
- Sleeman KL, Griffiths D, Shackley F, Diggle L, Gupta S, Maiden MC, Moxon ER, Crook DW, Peto TEA. 2006. Capsular serotype-specific attack rates and duration of carriage of *Streptococcus pneumoniae* in a population of children. *J Infect Dis* 194:682–688. <http://dx.doi.org/10.1086/505710>.
- Hanage WP, Kajjalainen TH, Syrjänen RK, Auranen K, Leinonen M, Mäkelä PH, Spratt BG. 2005. Invasiveness of serotypes and clones of *Streptococcus pneumoniae* among children in Finland. *Infect Immun* 73:431–435. <http://dx.doi.org/10.1128/IAI.73.1.431-435.2005>.
- Li Y, Weinberger DM, Thompson CM, Trzciński K, Lipsitch M. 2013. Surface charge of *Streptococcus pneumoniae* predicts serotype distribution. *Infect Immun* 81:4519–4524. <http://dx.doi.org/10.1128/IAI.00724-13>.
- Chewapreecha C, Harris SR, Croucher NJ, Turner C, Marttinen P, Cheng L, Pessia A, Aanensen DM, Mather AE, Page AJ, Salter SJ, Harris D, Nosten F, Goldblatt D, Corander J, Parkhill J, Turner P, Bentley SD. 2014. Dense genomic sampling identifies highways of pneumococcal recombination. *Nat Genet* 46:305–309. <http://dx.doi.org/10.1038/ng.2895>.
- Marks LR, Reddinger RM, Hakansson AP. 2012. High levels of genetic recombination during nasopharyngeal carriage and biofilm formation in *Streptococcus pneumoniae*. *mBio* 3(5):e00200-12. <http://dx.doi.org/10.1128/mBio.00200-12>.
- Andam CP, Hanage WP. 2015. Mechanisms of genome evolution of *Streptococcus*. *Infect Genet Evol* 33:334–342. <http://dx.doi.org/10.1016/j.meegid.2014.11.007>.
- Kamng'ona AW, Hinds J, Bar-Zeev N, Gould KA, Chaguza C, Msefula C, Cornick JE, Kulohoma BW, Gray K, Bentley SD, French N, Heyderman RS, Everett DB. 2015. High multiple carriage and emergence of *Streptococcus pneumoniae* vaccine serotype variants in Malawian children. *BMC Infect Dis* 15:234. <http://dx.doi.org/10.1186/s12879-015-0980-2>.
- Zerbino DR, Birney E. 2008. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res* 18:821–829. <http://dx.doi.org/10.1101/gr.074492.107>.

31. Zerbino DR. 2010. Using the Velvet *de novo* assembler for short-read sequencing technologies. *Curr Protoc Bioinformatics* Chapter 11:Unit 11.5. <http://dx.doi.org/10.1002/0471250953.bi1105s31>.
32. Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MTG, Fookes M, Keane JA, Parkhill J. 2015. Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* 31:3691–3693. <http://dx.doi.org/10.1093/bioinformatics/btv421>.
33. Corander J, Waldmann P, Sillanpää MJ. 2003. Bayesian analysis of genetic differentiation between populations. *Genetics* 163:367–374.
34. Cheng L, Connor TR, Sirén J, Aanensen DM, Corander J. 2013. Hierarchical and spatially explicit clustering of DNA sequences with BAPS software. *Mol Biol Evol* 30:1224–1228. <http://dx.doi.org/10.1093/molbev/mst028>.
35. Croucher NJ, Page AJ, Connor TR, Delaney AJ, Keane JA, Bentley SD, Parkhill J, Harris SR. 2015. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. *Nucleic Acids Res* 43:e15. <http://dx.doi.org/10.1093/nar/gku1196>.
36. Abdullahi O, Karani A, Tigoi CC, Mugo D, Kungu S, Wanjiru E, Jomo J, Musyimi R, Lipsitch M, Scott JA. 2012. Rates of acquisition and clearance of pneumococcal serotypes in the nasopharynx of children in Kilifi District, Kenya. *J Infect Dis* 206:1020–1029. <http://dx.doi.org/10.1093/infdis/jis447>.
37. Smith T, Lehmann D, Montgomery J, Gratten M, Riley ID, Alpers MP. 1993. Acquisition and invasiveness of different serotypes of *Streptococcus pneumoniae* in young children. *Epidemiol Infect* 111:27–39. <http://dx.doi.org/10.1017/S0950268800056648>.
38. Cornick JE, Chaguza C, Harris SR, Yalcin F, Senghore M, Kiran AM, Govindpershad S, Ousmane S, Plessis MD, Pluschke G, Ebruke C, McGee L, Sigauque B, Collard J-M, Antonio M, von Gottberg A, French N, Klugman KP, Heyderman RS, Bentley SD, Everett DB, PACE Consortium. 2015. Region-specific diversification of the highly virulent serotype 1 *Streptococcus pneumoniae*. *Microb Genomics* 1:1–13. <http://dx.doi.org/10.1099/mgen.0.000027>.
39. Ritchie ND, Mitchell TJ, Evans TJ. 2012. What is different about serotype 1 pneumococci? *Future Microbiol* 7:33–46. <http://dx.doi.org/10.2217/fmb.11.146>.
40. Donkor ES, Bishop CJ, Gould K, Hinds J, Antonio M, Wren B, Hanage WP. 2011. High levels of recombination among *Streptococcus pneumoniae* isolates from the Gambia. *mBio* 2(3):e00040-11. <http://dx.doi.org/10.1128/mBio.00040-11>.
41. Hyams C, Yuste J, Bax K, Camberlein E, Weiser JN, Brown JS. 2010. *Streptococcus pneumoniae* resistance to complement-mediated immunity is dependent on the capsular serotype. *Infect Immun* 78:716–725. <http://dx.doi.org/10.1128/IAI.01056-09>.
42. Hyams C, Camberlein E, Cohen JM, Bax K, Brown JS. 2010. The *Streptococcus pneumoniae* capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms. *Infect Immun* 78:704–715. <http://dx.doi.org/10.1128/IAI.00881-09>.
43. Page AJ, De Silva N, Hunt M, Quail MA, Parkhill J, Harris SR, Otto TD, Keane JA. 2016. Robust high throughput prokaryote *de novo* assembly and improvement pipeline for Illumina data. *bioRxiv* <http://dx.doi.org/10.1101/052688>.
44. Boetzer M, Henkel CV, Jansen HJ, Butler D, Pirovano W. 2011. Scaffolding pre-assembled contigs using SSPACE. *Bioinformatics* 27: 578–579. <http://dx.doi.org/10.1093/bioinformatics/btq683>.
45. Nadalin F, Vezzi F, Policriti A. 2012. GapFiller: a *de novo* assembly approach to fill the gap within paired reads. *BMC Bioinformatics* 13:S8. <http://dx.doi.org/10.1186/1471-2105-13-S14-S8>.
46. Stajich JE, Block D, Boulez K, Brenner SE, Chervitz SA, Dagdigian C, Fuellen G, Gilbert JG, Korf I, Lapp H, Lehväslaiho H, Matsalla C, Mungall CJ, Osborne BI, Pocock MR, Schattner P, Senger M, Stein LD, Stupka E, Wilkinson MD, Birney E. 2002. The Bioperl toolkit: Perl modules for the life sciences. *Genome Res* 12:1611–1618. <http://dx.doi.org/10.1101/gr.361602>.
47. Croucher NJ, Harris SR, Fraser C, Quail MA, Burton J, van der Linden M, McGee L, von Gottberg A, Song JH, Ko KS, Pichon B, Baker S, Parry CM, Lamberts LM, Shahinas D, Pillai DR, Mitchell TJ, Dougan G, Tomasz A, Klugman KP, Parkhill J, Hanage WP, Bentley SD. 2011. Rapid pneumococcal evolution in response to clinical interventions. *Science* 331:430–434. <http://dx.doi.org/10.1126/science.1198545>.
48. Stamatakis A. 2006. RAXML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22:2688–2690. <http://dx.doi.org/10.1093/bioinformatics/btl446>.
49. Tavaré S. 1986. Some probabilistic and statistical problems in the analysis of DNA sequences, p 57–86. *In* Lectures on mathematics in the life sciences, vol 17. American Mathematical Society, Providence, RI.
50. Hilty M, Wüthrich D, Salter SJ, Engel H, Campbell S, Sá-Leão R, de Lencastre H, Hermans P, Sadowy E, Turner P, Chewapreecha C, Diggle M, Pluschke G, McGee L, Köseoğlu Eser Ö, Low DE, Smith-Vaughan H, Endimiani A, Küffer M, Dupasquier M, Beaudoin E, Weber J, Brüggemann R, Hanage WP, Parkhill J, Hathaway LJ, Mühlemann K, Bentley SD. 2014. Global phylogenomic analysis of nonencapsulated *Streptococcus pneumoniae* reveals a deep-branching classic lineage that is distinct from multiple sporadic lineages. *Genome Biol Evol* 6:3281–3294. <http://dx.doi.org/10.1093/gbe/evu263>.
51. Letunic I, Bork P. 2011. Interactive Tree of Life v2: online annotation and display of phylogenetic trees made easy. *Nucleic Acids Res* 39: W475–W478. <http://dx.doi.org/10.1093/nar/gkr201>.
52. Cock PJ, Antao T, Chang JT, Chapman BA, Cox CJ, Dalke A, Friedberg I, Hamelryck T, Kauff F, Wilczynski B, de Hoon MJ. 2009. Biopython: freely available Python tools for computational molecular biology and bioinformatics. *Bioinformatics* 25:1422–1423. <http://dx.doi.org/10.1093/bioinformatics/btp163>.
53. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo MA. 2010. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res* 20:1297–1303. <http://dx.doi.org/10.1101/gr.107524.110>.