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Characterization of the Type 8 Capsular Gene Cluster of *Streptococcus pneumoniae*

ROSARIO MUÑOZ,[1,2] MARTA MOLLERACH,[1,3] RUBENS LÓPEZ,[1] AND ERNESTO GARCÍA[1]*

Centro de Investigaciones Biológicas, CSIC,[1] and Pharma Mar,[2] Madrid, Spain, and Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina[3]

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The complete nucleotide sequence of the capsular gene cluster (*cap8*) responsible for the biosynthesis of the capsular polysaccharide of *Streptococcus pneumoniae* type 8 has been determined. The *cap8* gene cluster, located between the genes *dexB and aliA*, is composed of 12 open reading frames. A 14.7-kb DNA fragment embracing the *cap8* genes was sufficient to transform an unencapsulated type 3 *S. pneumoniae* strain to a strain with the type 8 capsule. A possible scenario for the evolution of pneumococcal types 2 and 8 is outlined.

Streptococcus pneumoniae is an important human pathogen that causes diseases such as pneumonia, bacteremia, meningitis, and otitis media. *S. pneumoniae* has a polysaccharide capsule that is considered the main virulence factor of this species and is involved in evasion of the host immune system (1). There are 90 distinct capsular serotypes (19), but most cases of pneumococcal pneumonia appear to be caused by a subset of 23 serotypes (11, 44). Efforts to develop a new generation of polysaccharide-protein conjugate vaccines are currently being made (8) because of the limited efficacy of the currently used 23-valent polysaccharide-based vaccine in infants as well as in immunocompromised patients. Nevertheless, technical, immunological, and economic reasons restrict the number of different capsular polysaccharides that can be incorporated into a conjugate vaccine (8). Moreover, it has been predicted that when several serotypes interact epidemiologically, as observed in *S. pneumoniae*, vaccination against one capsular type may increase carriage of a second type more than it decreases carriage of the first (29). Other possible long-term limitation to the use of conjugate vaccines is the well-known capacity of *S. pneumoniae* to change its capsular type via in vivo genetic transformation under laboratory conditions (15, 39) as well as in nature (10). Given all of these grounds, it is clear that an understanding of the molecular organization of the genes involved in capsular polysaccharide biosynthesis may facilitate the identification of potential targets and the design of capsule biosynthesis inhibitors. In this context, we have recently proposed that GalU, a pneumococcal UTP:glucose-1-phosphate uridylyltransferase that catalyzes the formation of UDP-glucose (UDP-Glc), represents one of such targets, since UDP-Glc is a key component in the biosynthetic pathway of pneumococcal capsular polysaccharides containing Glc, galactose (Gal), and/or glucuronic acid (GlcA) or galacturonic acid (32).

At present, nucleotide sequence data are available only for a limited number of types, namely, 1, 2, 3, 14, 19F, 19A, 19B, 23F, and 33F (4, 20, 24, 30, 33–36, 38, 40). Type 3 is the only case where the complete biosynthetic pathway for the formation of a capsular polysaccharide has been determined. Only two type 3-specific genes (*cap3A* and *cap3B*) are required for capsular biosynthesis (4). Cap3A is a UDP-Glc dehydrogenase (UDP-GlcDH) that converts UDP-Glc into UDP-GlcA (3, 5), whereas *cap3B* encodes a processive polysaccharide synthase (6). It has long been recognized that type 3 and type 8 polysaccharides cross-react (16–18). Structural analyses of both polysaccharides revealed that they have similar compositions; that is, one half of the type 8 polysaccharide consists of cellobiuronic acid (21), the repeating unit of type 3 capsule (41) (Fig. 1A). Type 8 polysaccharide is included in the 23-valent vaccine (42), and pneumococci of this serotype ranked ninth among sterile-site isolates (44). Furthermore, the relative risk of disease with type 8 pneumococcal strains increases at middle age. We describe here the complete nucleotide sequence of the *cap8* locus of a type 8 clinical isolate of *S. pneumoniae*.

The type 8 clinical strain of *S. pneumoniae* 6028, isolated in a Spanish hospital in 1995 from the blood of a patient, was kindly provided by A. Fenoll (Pneumococcus Reference Laboratory, Instituto de Salud Carlos III, Majadahonda, Spain). The unencapsulated ($S3^-$) mutant strain M24 (*cap3A*) (5, 12) was used as the recipient for intertype transformation. All of the experimental procedures used in this work have been recently described (30). These include conditions for growth, transformation, and serotyping of *S. pneumoniae*, DNA isolation and amplification by PCR, determination of the nucleotide sequence, and computer analysis of DNA and protein sequences. To amplify long fragments of DNA we used the oligonucleotide primers DEX86 (5′-TTGAACATATCGGTC TTCAGTATCAGGAAGGTCAGCC-3′) and D41 (5′-ACTG CCGCGTATTCTTCACC-3′), which are located, respectively, in the 5′ region of *dexB* (30) and in the complementary strand of the sequence (EMBL database accession no. Z83335) corresponding to the *aliA* gene in type 1 DNA (38).

According to results obtained previously for other serotypes (13, 14), the gene cluster (*cap*) responsible for pneumococcal capsular synthesis is located between *dexB* and *aliA*, two genes that do not participate in capsule formation. Consequently, we amplified DNA prepared from strain 6028 using oligonucleotide primers DEX86 and D41, and a DNA fragment of about 15 kb was obtained. When this fragment was used to transform the $S3^-$ pneumococcal strain M24, encapsulated type 8 transformants were found (data not shown), confirming that we were dealing with the locus responsible for type 8 capsular polysaccharide biosynthesis. Combined PCR and restriction mapping of the DNA prepared from one of these type transformants revealed the integration of the 12 type 8 genes into the type 3 recipient DNA (unpublished data). Sequence analysis of the amplified DNA fragment (14,730 bp) showed the presence of 12 complete and 2 partial open reading frames

* Corresponding author. Mailing address: Centro de Investigaciones Biológicas, CSIC, Velázquez 144, 28006 Madrid, Spain. Phone: (34) 81-5611800. Fax: (34) 91-5627518. E-mail: e.garcia@cib.csic.es.

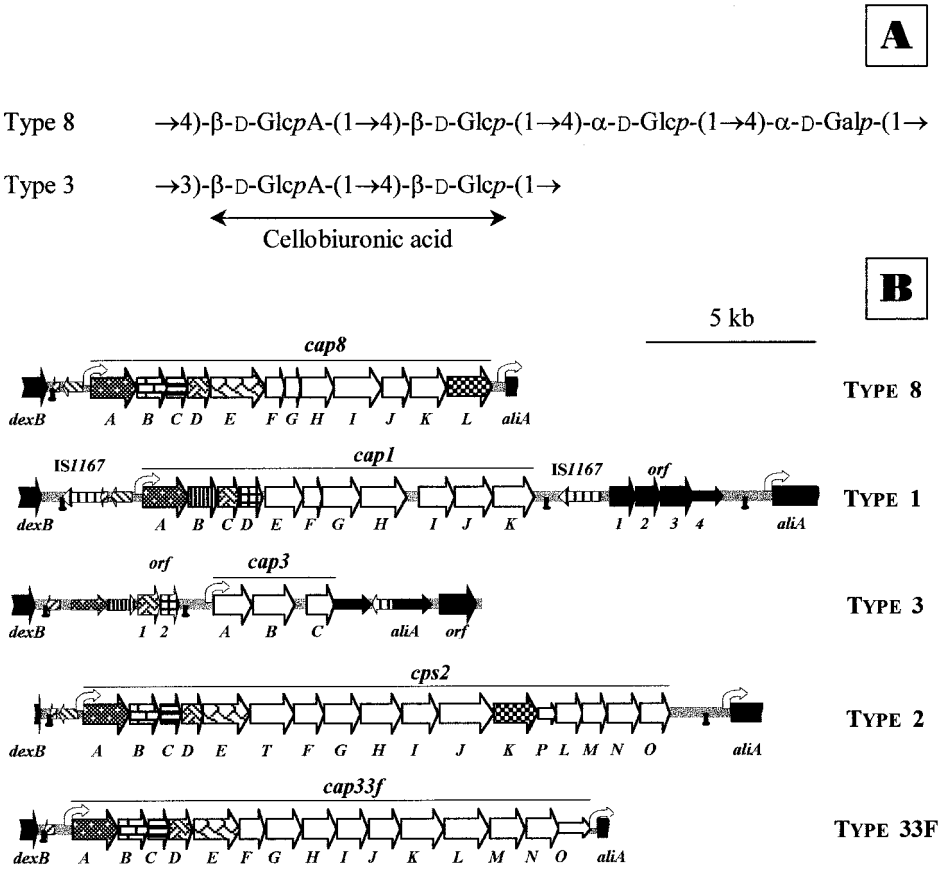


FIG. 1. (A) Structure of the repeating unit of the type 8 pneumococcal capsular polysaccharide (21). The corresponding structure of the type 3 capsule (41) is shown for comparison. (B) Genetic organization of the *S. pneumoniae* 6028/95 strain containing the *cap8* genes. The capsular gene clusters of types 1 (38), 2 (20), 3 (4), and 33F (30) are compared with type 8 in the text and are also included here. Small arrows correspond to interrupted genes. White and dashed arrows indicate capsular genes and putative insertion sequence elements, respectively. Capsular genes showing more than 90% identical nucleotides in comparisons of sequences of different serotypes are represented by identical shading. The locations of promoters (↱) and putative transcription terminators (🌢) are indicated.

(ORFs) (Fig. 1B). The two incomplete ORFs correspond to the genes *dexB* and *aliA*, as expected. The whole organization of the *cap8* locus closely matches that of *cap* (*cps*) loci previously studied (13, 14). However, significant differences were found when *cap8* and *cap3* gene clusters were compared (Fig. 1B), i.e., (i) between *dexB* and the *cap8* promoter (*cap8p*) there is a 351-bp region that is absent in type 3 DNA; this sequence might correspond to an insertion sequence-like element on the basis of sequence similarities (20); (ii) the *cap8p* region is intact and presumably functional (30), whereas in type 3 DNA, the −10 box of the promoter is completely missing (4); and (iii) the ORFs *cap8A* through *cap8D* are neither deleted nor mutated, in contrast to the situation reported for type 3 DNA.

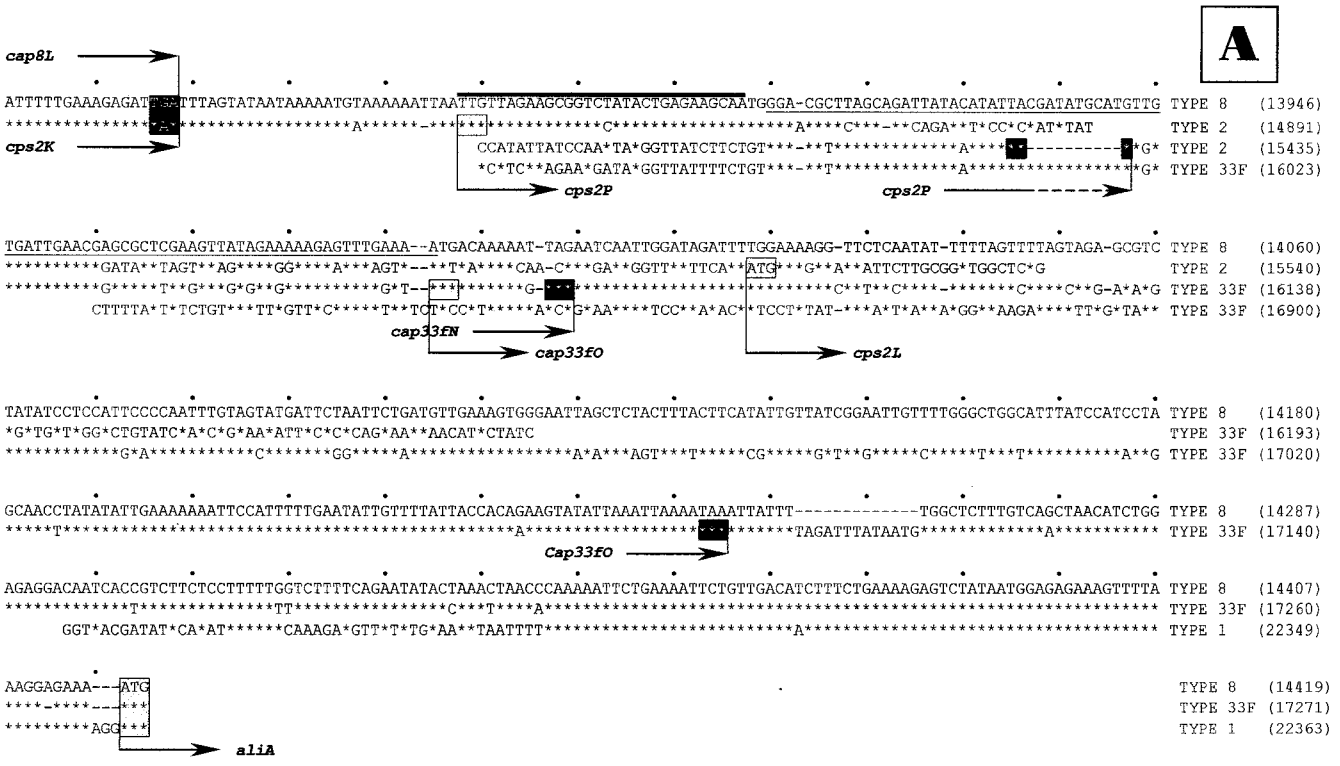
The fifth ORF of the cluster (*cap8E*) putatively codes for an undecaprenyl-phosphate Glc-1-phosphate transferase since the product of this gene is 84% similar to Cps14E, one of the few capsular gene products of *S. pneumoniae* that have been biochemically characterized (23). The highest similarities (>90% identity) were found when Cap8E was compared with Cps2E (20), Cps19aE (34), Cps23fE (33, 40), or Cap33fE (30) (Table 1). The genes *cap8G* and *cap8H* appear according to sequence similarities to encode glycosyltransferases (Table 1). Cap8F and Cap8G are similar to Cps14F and Cps14G, respectively. Cps14G is a β-1,4-Gal transferase responsible for the second step in the subunit synthesis of pneumococcal capsular polysaccharide type 14, that is, the transfer of Gal to lipid-linked Glc (25). The function of Cps14F is not clear, although the reduced glycosyltransferase activity detected in *Escherichia coli* transformants expressing *cps14E* and *cps14G* but lacking *cps14F* suggested that Cps14F has an enhancing role in glycosyltransferase activity (25). Cap8F and Cap8G also have sequence similarity with Orf5 and Orf6, two proteins involved in the synthesis of K40 capsular polysaccharide of *E. coli* that have been suggested to represent, respectively, the N- and C-terminal domains of a β-GlcA transferase (2). Moreover, it has been proposed that Orf6 encodes the catalytic domain of the enzyme since it contains a conserved amino acid (aa) sequence, termed domain A, and two separated catalytic Asp residues characteristic of β-glycosyltransferase (43). These features are also present in Cap8G, suggesting that this protein might be a β-GlcA transferase.

Cap8I is predicted to be a 55.3-kDa protein having 12 membrane-spanning regions. This finding together with sequence similarities (Table 1) strongly suggests that this protein is involved in transport of the type 8 repeating unit. Cap8K also appears to be an integral membrane protein because nine transmembrane segments can be predicted. Moreover, sequence comparison (Table 1) suggests that Cap8K may be the type 8 polysaccharide polymerase. The *cap8J* gene putatively codes for a 28.4-kDa protein of unknown function. A similar putative protein has been found in the capsular gene cluster of type 14 pneumococci (25).

TABLE 1. Type 8 capsular locus-encoded proteins: properties and similarities to proteins in the databases

Type 8 gene	Location in nucleotide sequence	Predicted protein (no. of aa/no. of kDa)	GRAVY[a] score	Proposed function or product	Similar polypeptide(s) (no. of aa)	Database accession no.	Degree of identity (%)/ degree of similarity (%)	Organism
cap8E	5782–7149	455/52.0	0.159	Undecaprenyl-phosphate glucose-1-phosphate transferase	Cap33fE (455)	O86888	96/97	*S. pneumoniae* type 33F
					Cps2E (455)	AAD10174	95/96	*S. pneumoniae* type 2
					Cps23fE (455)	AAC69528	94/95	*S. pneumoniae* type 23F
					Cps19aE (453)	AAC78667	92/95	*S. pneumoniae* type 19A
cap8F	7129–7633	165/19.5	0.002	Unknown	Orf5 (161)	O30630	37/58	*E. coli*
					Cps14F (149)	P72514	35/56	*S. pneumoniae* type 14
cap8G	7634–8113	159/18.2	−0.053	β-GlcA transferase	Cps14G (167)	P72515	34/60	*S. pneumoniae* type 14
					Orf6 (153)	O30631	33/52	*E. coli*
cap8H	8126–9190	354/40.0	−0.114	Galactosyltransferase	Cap33fG (346)	O86890	27/51	*S. pneumoniae* type 33F
					PH1844 (381)	O59512	30/52	*Pyrococcus horikoshii*
cap8I	9190–10650	486/55.3	0.818	Oligosaccharide repeat unit transporter	Cps19bJ (481)	O07870	33/56	*S. pneumoniae* type 19B
					Cps14L (487)	O07342	26/43	*S. pneumoniae* type 14
					Cap33fL (471)	O86895	25/52	*S. pneumoniae* type 33F
cap8J	10663–11391	242/28.4	−0.381	Unknown	C17G8.11C (356)	Q10323	35/49[b]	*Schizosaccharomyces pombe*
					Cps14K (282)	O07341	32/50[b]	*S. pneumoniae* type 14
cap8K	11375–12538	387/46.0	0.822	Polysaccharide polymerase	Cps23fG (397)	AAC69530	21/41	*S. pneumoniae* type 23F
cap8L	12607–13845	412/46.5	−0.250	UDP-GlcDH	Cps2K (412)	AAD10180	96/98	*S. pneumoniae* type 2
					Cap1K (410)	P96482	78/90	*S. pneumoniae* type 1
					Cap3A (394)	Q57346	57/74	*S. pneumoniae* type 3

[a] Grand average of hydropathy (26).
[b] Sequence similarity is restricted to the N-terminal part of the protein (aa positions 1 to 100).



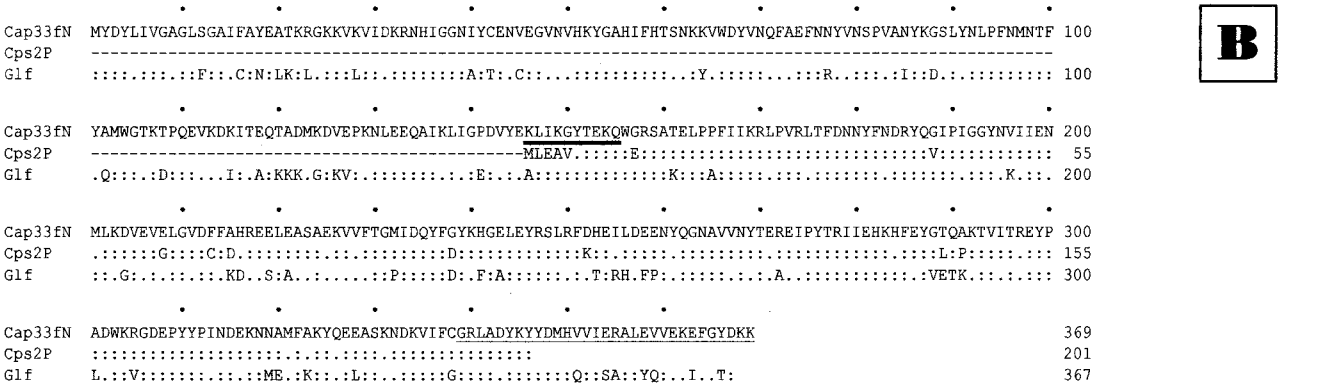


FIG. 2. (A) Multiple sequence alignment of the region located between the 3′ end of the *cap8* gene cluster and the *aliA* gene. Asterisks indicate identical nucleotides, and hyphens indicate gaps introduced to maximize similarity. Gray and black boxes indicate initiation and termination codons, respectively. Numbers at the right of each line correspond to the nucleotide positions of the sequences included in the data banks. (B) Multiple sequence alignment of the *cap33fN*, *cps2P*, and *glf* gene products. Colons and dots indicate identical and conserved amino acid residues, respectively. The amino acid sequences highlighted with either a thick line or underlining correspond to the translation of the corresponding sequence shown in panel A. Numbers on the right indicate amino acid positions.

Cap8L is a 412-aa UDP-GlcDH. Interestingly, the *cap8L* gene is 92% identical to the *cps2K* gene, which encodes the type 2 UDP-GlcDH. This was unexpected since, to date, the pneumococcal genes encoding this type of enzyme, namely, *cap3A* (5), *cap1K* (38), and *cps2K* (20), were noticeably divergent. Actually, the evolutionary distance between *cap1K* and *cap3A* is similar to that found between any of these pneumococcal genes and the *hasB* gene, which encodes a UDP-GlcDH of *Streptococcus pyogenes* (37).

The 573-bp-long *cap8L-aliA* intergenic region is one of the shortest segments found so far in this flanking region of the capsular gene clusters of *S. pneumoniae* (Fig. 2A). It is noteworthy that the similarity between *cap8L* and *cps2K* extends beyond the corresponding termination codons as far as the proposed 5′ end of the following gene of the *cps2* gene cluster (*cps2P*) (20). The proposal that Cps2P, suggested to be a UDP-galactopyranose (UDP-Gal*p*) mutase, participates in the biosynthesis of type 2 capsule is controversial since Gal*f* is not a structural component of this polysaccharide (45). In fact, a close examination of the nucleotide sequences of types 2, 33F, and 8 capsular gene clusters showed that *cps2P* is most probably a deleted version of an ancestral gene. This was particularly evident when Cps2P (201 aa residues), Cap33fN (369 aa residues) (30), and the *E. coli* UDP-Gal*p* mutase Glf (367 aa

residues) (27) were aligned (Fig. 2B). Csp2P apparently lacks the 151 N-terminal as well as the 23 C-terminal aa residues found in the other two proteins examined. Thus, the concept that *cps2P* starts with a TTG codon (20) cannot be supported. The corresponding region in type 8 pneumococci contains remnants of a gene coding for the 10 N-terminal aa residues of Cps2P (Fig. 2A and B) and the 32 C-terminal residues of Cap33fN (Fig. 2A and B). Figure 2A also shows that *cps2P* has undergone a 10-bp deletion that caused the appearance of a premature TAG termination codon and that the nucleotide sequence between positions 13906 and 14050 is very similar to the region containing the 3′ and 5′ ends of genes *cap33fN* and *cap33fO*, respectively (Fig. 1B). Further downstream (from position 14060 to 14255) the sequence was very similar to that of the 3′ end of the gene *cap33fO*, whereas from position 14344 to the ATG initiation codon of *aliA*, the type 8 sequence is almost identical to that of pneumococcal types 1, 2, 19F, 19A, and 23F, although only the sequence corresponding to type 1 is shown in Fig. 2A.

Pneumococcal polysaccharides of types 2, 19F, 19A, and 23F contain rhamnose (Rha) as a structural component of their capsular polysaccharides (45). On the other hand, it is noticeable that the four genes involved in the synthesis of dTDP-Rha were always found to be located at the right end of the capsular gene cluster (13). Interestingly, these four genes were also found to be located downstream of the *cap1* gene cluster in up to 19 different type 1 pneumococcal isolates examined (38) (indicated as *orf1* through *orf4* in Fig. 1B), although Rha is not a component of the type 1 polysaccharide. It has been suggested that the ancestor of the type 1 strains of *S. pneumoniae* might have been a strain of a Rha-containing serotype which, upon natural transformation with DNA from an unknown origin, retained the recipient dTDP-Rha-encoding genes, possibly as a consequence of an abnormal transformation event (38). In recent years, transformation of pneumococcal types has been documented as the underlying mechanism of capsular shifting in various clinical isolates (7, 9, 22, 31). Recently, the multiresistant Spanish clone 23F (MMSp23F) was shown to be transformed, on at least four separate occasions, to type 19F through recombination events that usually took place upstream of *dexB* and downstream of *aliA*. In two cases, however, the recombinational crossover point was identified within the genes involved in the synthesis of dTDP-Rha (10). Such transformation events can also be recognized by noting the presence of remnants, as in type 1 pneumococci, where an IS*1167*-like element is located between the 3′ end of the *cap1* cluster and the four genes originally involved in dTDP-Rha biosynthesis (38) (Fig. 1B). It is conceivable that the *cps2P* gene of type 2 pneumococci and part of the region located downstream of *cap8L* might also represent, respectively, remnants of the ancestors of type 2 and type 8 *S. pneumoniae* isolates. In a putative scenario, a type 33F pneumococcus might have been the parent of the contemporary type 8 strains whereas a strain having both Gal*f* and Rha in its capsule might have generated type 2 pneumococcal isolates. Both of these sugars are in fact components of the capsular polysaccharides of several pneumococcal types, i.e., 17A, 22F, and 31 (45). It is interesting that these capsules also contain GlcA in addition to Gal*f* and Rha.

The flexibility of pneumococci in exchanging capsular types is important since serious pneumococcal diseases can be caused by isolates expressing a considerable number of the 90 different capsular polysaccharides (44). The conserved organization of the *cap8* locus with respect to the *cap* loci previously reported undoubtedly facilitates switching of *cap8* to other capsular gene clusters. In this sense, the results reported here add most-needed information on genetic variability of the genes governing capsule formation in types that are potential candidates for the conjugate vaccine formulations currently under experimentation. Frequent recombinational events occurring at the capsular polysaccharide biosynthesis loci facilitate clonal variability and should represent a noticeable limitation for a worldwide use of these newly developed vaccines. In trials of pneumococcal vaccines type replacements which may increase the incidence of disease from serotypes not included in the vaccine have already occurred (28). A detailed molecular knowledge of the different pneumococcal types might provide insights in future approaches to controlling this variability by selecting the most appropriate strains to diminish serotype replacement.

Nucleotide sequence accession number. The nucleotide sequence of the *cap8* locus has been deposited in the EMBL, GenBank, and DDBJ databases under nucleotide sequence accession no. AJ239004.

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