Evolution of the capsular gene locus of *Streptococcus pneumoniae* serogroup 6

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Streptococcus pneumoniae expressing serogroup 6 capsules frequently causes pneumococcal infections and the evolutionary origins of the serogroup 6 strains have been extensively studied. However, these studies were performed when serogroup 6 had only two known members (serotypes 6A and 6B) and before the two new members (serotypes 6C and 6D) expressing *wciN*$_\beta$ were found. We have therefore reinvestigated the evolutionary origins of serogroup 6 by examining the profiles of the capsule gene loci and the multilocus sequence types (MLSTs) of many serogroup 6 isolates from several continents. We confirmed that there are two classes of *cps* locus sequences for serogroup 6 isolates. In our study, class 2 *cps* sequences were limited to a few serotype 6B isolates. Neighbour-joining analysis of *cps* sequence profiles showed a distinct clade for 6C and moderately distinct clades for class 1 6A and 6B sequences. The serotype 6D *cps* profile was found within the class 1 6B clade, suggesting that it was created by recombination between 6C and 6B *cps* loci. Interestingly, all 6C isolates also had a unique *wzy* allele with a 6 bp deletion. This suggests that serotype switching to 6C involves the transfer of a large (>4 kb) gene segment that includes both the *wciN*$_\beta$ allele and the 'short' *wzy* allele. The MLST studies of serotype 6C isolates suggest that the 6C *cps* locus is incorporated into many different pneumococcal genomic backgrounds but that, interestingly, 6C *cps* may have preferentially entered strains of the same genomic backgrounds as those of serotype 6A.

INTRODUCTION

Streptococcus pneumoniae (pneumococcus) is a common colonizer of the human nasopharynx, yet it is an important human pathogen responsible for several diseases, mainly in children, the elderly and the immune-compromised (Lynch & Zhanel, 2009). Using a polysaccharide (PS) capsule, of which there are at least 93 structurally distinct types (Bratcher *et al.*, 2010; Calix & Nahm, 2010; Henrichsen, 1995; Park *et al.*, 2007b), this bacterium is able to shield its surface from recognition by the host innate immune system, thereby making the capsule a potent colonization/virulence factor (Avery & Dubos, 1931; Bogaert *et al.*, 2004). Some capsule types (serotypes) are more prevalent in disease than others, with the serogroup 6 strains, which include serotypes 6A, 6B, 6C and 6D, being more commonly isolated from infections than the majority of other serotypes. Vaccination strategies in use today target the pneumococcal capsule from the most prevalent serotypes, and most pneumococcal vaccines, including the widely used 7-valent conjugate vaccine (PCV-7), contain the serotype 6B PS.

Because of its clinical importance, the evolution of the serogroup 6 strains has been previously studied in detail (Mavroidi *et al.*, 2004; Robinson *et al.*, 2002). Serogroup 6 capsular PS synthesis loci (*cps*) encode 14 ORFs and range in size from ~17 to ~19.1 kb due to variations in the non-coding regions found at either end (Fig. 1) (Mavroidi *et al.*, 2004, 2007; Park *et al.*, 2007a). Like most serotypes, the central, serotype-specific *cps* region of all serogroup 6 strains has a G+C content lower than the pneumococcal background, and three of these genes (*wciP*, *wzy* and *wzx*) are highly specific to serogroup 6 (Mavroidi *et al.*, 2004). Using DNA sequences of selected parts of these serogroup

Abbreviations: MLST, multilocus sequence typing; PCV-7, 7-valent conjugate vaccine; PS, polysaccharide; ST, sequence type.

The GenBank/EMBL/DDBJ accession number for the entire *cps* locus of a serotype 6D isolate is HM171374.

A supplementary table and a supplementary figure are available with the online version of this paper.

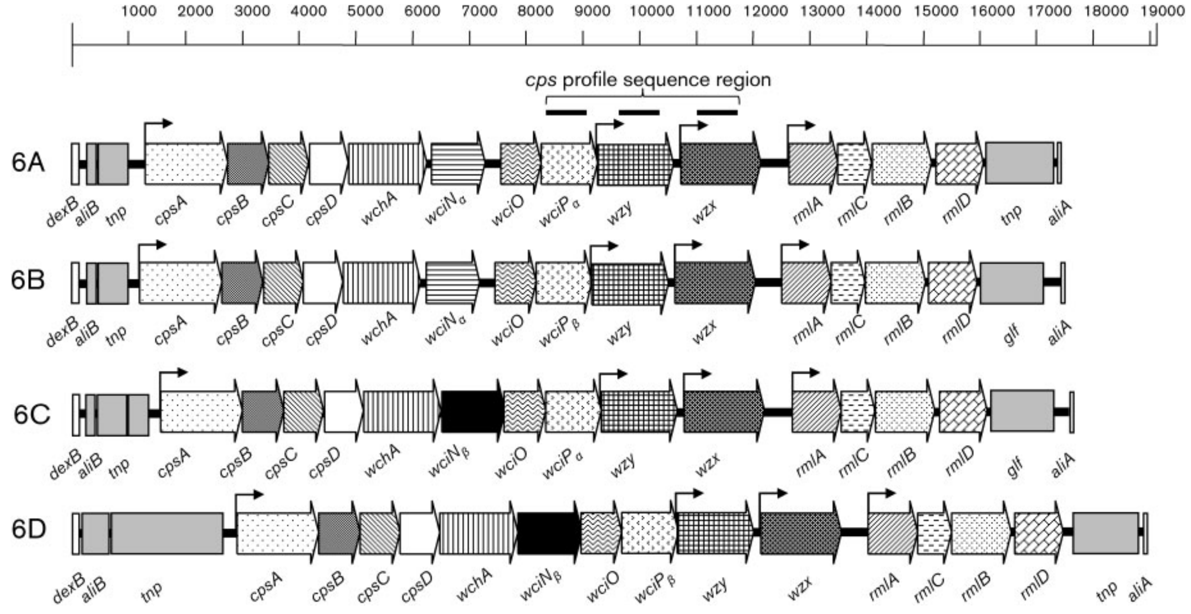

1000 2000 3000 4000 5000 6000 7000 8000 9000 10000 11000 12000 13000 14000 15000 16000 17000 18000 19000
cps profile sequence region
6A
dexB aliB tnp cpsA cpsB cpsC cpsD wchA wciNα wciO wciPα wzy wzx rmlA rmlC rmlB rmlD tnp aliA
6B
dexB aliB tnp cpsA cpsB cpsC cpsD wchA wciNα wciO wciPβ wzy wzx rmlA rmlC rmlB rmlD glf aliA
6C
dexB aliB tnp cpsA cpsB cpsC cpsD wchA wciNβ wciO wciPα wzy wzx rmlA rmlC rmlB rmlD glf aliA
6D
dexB aliB tnp cpsA cpsB cpsC cpsD wchA wciNβ wciO wciPβ wzy wzx rmlA rmlC rmlB rmlD tnp aliA

Fig. 1. Capsule gene loci of 6A (GenBank accession no. CR931638), 6B (GenBank accession no. CR931639), 6C (GenBank accession no. EF538714) and 6D (GenBank accession no. HM171374) strains. All ORFs involved in capsule synthesis are shown as horizontal arrows, and their direction indicates the transcriptional orientation. The three regions of the *wciP* (645 bases), *wzy* (492 bases) and *wzx* (477 bases) genes used for *cps* profiling are shown as three black bars above the 6A *cps* diagram. The *wciN* and *wciP* alleles are indicated by α and β. The size of a gene fragment from the beginning of *wciN*$_\beta$ to the end of *wzx* is about 4130 bases. Bent arrows indicate potential transcription sites.

6-specific genes to create a '*cps* profile', Mavroidi *et al.* (2004) showed that the only consistent genetic difference between serotypes 6A and 6B was a single nonsynonymous polymorphism in the *wciP* gene: the *wciP*$_\alpha$ gene of serotype 6A has a G at nucleotide 584 while *wciP*$_\beta$ of 6B has an A (Mavroidi *et al.*, 2004). They also showed that the serogroup 6 *cps* loci can be divided into two distinct classes based on the presence of an INDEL sequence (Mavroidi *et al.*, 2004). The majority of 6A and 6B strains do not have an INDEL and fall into class 1. In contrast, some 6B isolates and very few 6A isolates have an INDEL and fall into class 2. The capsule loci from the different classes have a 5.4 % sequence divergence whereas those of class 1 differ by only 1–2 % (Mavroidi *et al.*, 2004).

Since these studies were published, two new serogroup 6 members have been discovered. One new serotype, 6C, discovered in 2007 is serologically similar to 6A, but has a glucose residue replacing the galactose residue in the 6A PS (Park *et al.*, 2007b). The 6C *cps* is 98 % similar to the 6A *cps* except that it contains a unique *wciN* gene (referred to as *wciN*$_\beta$) which does not have significant sequence homology with other pneumococcal genes, including the *wciN*$_\alpha$ gene of 6A and 6B (Fig. 1) (Park *et al.*, 2007a). While PCV-7, which contains 6B PS, has been shown to reduce the occurrence of both carriage and invasive disease resulting from vaccine-related serotype 6A, serotype 6C seems to be able to evade this cross-reactive protection afforded by the vaccine and is, therefore, increasing in prevalence (Carvalho Mda *et al.*, 2009; Leach *et al.*, 2009; Nahm *et al.*, 2009; Park *et al.*, 2008; Tocheva *et al.*, 2010). Also, serotype 6D was recently described (Bratcher *et al.*, 2010; Jin *et al.*, 2009) and can be genetically distinguished from serotype 6C by the *wciP* gene (Bratcher *et al.*, 2009). Specifically, serotype 6C contains a *wciP*$_\alpha$ gene whereas serotype 6D has a *wciP*$_\beta$ gene. In view of these recent discoveries in serogroup 6, we have reinvestigated the genetic evolution of serogroup 6 strains by studying the origins of these new serotypes as well as the mechanism of expansion of serotype 6C.

METHODS

PCR, sequencing and sequence analysis. Genomic DNA was purified from pneumococci using phenol/chloroform extraction and was amplified by PCR as described previously (Mavroidi *et al.*, 2004) using appropriate primers and PCR mixture. Primer sequences are shown in Table 1. The PCR mix contained 37.5 µl sterile water, 1 µl genomic DNA, 2 µl each primer (5 pmol), 2 µl 10 mM dNTP, 5 µl 10× LA *Taq* buffer solution and 0.5 µl LA *Taq* polymerase (2.5 U µl^{-1}; Takara). The DNA sequence of the PCR product was determined by the Genomics Core Facility at University of Alabama at Birmingham.

DNA sequences of selected parts of the *wciP*, *wzy* and *wzx* genes were subjected to *cps* profiling studies using previously described approaches (Mavroidi *et al.*, 2004). Alleles were assigned according to the designations previously used, and new alleles were given arbitrarily numbered designations. The sequences of *wciP*, *wzy* and

Table 1. List of primers used in the study

Primer name (direction)*	Location	Sequence	Source or reference
5117 (F)	*wchA*	5′-ATCAAGTGGTATTGGAAGCGGG	This study
5386 (F)	*wciN*$_\beta$	5′-CTGCTTTCCAAAGAGTTCG	This study
5106 (F)	*wchA*	5′-TACCATGCAGGGTGGAATGT	Park *et al.* (2007a)
5108 (F)	*wciP*	5′-ATGGTGAGAGATATTTGTCAC	Mavroidi *et al.* (2004)
5140 (F)	*wzy*	5′-CCTAAAGTGGAGGGAATTTCG	Mavroidi *et al.* (2004)
5141 (F)	*wzx*	5′-TTCGAATGGGAATTCAATGG	Mavroidi *et al.* (2004)
3386 (R)	*wciN*$_\beta$	5′-TAATATACCTATCAACTCCACCGC	This study
3102 (R)	*wciP*	5′-CTGGCATGTCATCTTTAGAAAA	This study
3101 (R)	*wciO*	5′-CCATCCTTCGAGTATTGC	Park *et al.* (2007a)
3107 (R)	*wciP*	5′-AGCATGATGGTATATAAGCC	Mavroidi *et al.* (2004)
3143 (R)	*wzy*	5′-CCTCCCATATAACGAGTGATG	Mavroidi *et al.* (2004)
3144 (R)	*wzx*	5′-GCGAGCCAAATCGGTAAGTA	Mavroidi *et al.* (2004)

*F, Forward; R, reverse.

wzx were concatenated, and the concatenated sequences were subjected to neighbour-joining analysis (Saitou & Nei, 1987) to investigate the evolutionary relationship among the *cps* loci of different serogroup 6 isolates. All evolutionary trees were drawn using MEGA4 (Tamura *et al.*, 2007). Pairwise/evolutionary differences were computed using the maximum composite likelihood method in MEGA4 (Tamura *et al.*, 2004, 2007), and in both analyses, positions containing gaps were eliminated from the dataset (complete deletion option). The percentages of replicate trees in which the associated sequences clustered together in the bootstrap test (500 replicates) are reported as the bootstrap values.

wciN and flanking regions from serotype 6A (CR931638), a class 2 6B (AF246897), 6C (EF538714), 33F (CR931702) and 4 (CR931635) sequences were analysed for possible recombination events. Recombinational analysis was performed by the RDP (recombination detection program) method (Martin & Rybicki, 2000) using RDP3 (Martin *et al.*, 2005). For the recombination analysis, two sequences were created by randomly inserting the *wciN* gene or the *wciN* with its flanking regions into one insertion site of the serotype 4 *cps* locus in order to create a 'mock foreign source' for the *wciN* gene.

PCR amplicons were sequenced and the sequences were subjected to multilocus sequence typing (MLST) analysis as described previously (Enright & Spratt, 1998). Known alleles were then identified using the pneumococcal MLST website (http://spneumoniae.mlst.net), and numbers were assigned to new alleles by the database curator. All the MLST data listed in Table 1 have been submitted to the online pneumococcal MLST database. Evolutionary relations among MLST types were determined with the eBURST algorithm of the Department of Infectious Disease Epidemiology at Imperial College, London, as described by Enright & Spratt (1998).

Pneumococcal isolates. Fifty-seven isolates were collected between 1999 and 2008 from four different continents (Table 2). Twenty-four of the isolates were 6A, 25 were 6C, 6 were 6B and 2 were 6D. These 57 isolates were subjected to *cps* profiling as well as MLST studies. To supplement the *cps* profiling studies, we studied an additional 12 6B isolates that were already in our collection. The resulting panel of 69 isolates included 18 isolates from Asia, 18 from Europe, 14 from North America, and 19 from South America. Full information on the additional 12 6B isolates is provided in Supplementary Table S1 (available with the online version of this paper).

RESULTS

wciN and flanking region sequence variations among 6C and 6D isolates

The *wciN*$_\beta$ gene was sequenced from 25 new 6C isolates (Fig. 2, Table 2), which were obtained from four different continents over a 10 year period. In addition, we included data from the 6C strain (CHPA388) (Fig. 2) for which the entire *cps* locus has been sequenced and published (GenBank accession no. EF538714) for comparison. By comparing the sequences, all the variations were found at 17 (1.51 %) of the 1125 bases in *wciN*$_\beta$ of 6C serotype. The total sequence variation is also very small: 0.11 %, 31 bases differed out of a total of 29 250 (=26 × 1125) bases. This difference is consistent with the heterogeneity observed for the *cps* sequences (GenBank accession nos AF246898, AY078347 and CR931638) of three 6A isolates (0.1–0.2 %), which presumably represent randomly chosen isolates. Furthermore, nine clinical isolates collected from three different continents (Europe, North America and South America) over an 8 year period have exactly the same nucleotide sequences for 2782 bases including *wciN*$_\beta$ and flanking regions (Fig. 2, top 9 rows). Thus, it is likely that *wciN*$_\beta$ was introduced to the serogroup 6 *cps* locus from a foreign, probably non-pneumococcal, source only once.

Our previous study (Park *et al.*, 2007a) suggested that the insertion of *wciN*$_\beta$ may have been facilitated by two clearly identifiable flanking regions (grey shading in Fig. 2), which are about 300 and 110 bases long in the 5′ and 3′ regions, respectively. The flanking regions were defined by an intermediate level of genetic similarity (80–90 %) when comparing 6A and 6C *cps* loci, while the central region (i.e. the foreign gene) has no homology (29 % similarity) and outside of the flanking regions is highly similar (>98 %) (Park *et al.*, 2007a). These flanking regions and their margins would vary among 6C isolates if the foreign gene was introduced multiple times to form serotype 6C *cps*. We

Table 2. Pneumococcal isolates used for the study

All isolates are from this study except MNZ595 and MNZ604, which are from the study by Bratcher *et al.* (2009).

Strain	Location	Year isolated	Serotype	Clonal complex	ST	*cps* profile data		
						wciP	***wzy***	***wzx***
MNZ680	S. America	2001	6A	CC176	4598	2	8	1
MNZ616	S. America	2004	6A	CC176	4598	2	8	1
MNZ631	S. America	2003	6A	CC176	4598	2	1	1
MNZ632	S. America	2002	6A	CC176	4598	2	1	1
MNZ681	S. America	2001	6A	CC176	4600	2	1	1
MNZ664	S. America	1999	6A	CC176	4622	2	1	1
MNZ208	Europe	1999	6A	Singleton	2611	2	6	1
MNZ446	N. America	2007	6A	CC2090	376	2	6	1
MNZ428	N. America	2007	6A	CC2090	1538	2	6	1
MNZ677	S. America	1999	6A	CC315	1093	2	6	1
MNZ218	Europe	2001	6A	CC395	327	15	6	1
MNZ459	N. America	2007	6A	CC460	460	2	6	1
MNZ497	N. America	2004	6A	CC460	460	1	1	1
MNZ479	N. America	2004	6A	CC460	460	1	13	1
MNZ239	Europe	2008	6A	CC473	813	2	1	1
MNZ471	N. America	2007	6A	CC473	1876	14	1	1
MNZ462	N. America	2007	6A	CC473	1876	14	1	1
MNZ226	Europe	2003	6A	CC473	4595	2	1	3
MNZ212	Europe	1999	6A	CC490	4363	2	1	1
MNZ222	Europe	2002	6A	CC690	690	14	1	1
MNZ28	Asia	2008	6A	CC81	81	2	1	1
MNZ13	Asia	2008	6A	CC81	81	2	1	1
MNZ19	Asia	2008	6A	CC81	282	2	1	1
MNZ17	Asia	2008	6A	Singleton	4620	2	1	1
MNZ31	Asia	2008	6B	CC176	4604	16	15	1
MNZ59	Asia	2008	6B	CC176	4605	16	15	1
MNZ02	Asia	2008	6B INDEL	CC180	1624	8	14	7
MNZ39	Asia	2008	6B INDEL	CC90	90	8	7	7
MNZ62	Asia	2008	6B INDEL	CC90	90	8	14	7
MNZ60	Asia	2008	6B INDEL	CC90	4606	8	14	7
MNZ440	N. America	2007	6C	CC1379	1379	13	10	1
MNZ613	S. America	2002	6C	CC1379	1379	9	10	1
MNZ472	N. America	2007	6C	CC1379	1379	13	10	1
MNZ595	S. America	2001	6C	CC1379	4599	13	10	1
MNZ480	N. America	2004	6C	CC1390	1390	13	12	1
MNZ488	N. America	2004	6C	CC1390	1390	13	10	1
MNZ435	N. America	2007	6C	CC1390	1390	13	10	1
MNZ458	N. America	2007	6C	CC1439	4602	13	10	1
MNZ219	Europe	2002	6C	CC1715	1715	9	10	1
MNZ240	Europe	2008	6C	CC176	2689	9	10	1
MNZ23	Asia	2008	6C	CC176	4596	9	10	12
MNZ34	Asia	2008	6C	CC176	4596	9	10	12
MNZ38	Asia	2008	6C	CC176	4597	9	10	12
MNZ604	S. America	1999	6C	CC3854	4601	9	10	1
MNZ597	S. America	2002	6C	CC3854	4601	9	10	1
MNZ683	S. America	2001	6C	CC3854	4601	9	10	1
MNZ592	S. America	2003	6C	CC3854	4601	9	10	1
MNZ678	S. America	1999	6C	CC3854	4621	9	10	1
MNZ221	Europe	2002	6C	CC395	395	9	10	1
MNZ213	Europe	1999	6C	CC395	395	9	10	1
MNZ432	N. America	2007	6C	CC473	473	9	10	11
MNZ16	Asia	2008	6C	Singleton	855	9	10	11

Table 2. cont.

Strain	Location	Year isolated	Serotype	Clonal complex	ST	*cps* profile data		
						wciP	*wzy*	*wzx*
MNZ18	Asia	2008	6C	Singleton	855	9	10	11
MNZ227	Europe	2004	6C	Singleton	3531	9	11	1
MNZ209	Europe	1999	6C	Singleton	4603	9	11	1
MNZ21	Asia	2008	6D	CC81	282	5	1	1
MNZ22	Asia	2008	6D	CC81	282	5	1	1

have therefore determined the sequence of the *wciN*$_\beta$ flanking regions from the 26 6C isolates and the two 6D isolates from Korea (Fig. 2). We also included the sequences of two 6D isolates from Fiji available from GenBank in the analysis (Fig. 2) (Jin *et al.*, 2009). When the 410 bases in the two flanking regions from the 30 isolates were compared, only five bases varied, and all of these are located in the middle of the flanking regions, suggesting no variation at the margin. Thus, analysis of flanking regions also supports a single incorporation of the *wciN*$_\beta$ gene in the serogroup 6 *cps*.

Interestingly, the 5′ flanking region is very similar to the corresponding region of pneumococcal serotype 33F *cps* and the 3′ flanking region is very close to the corresponding region of a class 2 6B strain *cps*, as shown at the bottom of Fig. 2. Potential recombination events that could have produced the 6C *wciN* and its flanking regions were investigated with the RDP method. The analysis suggests that generation of the 6C *cps* locus would involve complex recombination events requiring sources for the *wciN*$_\beta$ and the 'short' *wzy* in addition to 33F and class 2 serotype 6B *cps*.

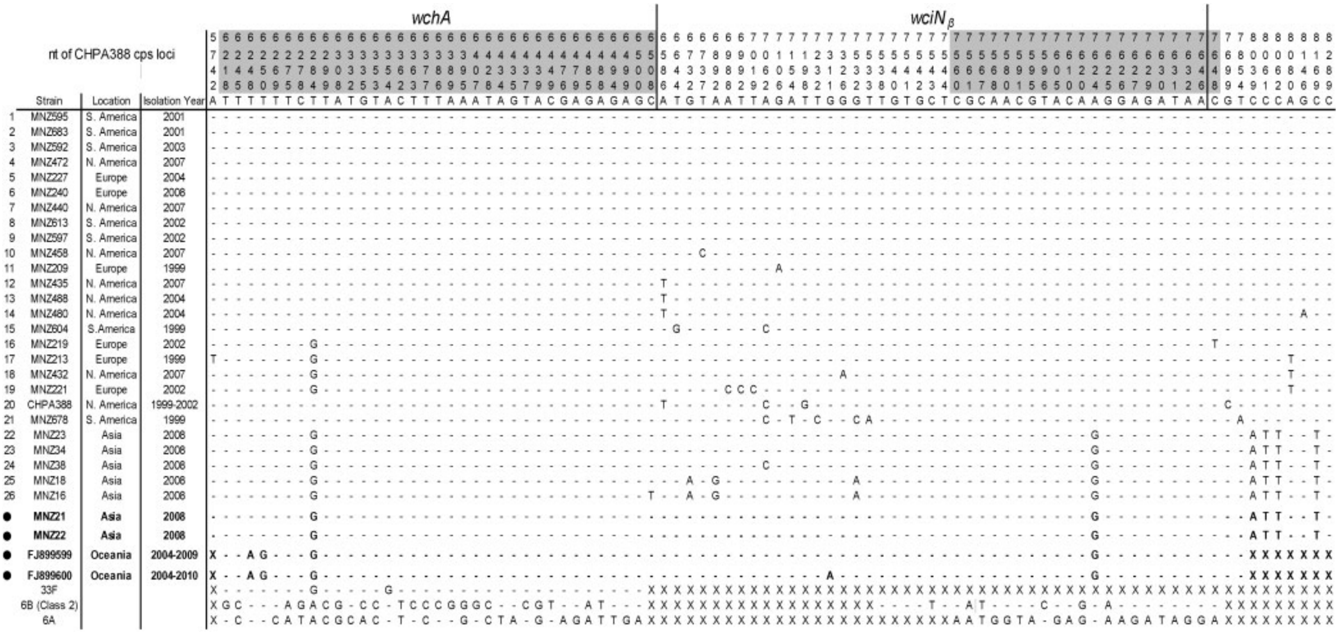


Fig. 2. Sequence diversity of the *wciN*$_\beta$ and its flanking regions for serotype 6C and 6D strains. The DNA sequence was determined from base 5671 to base 8452. The consensus sequence (top line) and the numbering system (numbers are given vertically) are based on the 6C *cps* sequence (GenBank accession no. EF538714). Heavy vertical bars at the top indicate the two ends of the *wciN*$_\beta$ ORF. The flanking regions of the 'foreign gene' that produced *wciN*$_\beta$ are shaded (between bases 6209 and 6508 and bases 7545 and 7655). Most sequences were from serotype 6C isolates except for the four sequences from 6D isolates (marked by ●). One of 26 6C sequences is a published sequence (CHPA388, GenBank accession no. EF538714). Two Oceania 6D sequences are from GenBank (accession nos FJ899599 and FJ899600). X, No corresponding sequences. Sequences from 33F (GenBank accession no. CR931697) and a class 2 6B strain (GenBank accession no. AF246897) are included to show the similarity in the 5′ and 3′ flanking regions, respectively. The sequence for 6A (GenBank accession no. CR931638) is shown for comparison at the bottom.

Analysis of the capsule gene loci of 6C and 6D isolates

Recently, isolates expressing serotype 6D were discovered in nature. To examine the evolutionary relationship of serotype 6D with the other three serotypes of serogroup 6, we determined the sequence of the entire *cps* locus of a serotype 6D isolate (GenBank accession no. HM171374). As illustrated in Fig. 1, the 6D *cps* is bound by *dexB* and *aliA*, as are the other capsule gene loci (García *et al.*, 2000), and has transposase-like regions at each end. Between these transposase-like regions, the 6D *cps* has the same 14 functional genes found in the other serogroup 6 *cps* loci. In addition, the sequence of the 6D *cps* locus is almost identical (98.6 % identity in the 14 933 bp that include all the ORFs) to that of 6C (GenBank accession no. EF538714) except for the known difference in the *wciP* gene and differences in the non-coding regions flanking the *cps* locus. Similar to other members of serogroup 6, the G + C content of the region from *wciN*$_{\beta}$ to *wzy* for 6D is only 31 %, which is lower than the rest of the pneumococcal genome (39 %) (Tettelin *et al.*, 2001).

To study the evolutionary relationship between serotype 6C and serotypes 6A and 6B, we determined the *cps* profiles of the 57 isolates listed in Table 2 along with 12 archived 6B isolates. The *cps* profile was determined as previously described by sequencing a portion of three genes (*wciP*, *wzy* and *wzx*) that are common in all serogroup 6 capsule gene loci (Mavroidi *et al.*, 2004). Our study identified 11 new alleles (shown in Fig. 3c): four for *wciP*, five for *wzy* and two for *wzx*. Interestingly, *wzy* alleles 10 (Mavroidi *et al.*, 2004), 11 and 12 are used only by serotype 6C isolates and have a very distinct 6 bp deletion (Fig. 3c).

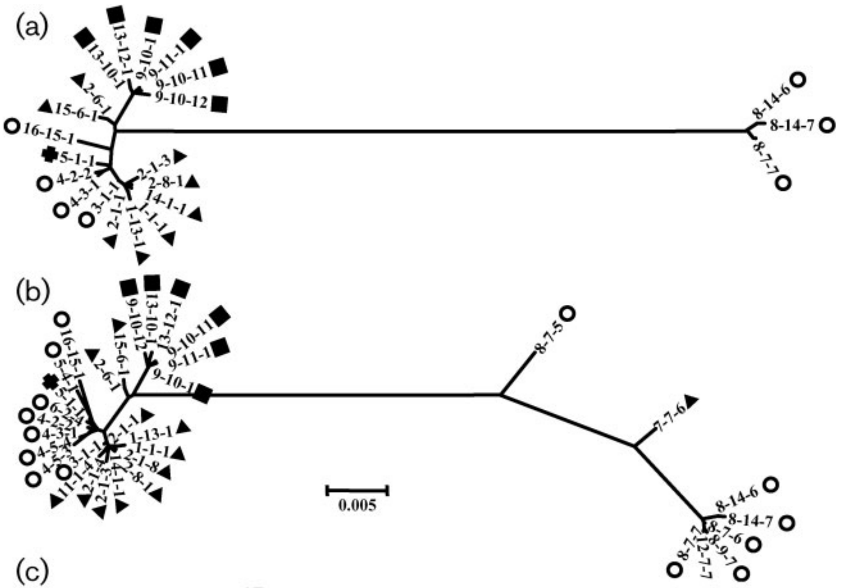
When the three (*wciP*, *wzy* and *wzx*) sequences of each isolate were concatenated and the evolutionary tree was determined for the 69 isolates using the neighbour-joining method (Fig. 3a), the class 2 isolates (i.e. isolates with an INDEL) could be clearly separated from the class 1 isolates (isolates without an INDEL), as described previously (Mavroidi *et al.*, 2004), with 99 % bootstrap support and the genetic distance between them being greater than 5.4 %. When class 1 isolates were examined, the 6C strains formed a distinct clade (99 % bootstrap support) suggesting a single origin (Fig. 3a). The class 1 6A and 6B strains formed moderately distinct clades (79 % bootstrap support) and a common ancestor for the two clades could not be excluded (Mavroidi *et al.*, 2004). The genetic distance between class 1 6A and 6B isolates was >0.26 %, whereas the genetic distance of the 6C cluster from the 6A and 6B clusters was >0.78 % by pairwise differences computed using MEGA4 (Fig. 3a). Nevertheless, these clades are largely serotype-specific, and so we refer to the two clades by the dominant serotype in that clade. The two 6D isolates were nestled within the class 1 6B clade. The clustering pattern of the serotypes within clades did not change when additional published *cps* profile data (Mavroidi *et al.*, 2004) were merged with our data (Fig. 3b). Also, analysis of the clustering patterns of individual gene segments did not provide additional information (see Supplementary Fig. S1, available with the online version of this paper). The *cps* profile analysis provides additional support that all the 6C *cps* loci shared a distinct origin but the class 1 6A and 6B clades are less distinct.

While most isolates in one clade are of a single serotype, there are some exceptions. Two obvious exceptions are the serotype 6D isolates expressing profile 5-1-1, which is within the 6B cluster. While more serotype 6D isolates should be examined, the *cps* loci of these two 6D isolates were most likely generated when 6C and 6B *cps* recombined at a location between *wciN* and *wciP*. The other exception is a 6B strain expressing 3-1-1, which is located within the 6A cluster. A previous study suggested that this profile may have arisen from the 6A serotype by a mutation of the *wciP* gene (Mavroidi *et al.*, 2004). Additional outliers are the 6A strains expressing *cps* profiles 2-6-1 and 15-6-1. They have a relatively distinct *wzy* sequence containing four single nucleotide polymorphisms separating them from the class 1 6A and 6B sequences. These single nucleotide polymorphisms are shared by the 6C sequences, suggesting these outlier 6A strains may have been the source of the *wzy* sequence in the 6C strains.

The 6C cluster is associated with three very similar *wzy* alleles that have a 6 nt in-frame 'ATCGTG' deletion (Fig. 3c). This association was observed for the 25 6C isolates studied here and for 14 additional 6C isolates that were collected for our previous study (Park *et al.*, 2007a; unpublished data). In contrast, all 42 isolates expressing serotypes 6A and 6B have normal *wzy* alleles. Consequently, the 6C *cps* locus has two genetic markers (*wciN*$_{\beta}$ and the 'short' *wzy*) that are separated by about 4130 bp, which spans almost the entire stretch of serotype-specific genes in the *cps* locus. This finding suggests that the serotype switching event that results in expressing the 6C capsule involves a genetic transfer of not only *wciN*$_{\beta}$ but also a large piece of DNA including *wciN*$_{\beta}$ and the 'short' *wzy*.

Analysis of the genomic background of 6C and 6D clinical isolates

Since the introduction of conjugate pneumococcal vaccines, the prevalence of serotype 6C has greatly increased in several parts of the world. To investigate whether all or selected clones of serotype 6C were increasing in prevalence, we performed an MLST analysis on a global set of 6C isolates. Table 2 shows the sequence type (ST) and clonal complex of all of the pneumococcal isolates used in this study. The clonal complex of each isolate was identified by performing eBURST analysis using resources available at http://www.mlst.net. Our results show that some 6A isolates possess genetic backgrounds of ST81, ST376 and ST1538, which are associated with well-known antibiotic resistant clones (Spain^{23F}-1 and North Carolina^{6A}-23).



(c)

wciP

			1	2	2	2	3	4	4	4	4	4	5
	Associated	1	4	0	1	3	0	0	1	3	6	6	9
Allele no.	serotype(s)	5	5	5	2	1	3	3	3	2	1	5	0
1	6A	C	T	A	T	T	T	G	G	A	C	G	G
13	6C	A	.	C	C	.	A	.	.	G	.	A	.
14	6A	.	.	.	C	.	.	.	.	.	.	.	T
15	6A	.	.	.	C	.	.	.	.	.	T	.	.
16	6B	A	C	C	C	G	.	T	A	.	.	.	.

wzy

										1	1	1	1	1	1	1	1	1	2	2	2	2	2	3	3	3	3	3	3	3	4	4	4	4	4
	Associated		1	3	4	4	6	6	8	5	5	5	5	5	5	5	6	8	0	0	2	4	4	2	3	4	5	7	9	9	1	2	3	4	5
Allele no.	serotype(s)	2	4	1	4	7	3	7	2	1	2	3	4	5	6	7	5	1	5	8	8	6	9	5	0	6	2	0	0	2	0	6	8	2	3
1	6A/6B/6D	T	C	T	T	G	A	T	A	A	A	T	C	G	T	G	A	T	C	A	G	T	A	T	C	G	T	A	T	C	A	C	G	A	T
11	6C	.	T	C	C	.	G	.	.	.	_	_	_	_	_	_	.	.	.	.	A	.	.	.	.	.	G	G	.	.	.	T	.	G	.
12	6C	.	T	C	C	.	G	.	.	.	_	_	_	_	_	_	.	.	.	.	A	.	.	.	.	.	G	.	A	.	.	T	.	G	.
13	6A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
14	6B	G	T	C	.	A	G	C	G	G	.	.	.	.	.	.	.	G	T	G	A	G	G	C	.	A	A	.	.	A	G	T	T	G	G
15	6B	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	T	.	G	.	.	.	.	T	.	.	.

wzx

		1	3	3
	Associated	2	4	4
Allele no.	serotype(s)	9	3	5
1	6A/6B/6C/6D	C	T	A
11	6C	.	C	.
12	6C	T	.	T

Fig. 3. Neighbour-joining trees of unique *cps* profiles. (a) Tree constructed with 22 unique *cps* profiles from our study, which included 23 6A (▲), 18 6B (○), 25 6C (■) and 2 6D (+) isolates. Table 2 shows the *cps* profiles of all the strains included in our study. Note that three class 2 6B isolates are shown on the right of the tree and all class 1 strains are shown on the left. (b) Tree constructed with *cps* profile data from both our current study and a published study (Mavroidi *et al.*, 2004). Class 2 isolates are shown on the right of the tree and two cross-over strains (profiles 8-7-5 and 7-7-6) are shown in the middle. The bar represents a genetic distance of 0.5 %. (c) Polymorphic nucleotides for all new alleles of the *cps* genes (*wciP*, *wzy* and *wzx*). Allele 1 is shown at the top for comparison. Note that *wzy* alleles 11 and 12, which are associated with serotype 6C, have 6 base deletions (positions 152–157). –, No 6 bp deletions; ., identical bases.

Interestingly, ST282 expressed by serotype 6D is a single-locus variant of the Spain^{23F}-1 clone. The *cps* gene of serotype 6C is associated with STs in multiple clonal complexes, as others have also found (Carvalho Mda *et al.*, 2009; Jacobs *et al.*, 2009; Nunes *et al.*, 2009), but no 6C STs in this list could be linked with antibiotic resistant clones. However, it was shown that serotype 6C can have an antibiotic resistant ST (e.g. ST376) (Carvalho Mda *et al.*, 2009).

DISCUSSION

A previous study of *cps* profiles of serogroup 6 isolates revealed two classes of *cps* profiles (named classes 1 and 2) and only a small evolutionary separation between class 1 6A and 6B clades. Since that study had been performed before serotypes 6C and 6D were discovered, a fresh look at the evolutionary relationships amongst the serogroup 6 serotypes was warranted. We have now studied the *cps* profiles of many additional isolates, including those of serotypes 6C and 6D. In addition to confirming the two classes of profiles found earlier, our *cps* profile studies show three clades within class 1, with two moderately distinct clades for 6A and 6B, as seen previously, and a new clade associated with serotype 6C.

A surprising and unexpected finding is that all 25 6C isolates included in the current study have 'short' *wzy* alleles lacking ATCGTG. In contrast, all 42 isolates expressing serotypes 6A and 6B that were included in this study have the normal-sized *wzy*. When we studied an additional 14 6C isolates (Park *et al.*, 2007a), we found that all of them have the 'short' *wzy* alleles (unpublished data). Furthermore, Mavroidi *et al.* (2004) studied 102 serogroup 6 isolates before serotype 6C was discovered and described two serogroup 6 isolates as expressing a 'short' *wzy* allele. We were able to test one of the two isolates and found that it expressed serotype 6C (unpublished observation). Thus, the 'short' *wzy* allele is very strongly associated with serotype 6C *cps*. However, the 'short' *wzy* is not essential for expressing the serotype 6C capsule because we were previously able to convert a 6A serotype strain to a 6C strain by replacing *wciN*$_\beta$ alone, without replacing *wzy* (Park *et al.*, 2007a).

Our studies shed new insights into the origins of serotype 6C *cps*. Previously, we proposed that a *wciN*$_\beta$ gene of an unknown origin (about 1500 bases) was inserted into the 6A *cps* with help from the two flanking regions (Park *et al.*, 2007a). Although a recombination event involving the serotype 33F *cps* and class 2 serotype 6B *cps* was also considered, a more likely possibility is that 6C *cps* was created by transfer of a large foreign gene segment spanning *wciN*$_\beta$ and the 'short' *wzy* to pneumococcus. The source of the foreign gene is unclear at the moment but it is likely to have come from bacteria in the nasopharynx, where pneumococci normally reside. Oral streptococcal species are logical candidates since they commonly coexist with pneumococci in the nasopharynx, often have capsules like pneumococci (Mavroidi *et al.*, 2007) and may have served as the source for antibiotic-resistance genes for pneumococci (Guerin *et al.*, 2000; Hakenbeck *et al.*, 1998). As we find limited heterogeneity in the sequences of serotype 6C *cps*, serotype 6C was probably created once. Thus, we may be able to deduce the *cps* of the 6C founder. We believe that the founder may have had a *cps* profile of 9-10-1 based on the frequency and diversity of the *cps* alleles (Table 2). While the knowledge of the 6C founder sequence with two genetic markers may help its identification, the bacterial gene pool in the nasopharynx is very large, indicating that the identification of the exact source of 6C *cps* would not be simple.

While determining the origin of serotype 6C *cps* requires additional studies, the *cps* of the two Korean 6D isolates appears to have been created by a genetic recombination between serotypes 6B and 6C: the *cps* of these two serotypes may have recombined between *wciN* and *wciP*. However, more 6D isolates should be studied before we can conclude that all 6D isolates arose in this manner. Recently, 6D isolates were found in Fiji (Jin *et al.*, 2009) and Finland (unpublished information). The *cps* profile of the Finnish 6D is 5-1-4. This is almost identical to the Korean 6D *cps* profile since alleles 4 and 1 of *wzx* differ in only 1 nt. The Fijian 6D isolates also have *wciP* allele 5 (Jin *et al.*, 2009) as do the Korean 6D isolates. This suggests that serotype 6D *cps* may have been created once and spread throughout the world. Given its potentially wide distribution, it is interesting to note that reports of serotype 6D are currently very rare and that it was not found in several large screens of clinical isolates (Bratcher *et al.*, 2009; Carvalho Mda *et al.*, 2009; Hermans *et al.*, 2008; Jacobs *et al.*, 2010). One of the reasons for the rarity of serotype 6D may be difficulties in distinguishing between serotypes 6C and 6D. The presence or absence of 'short' *wzy* may be useful for distinguishing them because, unlike 6C, both Korean and Finnish 6D have normal-sized *wzy*.

While serotype 6C *cps* profiles have remained quite distinct from those of other strains of serogroup 6 (Fig. 3), our data as well as those from others (Carvalho Mda *et al.*, 2009; Jacobs *et al.*, 2009) show that serotype 6C is often associated with STs associated with other members of serogroup 6. For instance, many 6C isolates possess STs of 395, 473, 1379 and 1390, which are the most common STs for serotype 6A. While there are multiple potential explanations for this situation, we favour the explanation that the entire *cps* can easily move among different pneumococcal backgrounds as already shown for serotype 19A (Brueggemann *et al.*, 2007). This movement may be easy because both ends of the *cps* have transposase-like regions whose exact function is still not well understood but may facilitate *cps* exchanges. Another explanation may be that two penicillin-resistance genes found at two extremes of the *cps* locus may facilitate the retention of transfers involving the entire *cps*, as was shown experimentally (Trzciński *et al.*, 2004). However, serotype 6C appears to preferentially share its STs with those of serotype 6A but not 6B or other serotypes (e.g. serotype 19A). More studies are needed to explain how pneumococcal *cps* can so easily yet selectively move among different genetic backgrounds.

Our studies significantly clarify the evolutionary origins of serogroup 6 *cps* loci (Fig. 4). Previous studies suggested two independent origins of serogroup 6 *cps*: *cps* of class 1 and class 2 isolates (Fig. 4a). Although class 1 6A and 6B isolates could be grouped into two clades, the two clades were not so distinct, and class 1 6A and 6B isolates were presumed to

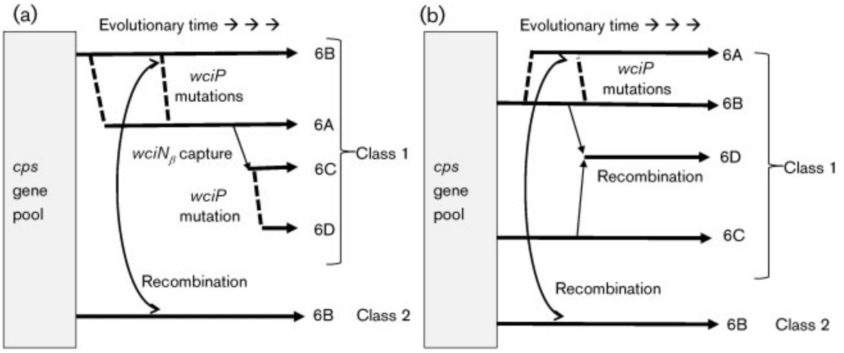


Fig. 4. Two models for the evolution of the serogroup 6 strains. (a) The model previously proposed by Mavroidi *et al.* (2004) modified to include the new serotypes 6C and 6D. (b) Proposed new model based on the new data. The nature of the '*cps* gene pool' from which the capsule genes have originated is undefined at the moment. Genetic recombination events are shown with thin arrows. Mutations are shown with dashed lines.

have a single origin since the two serotypes could interconvert with a single mutation (Fig. 4a) (Mavroidi *et al.*, 2004). Following the discovery of serotypes 6C and 6D, we then presumed that 6C *cps* arose from 6A *cps* by capturing the *wciN*$_\beta$ gene (Park *et al.*, 2007a) and that 6C *cps* became 6D *cps* by a somatic mutation (Fig. 4a). However, our data show that the clade for serotype 6C *cps* is distinct and it was probably produced by capturing a DNA fragment spanning the entire low G+C region from a currently ill-defined gene pool in the nasopharynx (Fig. 4b). Serotype 6D *cps* appears to have resulted from a recombination between 6B *cps* and 6C *cps* (Fig. 4b). These findings show that serogroup 6 *cps* loci, despite their high degree of sequence similarity, have at least three independent origins: for 6C *cps*, class 1 6B *cps* and class 2 6B *cps*. Perhaps these multiple independent origins are possible because the nasopharyngeal microbiome provides a large gene pool for pneumococcal *cps*, which allows *S. pneumoniae* to express a great variety of capsular structures.

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