A Single Gene (*tts*) Located outside the *cap* Locus Directs the Formation of *Streptococcus pneumoniae* Type 37 Capsular Polysaccharide: Type 37 Pneumococci Are Natural, Genetically Binary Strains

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Summary

The molecular aspects of the type 37 pneumococcal capsular biosynthesis, a homopolysaccharide composed of sophorosyl units (β-d-Glc-(1→2)-β-d-Glc) linked by β-1,3 bonds, have been studied. Remarkably, the biosynthesis of the type 37 capsule is driven by a single gene (*tts*) located far apart from the *cap* locus responsible for capsular formation in all of the types characterized to date in *Streptococcus pneumoniae*. However, a *cap37* locus virtually identical to the *cap33f* cluster has been found in type 37 strains, although some of its genes are inactivated by mutations. The *tts* gene has been sequenced and its transcription start point determined. Tts shows sequence motifs characteristic of cellulose synthases and other β-glycosyltransferases. Insertion of the *tts* gene into the pneumococcal DNA causes a noticeable genome reorganization in such a way that genes normally separated by more than 350 kb in the chromosome are located together in clinical isolates of type 37. Encapsulated pneumococcal strains belonging to 10 different serotypes (or serogroups) transformed with *tts* synthesized type 37 polysaccharide, leading to the formation of strains that display the binary type of capsule. Type 37 pneumococcus constitutes the first case of a natural, genetically binary strain and represents a novel alternative to the mechanisms of intertype transformation.

Key words: binary capsulation • capsule • glucosyltransferase • pneumococcus • sophorose

Microbial pathogens have developed a great variety of strategies to overcome host cell defenses and ensure their own survival and expansion. These strategies have become extremely accurate in the case of pathogens that have kept a close association with their host (1). *Streptococcus pneumoniae* (pneumococcus) has evolved as a microorganism highly adapted to and dependent on its human host and is currently considered the most dangerous vehicle, causing conditions from otitis media and sinusitis to pneumonia, septicemia, and meningitis (2). Pneumococcal disease accounts for more deaths than any other vaccine-preventable bacterial disease (3). The capsular polysaccharide has been identified as the main virulence factor of pneumococcus. There are at least 90 different capsular types, although only a subset of 23 types causes more than 90% of invasive disease worldwide (2). The use of a 23-valent polysaccharide-based vaccine has turned out to be quite limited to protecting those segments of the population that are extremely sensitive to invasion by pneumococcus (e.g., children under three years old and the elderly).

Recent studies have provided insights on the gene cluster (*cap*) involved in capsular formation in *S. pneumoniae*. This cluster has been characterized at a molecular level in the case of types 1, 3, 14, 19F, 19B, 23F, and 33F (4–11). All of the *cap* clusters characterized so far are placed between the *dexB* and *aliA* genes, with a central region embracing those genes responsible for the synthesis of the type-specific capsule and flanked by open reading frames (ORFs)[1] that share, in most cases, homology among all of the types described so far. The number of genes involved in type-specific capsule formation varies according to the chemical complexity of the capsule, whereas the biological role of the ORFs flanking the specific genes remains to be determined (12). More recently, we have found that *galU*, a gene located outside of the *cap* locus and encoding a uridine diphosphoglucose pyrophosphorylase, is essential for capsular polysaccharide biosynthesis, at least in type 1 and 3 pneumococci (13).

Shifting from one capsular type to another (intertype transformation) was suggested to happen in nature and has been repeatedly demonstrated in the laboratory (for review see reference 14). More recently, detailed molecular analysis of the *cap* locus has revealed that capsular changes are

[1]*Abbreviations used in this paper:* aa, amino acid(s); IS, insertion sequence; Ln, lincomycin; ORFs, open reading frames; PFGE, pulsed-field gel electrophoresis; *p*, gene promoter; UDP-Glc, uridine diphosphoglucose.

quite frequent between the most virulent clinical isolates of pneumococci (15, 16). The strategy used to carry out interype transformation is based on the complete interchange of large DNA fragments (from 14 to 22 kb long) between different capsular types, taking advantage of the similarity found in the ORFs flanking the capsular-specific genes. The frequent presence in the flanking regions of insertion sequence (IS) elements might also promote this type of interchange and suggests that the capsular cluster could behave as a pathogenicity island. In other bacterial pathogens, it has been suggested that ISs might facilitate the evolution and adaptation of microorganisms to their host's environment by using a kind of 'quantum leap' evolution that leads to rapid changes (17), as could be the case for the pneumococcal capsule.

In this paper, we describe a novel strategy used by *S. pneumoniae* to synthesize the type 37 capsule. This strategy implies the participation of a single gene (*tts*) to direct the formation of an abundant capsular envelope that is composed of sophorosyl units (β-d-Glc-(1→2)-β-d-Glc) interlinked through β-1,3 bonds (18). The *tts* gene responsible for the formation of this capsule was located outside of the *cap* cluster and characterized. Our work also illustrates an extremely simplified strategy that pneumococcus has developed to direct the formation of its main virulence factor, which contributes in a fundamental way to the survival of this pathogenic microorganism in humans.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions. We used the following unencapsulated laboratory *S. pneumoniae* strains: M24 ($S3^-$; reference 19), M29 ($S1^-$; reference 4), and M31 (Δ*lytA*; $S2^-$; reference 20). The type 37 clinical isolates were purchased from the Statens Seruminstitut (strain 7077/39) or provided by A. Fenoll (Spanish Pneumococcal Reference Laboratory, Majadahonda, Spain; strains 1235/89 and 975/96), who also provided most of the other encapsulated pneumococci used in this work. The number after the shill indicates the year of isolation of the corresponding strain. When working with *Escherichia coli*, strains DH5α (21) and C600 (22) were employed. Growth and transformation of laboratory strains of *S. pneumoniae* and *E. coli* was performed as previously described (13). Clinical pneumococcal isolates were transformed after the procedure of Håvarstein et al. (23) using a competence-inducing peptide provided by D.A. Morrison (Department of Biological Sciences, University of Illinois at Chicago, IL). *S. pneumoniae* clones obtained upon transformation with derivatives of pLSE1 (*tet ermC*; reference 24) were scored on blood agar plates containing 0.7 μg of lincomycin (Ln) per milliliter. Plasmid pLSE4 is a promoter-probe vector able to replicate in *S. pneumoniae* and *E. coli* that contains a promoterless *lytA* gene (25). Plasmid pUCE191 has been described elsewhere (5).

DNA Techniques and Plasmid Construction. DNA manipulations and standard molecular biological methods were performed as described by Sambrook et al. (22). *S. pneumoniae* DNA digested with either SmaI, SacII, or ApaI was analyzed by pulse-field gel electrophoresis (PFGE) using a contour-clamped homogeneous electric field DRII apparatus (Bio-Rad Labs.) as previously described (26). Primer-extension mapping of the transcription initiation site was carried out as previously described (4).

PCR amplifications were performed as previously described (11). Conditions for amplification were chosen according to the G plus C content of the corresponding oligonucleotides. The oligonucleotide primers mentioned in the text were: (OL82) 5′-TCAGCcCgGgTCATTATCAACCAAAC-3′; (OL83) 5′-CAAGTcccGGGATGCAGTTTATGC-3′; (D5) 5′-CAGCCcgGGCTTTTTCTGGATTGTAAAGACCATCCTG-3′; (D62) 5′-TTACGAGATGATCAACGTCCAAGAAGCGCTGGC-3′; (D90) 5′-CAGACCTTGTTTCTGACTCCAC-3′; (D91) 5′-ATCGTGTAGGTGCAGCTCCG-3′; (D101) 5′-TTTGACCAAGCTTACACTTCAG-3′; (D109) 5′-ATCGTAccGcGgAAACTGAAAAGAAGGATAG-3′; (D112) 5′-TCTCATATTCTAgaCTTCTTTTCAGTTTACAC-3′; and (D116) 5′-TCCTTACCATACaTCgATACTAAC-3′. The oligonucleotide primer OL62 (5′-CGCTTCATTCTGTACGGTTGAATGCGG-3′) has been previously described (4). Lowercase letters indicate nucleotides introduced to construct appropriate restriction sites. These are underlined.

Plasmid pDLP37 was constructed by cloning a 1.7-kb SphI–NheI DNA fragment of strain 1235/89 containing the *tts* gene into pUC19 previously digested with SphI and XbaI. Plasmid pDLP40 contains a 1.7-kb SphI–KpnI DNA fragment of pDLP37 embracing the *tts* gene, inserted into an EcoRI-deficient pUC18 previously treated with the same enzymes. The latter plasmid was constructed by digesting pUC18 with EcoRI, filling in with the Klenow (large) fragment of the *E. coli* DNA polymerase, and self-ligation. We used PCR to amplify the *ermC* gene from plasmid pLSE1 using oligonucleotide primers OL82 and OL83. This promoterless gene was digested with SmaI and cloned into EcoRI-digested pDLP40. Before ligation, the EcoRI site located in the *tts* gene had been filled in as described above. Plasmid pDLP41 was isolated among the erythromycin-resistant transformants of *E. coli* DH5α. Plasmid pDLP43, containing a promoterless *tts* gene placed downstream of the *tet* gene of the pLSE1 vector, was constructed as follows: DNA prepared from strain 1235/89 was PCR amplified using oligonucleotide primers D109 and D116. The amplified product was filled in, digested with ClaI, and ligated to pLSE1 previously treated with EcoRV and MspI.

NEBlot™ Phototope™ Kit (Millipore Corp.) was used to construct biotin-labeled probes and Phototope™ 6K Detection Kit (Millipore Corp.) was used for chemiluminescent detection. Southern blots, dot blots, and hybridizations were carried out according to the manufacturer's instructions.

Nucleotide Sequence and Data Analysis. DNA sequencing was carried out by using an Abi Prism 377™ DNA sequencer (Applied Biosystems, Inc.). DNA and protein sequences were analyzed with the Genetics Computer Group software package (version 9.0; reference 27) or using the programs indicated in the text that are available at the internet address specified below. Sequence similarity searches were performed using the EMBL/GenBank, SWISS-PROT, and PIR databases. Preliminary sequence data of the *S. pneumoniae* genome were obtained from The Institute for Genomic Research at http://www.tigr.org.

Miscellaneous Techniques. Pneumococcal transformants harboring pLSE4-derived plasmid were scored on Ln-containing plates using a filter technique to distinguish the LytA phenotype (28). Immunoagglutination using anti-R serum (29) or coagglutination assays with type antisera purchased from the Statens Seruminstitut were carried out as previously described (11). Typing by the Quellung technique was carried out by L. Vicioso (Spanish Pneumococcal Reference Laboratory, Majadahonda, Spain).

Nucleotide Sequence Accession Numbers. The sequence data reported here have been submitted to the EMBL/GenBank/DDBJ databases under accession numbers AJ131984 and AJ131985.

Results

Type 37 Pneumococcal Strains Possess a Cryptic cap33f Locus. Long PCR using oligonucleotide primers D62 (*dexB*) and D5 (*aliA*) and DNA prepared from three different type 37 pneumococcal clinical isolates produced 20-kb DNA fragments that were apparently identical to each other (Fig. 1 A). The amplified DNA fragment obtained from strain 1235/89 was completely sequenced (20,133 bp) and compared with the sequences available in the databases. High similarity (>97% identity) was found throughout the entire sequence between the *cap37* locus and the *cap33f* cluster recently described (reference 11; Fig. 1 B). Most interesting, mutations interrupting the reading frames were found in *cap37B*, *cap37E*, *cap37N*, and *cap37O*, suggesting that none of these genes is required for type 37 capsule biosynthesis.

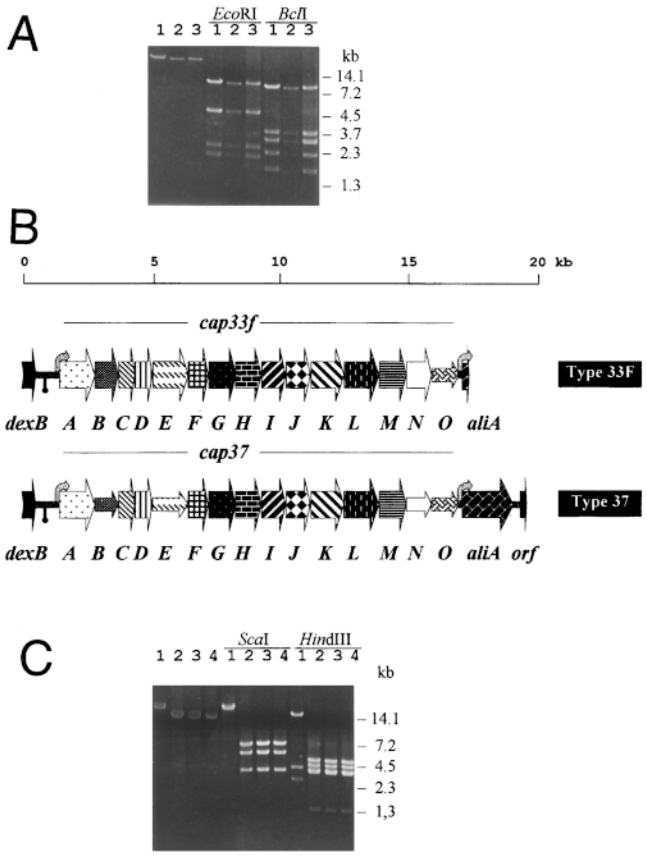

Figure 1. Molecular characterization of the capsular gene locus of type 37 *S. pneumoniae*. (A) Long PCR amplification and restriction enzyme analysis of three different type 37 isolate DNAs, namely, 1235/89 (lane 1), 975/96 (lane 2), and 7077/39 (lane 3). (B) Genetic organization of the *cap37* cluster of the 1235/89 strain. The gene organization of the *cap33f* locus (reference 11) is shown for comparison. Small arrows correspond to interrupted ORFs. Genes showing at least 95% identical nucleotides are indicated by identical shadings. The location of promoters (curved gray arrow) and of a putative transcription terminator (bold exclamation point) is indicated. (C) Type 37 laboratory transformants have not changed their recipient *cap3* locus. PCR-amplified and restricted DNAs were prepared from the type 37 strain 1235/89 (lane 1), the S3$^-$ M24 strain (lane 2), and two different S37$^+$ transformants of M24, strains DN2 (lane 3) and DN5 (lane 4). The positions of some of the BstEII-digested λ DNA fragments used as size standards are indicated at the right of A and C.

These mutations were confirmed by repeated sequencing (at least three times) of different PCR-amplified products. The great number of genes found in the *cap37* locus was unexpected, as type 37 polysaccharide is, as reported above, very simple and, in all the cases documented so far in the literature, there was a direct relationship between the size of the *cap* cluster and the chemical and structural complexity of the corresponding capsular polysaccharide (12). It would be conceivable, however, that the observed inactivation of some of the genes of the locus might result in a polysaccharide simpler than that of type 33F. If this was the case, transformation of *S. pneumoniae* with the 20-kb PCR fragment containing the *cap37* genes should have shifted the capsule type of the recipient strain to that of type 37. However, we never found type 37 transformants when using competent cells of strains M24 (S3$^-$) or M29 (S1$^-$) as recipient bacteria for the 20-kb type 37 DNA (data not shown). Moreover, when the *cap* locus from strains DN2 or DN5 (two independently isolated type 37 transformants of strain M24 obtained by using chromosomal DNA prepared from strain 1235/89) was amplified by PCR using oligonucleotides D62 and D5, the length as well as the restriction enzyme profile of the amplified PCR DNA fragments corresponded to that of the recipient S3$^-$ strain (M24) and not to the donor DNA (Fig. 1 C). In addition, no amplification was obtained using DNA from DN2 or DN5 and any pair of internal oligonucleotide primers designed on the basis of the *cap37* sequence (data not shown). Taken together, these results strongly suggested that additional genes located outside the *cap37* locus were required for transformation to the type 37 phenotype (S37$^+$).

A Single Gene (tts) Transforms S. pneumoniae to the S37$^+$ Phenotype. To localize the gene(s) responsible for the synthesis of the type 37 capsule, DNA prepared from strain 1235/89 was digested with several restriction endonucleases, and the fragments were separated by electrophoresis on 0.7% low-melting-point agarose gels. DNA fragments of various sizes were purified and used to transform competent cells of M24 (S3$^-$) to the type 37 capsule. S37$^+$ transformants were observed using as donor material fragments of ~7 kb when DNA from strain 1235/89 was digested with PstI. Afterwards, a ligation mixture containing 7-kb PstI DNA fragments from strain 1235/89 and PstI-digested pUCE191 was used to transform competent M24 cells. Several S37$^+$, Ln-resistant transformants were isolated, and one of them (strain C2) was used for subsequent study. Transformation experiments using chromosomal DNA prepared from strain C2 demonstrated that the *ermC* marker was genetically linked to the gene(s) responsible for the synthesis of the type 37 polysaccharide. Afterwards, C2 DNA was digested with restriction endonucleases without target sequences in pUCE191 (indicated by X in Fig. 2), namely BglII, EcoRV, Eco47III, MunI, or SpeI, diluted and self-ligated. The ligation mixture was used for PCR amplification with the direct and reverse M13/pUC primers. Amplified DNA fragments were found exclusively with the

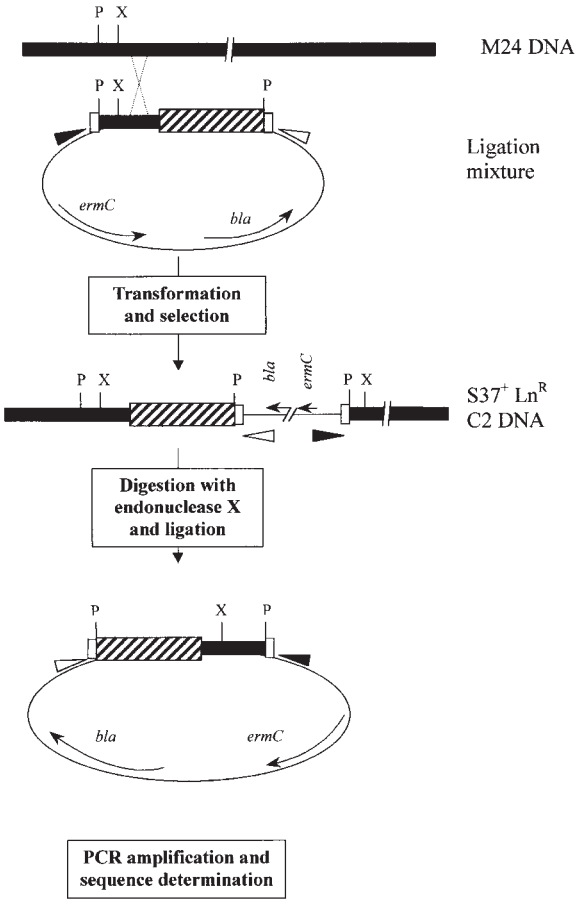

Figure 2. Schematic representation of the procedure used for determination of the sequence of the ends of the PstI restriction fragment containing the gene(s) responsible for type 37 capsular polysaccharide biosynthesis. The type 37–specific DNA is indicated by a hatched bar. The open rectangles depict the polylinker region of pUCE191. Open and solid triangles represent the direct and reverse M13/pUC oligonucleotide primers, respectively. Ln^R, Ln-resistant; P, PstI; X, any restriction site not present in pUCE191.

EcoRV and MunI digestions (not shown). Determination of the nucleotide sequence beyond the PstI sites served to design a pair of oligonucleotide primers (D90 and D91) that were used for PCR amplification of DNA prepared from strain 1235/89. Those primers produced a fragment of ~7 kb that was capable of transforming the $S3^-$ strain M24 to the $S37^+$ phenotype (not shown). In addition, identical fragments were produced when DNAs prepared from the type 37 strains 975/96 and 7077/39 were used as substrates for PCR amplification. These amplified DNA fragments were also able to transform the M24 strain to the type 37 capsule (not shown). The amplified DNA fragment obtained from strain 1235/89 was completely sequenced, and a schematic representation of the results is shown in Fig. 3 A. The nucleotide sequence of the PstI fragment (7,311 bp) was compared with a partial (and still preliminary) nucleotide sequence of the genome of a type 4 pneumococcal strain (see Materials and Methods). Surprisingly, from positions 1 to 1,479, the sequence matched part of contig sp_14 (Fig. 3 B), in particular that containing a gene (*gpmA*) putatively encoding a protein highly similar (64.3% identity and 76.6% similarity) to the phosphoglyceromutase (GpmA) of *Haemophilus influenzae*. However, from nucleotide 5,298 to the end of the PstI fragment, the sequence was virtually identical to part of contig sp_58 (that located immediately downstream of the TAA termination codon of the *metE* gene) and putatively codes for a protein that is 66% identical (80.7% similar) to the PyrDA dihydroorotate dehydrogenase of *Lactococcus lactis*, and for a partial ORF (*orfY*) of unknown function (Fig. 3 C). Upstream of the *pyrDA* gene, a 105-bp repeat element characteristic of *S. pneumoniae* (4) was found. There is no data indicating the distance between both contigs, but it can be estimated to be >22 kb, that is, the smallest distance between *gpmA* and the right end of contig sp_14. The apparently anomalous structure of the PstI fragment will be discussed in detail below.

From nucleotide 3,834 to 5,297 of the PstI fragment obtained from strain 1235/89 DNA, a copy of the IS element IS*1167* (30) was found (Fig. 3 A). The trp_{1167} gene should encode a defective transposase because it contains a frameshift mutation. From nucleotide position 3,706 to 3,833, the sequence is identical to that found 3 bp downstream of the TAA termination codon of *gpmA* in contig sp_14, strongly suggesting that this region represents the integration site of the type 37–specific sequences.

The only gene in the whole 7-kb PstI fragment from strain 1235/89 that showed no similarity to any other present in the *S. pneumoniae* database was named *tts*. Upstream of the ATG initiation codon, a putative promoter (*ttsp*) was found (TTGATA–17 bp–TATAAT). An extended −10 promoter motif, TtTG, characteristic of the −16 region of *S. pneumoniae* (31) was also observed. On the other hand, another copy of the 105-bp repeat element characteristic of *S. pneumoniae* (reference 4; see above) was located further upstream. Both repeats are 71.7% identical and oppositely oriented. The *tts* gene putatively codes for a protein of 509 amino acid (aa) residues with a predicted M_r of 58,888. Six transmembrane regions could be anticipated for Tts using different prediction programs, suggesting that the protein targets to the membrane. The aa sequence positions for these predicted transmembrane helices are A (aa 11–33), B (aa 45–63), C (aa 347–369), D (aa 378–400), E (aa 407–429), and F (aa 483–505). The central part of the protein is more hydrophilic and is predicted to reside in the cytoplasm and contain the catalytic site(s). Two independent predicting methods (SignalP V1.1 and PSORT) were used to test whether Tts possesses a signal peptide, and both methods strongly suggested that this was indeed the case. The possible cleavage site was predicted to be located between residues 36 and 37 or 32 and 33 depending on the program used. The putative signal peptide coincides with transmembrane helix A. On the other hand, we have also determined the complete nucleotide sequence of the *tts* gene of the other two clinical type 37 isolates, strains 7077/39 and 975/96, and observed that the three *tts* genes were identical (not shown). As the type 37 clinical strains studied here were isolated in different geographic locations and one of them as early as in

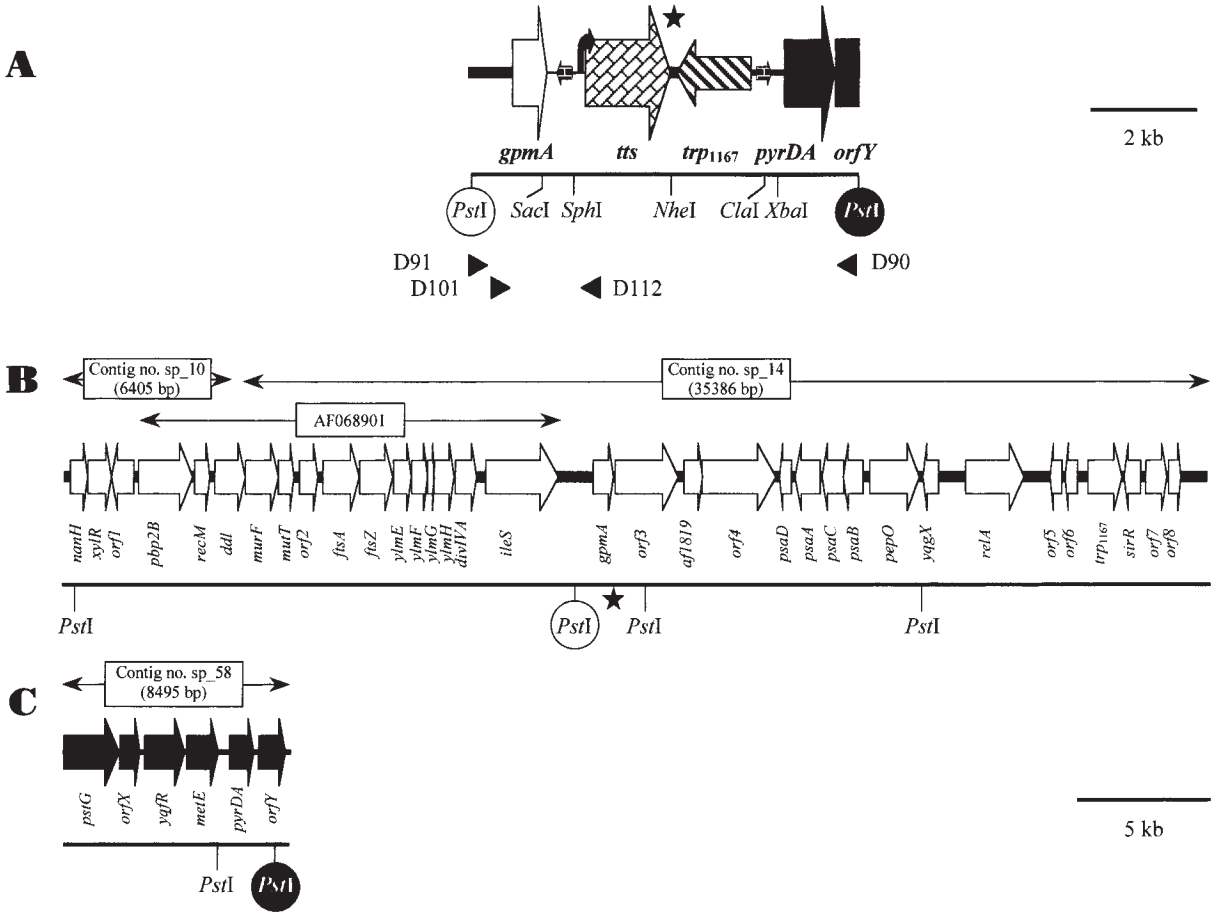

Figure 3. Genetic organization of the type 37–specific polysaccharide synthase gene (*tts*) and its surrounding regions. (A) Localization of *tts* and flanking genes in type 37 clinical strains. The thin hatched arrow corresponds to the interrupted gene encoding the IS*1167* transposase. The small divergent arrows show the location of a 105-bp repeat element characteristic of pneumococcus. The black star indicates the position of a 128-bp sequence where integration of the *tts* gene occurred. The location of *ttsp* is indicated (black curved arrow). Several pertinent restriction sites and oligonucleotide primers (triangles) are depicted. B and C show the gene structure of several contigs of the *S. pneumoniae* chromosome as deduced from a preliminary sequence (see text). Whenever possible, genes are identified by the designation of their most similar homologue. The nucleotide sequence of the indicated DNA fragment that has been recently reported (54) is available from EMBL/GenBank/DDBJ under accession number AF068901. The PstI sites flanking the *tts* gene and relevant to this study are circled.

1939, this finding illustrates the noticeable genetic stability of the *tts* gene.

To ascertain that the *tts* gene is responsible for the synthesis of the type 37 capsule, insertion-inactivated mutants were constructed using pDLP41 to transform competent cells of the S37$^+$ pneumococcal strain DN2. Plasmid pDLP41 contains the gene *ermC* inserted into the *tts* gene (see Materials and Methods). One of the Ln-resistant transformants was used for further study (strain DN21). The accuracy of the construction was checked by restriction analysis of the PCR-amplified products of DN21 and DN2 DNAs using oligonucleotide primers D90 and D91 (Fig. 4). Cells of strain DN21 were shown to be unencapsulated, as deduced from the failure of the type 37 antiserum to agglutinate them. Moreover, these transformants deposited at the bottom of the test tube when grown in liquid medium and agglutinated with anti-R serum (not shown). On the other hand, when competent DN21 cells were transformed with pDLP43 containing exclusively *tts* gene cloned into pLSE1, S37$^+$ transformants were isolated (not shown). All of these results indicated that Tts is the type 37–specific polysaccharide synthase.

Identification of the tts Promoter and the Transcription Start Point. To determine whether the proposed promoter sequence (see above) actually represents *ttsp*, a DNA fragment containing the putative promoter was amplified using oligonucleotide primers D101 and D112 (Fig. 3 A). After digestion with SphI and XbaI, the fragment (198 bp) was ligated to pLSE4 previously treated with the same enzymes and used to transform competent cells of the pneumococcal M31 strain. Plasmid pLSE4 is a promoter-probe vector able to replicate in *S. pneumoniae* and *E. coli* that contains a promoterless *lytA* gene (25). LytA$^+$ cells, detected among the Ln-resistant M31 (Δ*lytA*) transformants, contained a recombinant plasmid designated pDLP36. Crude sonicated extracts of M31 cells harboring pDLP36 contained LytA activity (~12 U/mg of protein; data not shown), which proved the presence of a functional promoter in the cloned fragment.

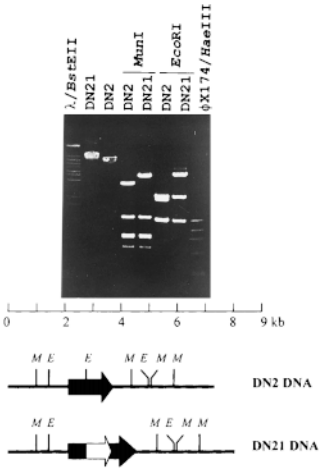

Figure 4. Agarose gel electrophoresis demonstrating the insertion–duplication mutagenesis of the *tts* gene. Strain DN21 contains the *ermC* gene (open arrow) inserted into the DN2 *tts* gene (solid arrow). The wild-type and mutant genes were PCR amplified using oligonucleotides D90 and D91 and digested with MunI (*M*) or EcoRI (*E*).

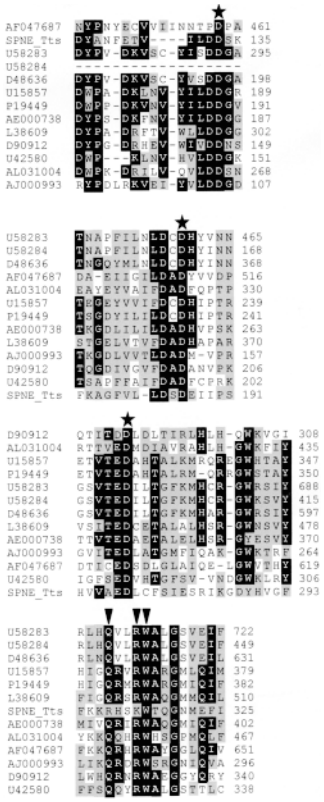

Figure 6. Computer-generated alignment (PILEUP) of selected regions of the Tts synthase (SPNE_Tts) and several cellulose synthases and other glucosyltransferases. Stars indicate the conserved aspartic acid residues, and solid triangles indicate the QXXRW motif reported to be critical for UDP-Glc binding and/or catalysis (34). Residues in black boxes indicate aa residues identical in at least 7 out of the 13 proteins aligned. Conserved aa substitutions are shown in gray. The accession numbers of the selected proteins are also indicated: U58283 (*Gossypium hirsutum* CelA1); U58284 (*G. hirsutum* CelA2); D48636 (*Oryza sativa* CelA); U15857 (*A. xylinum* AcsAII): P19449 (*A. xylinum* BcsA); L38609 (*A. tumefaciens* CelA); AE000738 (*Aquifex aeolicus* BcsA); AL031004 (*Arabidopsis thaliana* ATF28M20); AF047687 (*Bradyrhizobium japonicum* NdvB); AJ000993 (*L. lactis* OrfD); D90912 (*Synechocystis* subspecies S11377); and U42580 (*Paramecium bursaria* Chlorella virus A473L).

To demonstrate that *ttsp* was actually located in this region, the transcription start point was mapped by primer extension of the oligonucleotide OL62. This analysis (Fig. 5) showed that the transcription of the *tts* gene initiates 9 nucleotides after the −10 consensus sequence.

Tts Appears To Be a β-Glucosyltransferase. The deduced aa sequence of the *tts* gene was compared with the sequences available in the databases. Using COG (Clusters of Orthologous Groups) analysis (32), sequence similarities suggested that Tts might be a member of the group of glycosyltransferases involved in cell wall biogenesis, whereas BLASTP showed moderate similarity with cellulose synthases. In particular, Tts exhibits significant similarities (Fig. 6) in the regions recently shown to be highly conserved among plant as well as bacterial cellulose synthases and several other glucosyltransferases (33). These conserved motifs have previously been suggested to be critical for catalysis and/or binding of the substrate uridine diphosphoglucose (UDP-Glc; reference 34).

Genomic Reorganization Caused by Intertype Transformation in Type 37 Pneumococcal Strains. The *tts* gene from the type 37 clinical strains has been shown to reside in a 7-kb PstI fragment that, apparently, might be the result of a profound reorganization of the genome. This assumption was based on the finding that the genes flanking *tts* reside in two different contigs, namely sp_14 and sp_58, that are located far apart on the partially sequenced genome of a type 4 pneumococcal strain. This also appears to be the case for the laboratory strain M24, a late descendant of the classical R6 strain (19), as repeated attempts to amplify M24 DNA using oligonucleotides D90 and D91 and the long PCR technique were unsuccessful (data not shown). On the other hand, PCR amplification experiments using DNA prepared from either DN2 or DN5, two type 37 transformants of the M24 strain, and the same oligonucleotide primers only rendered a PCR product in the case of DN2 DNA. Interestingly, restriction enzyme analysis showed that the amplified DN2 DNA fragment was identical to that of the 7-kb PstI fragment of the parental clinical strain 1235/89 DNA (not shown).

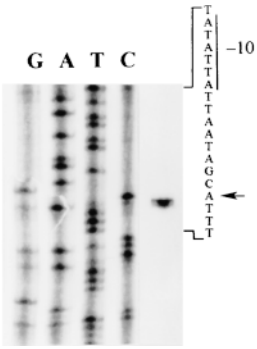

Figure 5. Primer-extension mapping of the transcription initiation site for the *tts* gene. Total RNA was extracted from a culture of M31 harboring pDLP36. The final products were loaded on a 6% polyacrylamide 7 M urea sequencing gel, in parallel with a sequencing reaction using the same oligonucleotide primer (OL62) and pDLP36. The major extended product is indicated by an arrow, and the −10 consensus sequence of *ttsp* is also shown. Note that the indicated sequence corresponds to the coding strand.

PFGE is a powerful tool to distinguish among isolates of *S. pneumoniae* due to the great polymorphism exhibited by the DNAs of different pneumococcal strains (35). Unfortunately, this polymorphism precludes the use of DNA prepared from clinical isolates to directly locate any gene, because only the physical map of the Avery's R6 strain (36) has been worked out (37, 38). As previously reported (26), two different DNA fragments were generated by digestion of M24 DNA with either ApaI or SacII with respect to those produced in R6 DNA, whereas both strains have identical SmaI profiles. Fig. 7 A shows a partial physical/genetic map of the M24 chromosome. When analyzed by PFGE, identical profiles were observed for M24 and DN5 DNAs digested with ApaI, SacII, or SmaI (Fig. 7 B). However, DN2 DNA showed altered bands with all three enzymes used, indicating that genomic reorganization did occur during transformation of the $S3^-$ recipient strain M24 to the $S37^+$ phenotype. It should be stressed that, for in-

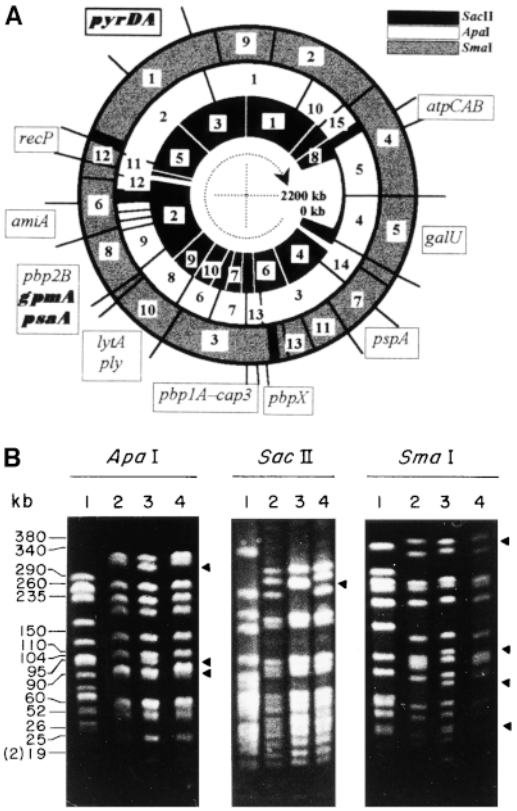

Figure 7. (A) Partial physical and genetic maps of the *S. pneumoniae* strain M24 DNA. The locations of most of the restriction fragments and the genetic markers are taken from Gasc et al. (37). The genes *gpmA* and *pyrDA* that flank the *tts* gene in type 37 clinical isolates of *S. pneumoniae* have been localized in this work and are shown in bold. The *psaA* gene, located downstream of *gpmA*, has also been mapped here and is shown in bold. (B) PFGE of the DNAs obtained from strains 1235/89 (lane 1), M24 (lane 2), DN2 (lane 3), and DN5 (lane 4) digested with ApaI, SacII, or SmaI. Solid triangles indicate DNA fragments characteristic of DN2 DNA.

stance, the SacII fragment number 3 (~260 kb) of M24 and DN5 DNAs is converted, in DN2 DNA, into a 290-kb fragment that superimposes on the original SacII fragment number 2 of M24 and DN5 DNA. This reorganization does not affect the *cap3* recipient cluster as shown above and might involve those fragments where contigs sp_14 and sp_58 are located. To test this hypothesis, chromosomal DNAs prepared from M24, DN2, and DN5 were digested with ApaI, SacII, or SmaI, subjected to PFGE, blotted, and hybridized with different biotin-labeled probes (Table I). The probes used contained internal fragments of the genes *tts*, *gpmA*, *psaA*, or *pyrDA* (see gene locations in Fig. 3). First of all, we localized the genes *gpmA* (contig sp_14) and *pyrDA* (contig sp_58) in the *S. pneumoniae* M24 chromosome and observed that they map at very distant positions (Table I and Fig. 7 A). As expected, the location of *gpmA* matched that of the previously mapped *pbp2B* gene (36) that is located only 15 kb upstream of *gpmA* according to recent sequence data (Fig. 3 B). These results also showed that contigs sp_14 and sp_58 are located very far apart in the *S. pneumoniae* chromosome. In fact, these contigs are separated by at least 380 kb, the sum of the sizes of the intervening macrorestriction fragments (Fig. 7 A).

Different hybridization bands were observed when comparing DN2 and DN5 DNAs (Table I), in agreement with the different chromosomal location of the *tts* gene in both strains. Moreover, apart from the hybridization band of DN5 DNA with the type 37–specific *tts* probe, the hybridization patterns of M24 and DN5 DNAs were identical, strongly suggesting that a large chromosome reorganization had not taken place in DN5 as a consequence of transformation of M24 to the $S37^+$ phenotype. In fact, combined PCR amplification experiments and sequence determination showed that, in DN5 DNA, the *tts* gene integrated between *gpmA* and *orf1819* (Fig. 3 B), as 2,400 out of 2,412 bp of the intervening *orf3* gene were lost (data not shown). In the type 37 DN2 transformant, however, we found that *gpmA* moved from its original position to that where *pyrDA* resides (Table I). Moreover, this reorganization also affected some genes located downstream of *gpmA*, as deduced from the finding that *psaA* that is located ~7 kb downstream of *gpmA* in the *S. pneumoniae* genome (Fig. 3 B) hybridizes with a novel SmaI fragment (number 7) in DN2 DNA (Table I and Fig. 7 B).

To investigate whether the IS element located downstream of *tts* might be involved in the reorganization of the

Table I. *Mapping of the Integration Site of the Gene Coding for the Tts Synthase*

Enzyme	Probe containing an internal fragment of											
	gpmA			*psaA*			*tts*			*pyrDA*		
	M24	DN2	DN5	M24	DN2	DN5	M24	DN2	DN5	M24	DN2	DN5
ApaI	9	2	9	9	8*	9	−	2	9	2	2	2
SacII	2	2 + 3*	2	2	2 + 3*	2	−	2 + 3*	2	3	2 + 3*	3
SmaI	8	1	8	8	7*	8	−	1	8	1	1	1

Numbers represent the restriction fragments separated by PFGE (Fig. 7 A) that hybridize with labeled probes containing the indicated genes. −, No hybridization signal.
*Restriction fragments not present in M24 DNA (Fig. 7 B).

genome, type 37 transformants of the M24 strain were obtained by using, as donor DNA, a 4.1-kb SacI–ClaI fragment containing the *tts* gene, the IS*1167* element, and the last 140 nucleotides of *gpmA* (Fig. 3 A). Five independently isolated type 37 transformants were tested using a combination of PCR amplification and Southern blot analysis (not shown). All of them turned out to be identical and appeared to have arisen by homologous recombination between the 3′ end of *gpmA* and the 128-bp region located immediately downstream of the *tts* gene (represented by a star in Fig. 3, A and B) without any additional genome rearrangement. Moreover, all of the transformants had lost the IS*1167* element. Although the number of transformants studied is limited, these results suggest that the sequences flanking the *tts* gene are more relevant for successful transformation than the IS element itself.

Construction of Binary Encapsulated Strains of S. pneumoniae. Apart from the natural type 37 strains, only *cap3A* unencapsulated pneumococcal mutants had been used in this study as recipients for intertype transformation experiments. Consequently, we were interested to know whether the *tts* gene could code for the biosynthesis of type 37 capsule in pneumococcal isolates of different types. *S. pneumoniae* strains belonging to serotypes (or serogroups) 1, 2, 5, 6, 8, 9, 19, 33A, 33B, or 33F were incubated with DNA prepared from strain C2, and Ln-resistant transformants were scored in blood agar plates. Selected clones were then analyzed for capsulation using both the Quellung reaction and coagglutination assays. All of the clones tested showed two capsules, that of the recipient strain and the type 37 capsule encoded by the transforming donor DNA (not shown).

Discussion

It is noteworthy that the three clinical strains of *S. pneumoniae* studied here, one recovered in Denmark in 1939 shortly after the first isolation of a type 37 strain (39) and the other two in Spain in 1989 and 1996, respectively, contain in their chromosomes nearly identical and mutated *cap33f* loci placed between the *dexB* and *aliA* genes (Fig. 1). This *cap33f* locus appears to be silent in all type 37 strains, as measurable amounts of serogroup 33 polysaccharide were not found (data not shown). The finding that no $S37^+$ transformants could be identified when the *cap33f* locus was PCR amplified and used as donor DNA to transform unencapsulated recipient cells suggested that the gene(s) responsible for the synthesis of the type 37 capsular polysaccharide might be located elsewhere in the genomes of type 37 strains.

In this paper, we show that a single gene, designated *tts* and located in a 7.3-kb PstI DNA fragment common to all of the clinical type 37 isolates (Fig. 3 A), is responsible for the synthesis of the type 37 capsular polysaccharide. The Tts protein coded by the *tts* gene appears to be an integral membrane protein having a potentially cleavable signal peptide. As the type 37 polysaccharide has two different β-glucosidic linkages, 1,2 and 1,3 (18), Tts should catalyze both kinds of linkages. There is increasing evidence showing that this property is not so unusual as previously envisaged. Type 3 pneumococcal Cap3B synthase (40) and the HasA hyaluronan synthase of *Streptococcus pyogenes* (41) provide examples of dual enzymatic activity. More recently, Griffiths et al. (42) have demonstrated that KfiC, an enzyme involved in the synthesis of the *E. coli* K5 capsule, is a bifunctional enzyme with both α- and β-glycosyltransferase activities responsible for the sequential addition of glucuronic acid and *N*-acetylglucosamine to the growing polysaccharide chain. Interestingly, it has been possible to produce a truncated protein lacking only one of the two transferase activities (42). If a similar situation could be demonstrated for the Tts synthase, it might be possible to construct *tts* mutants lacking the 1,2-glucosyltransferase activity that would produce a callose-containing capsular polysaccharide (β-1,3-glucan). Nevertheless, it should be emphasized that this type of capsule has never been reported in *S. pneumoniae*.

The type 37 synthase shows sequence signatures known to be characteristic of bacterial and plant cellulose synthases and other β-glycosyltransferases (33; Fig. 6). Currently, it is not known whether genes other than *tts* and those common to all pneumococci might cooperate in the capsular synthetic process as reported, for example, for the *Acetobacter xylinum* cellulose synthase, the only well characterized cellulose synthase that comprises at least one putatively regulatory subunit in addition to the catalytic subunit (34). Also, we lack sufficient biochemical information to speculate about whether the Tts synthase is responsible for direct polymerization of glucan from UDP-Glc, as proposed for *A. xylinum*, or whether it might catalyze the synthesis of a lipid-Glc precursor as suggested for the CelA protein of *Agrobacterium tumefaciens* (34).

Transformation of a laboratory strain (M24) with type 37 chromosomal DNA produced at least two categories of strains. In one of them, the DN2 strain has suffered a noticeable genomic reorganization, as genes separated for at least 380 kb in the genome of the recipient strain (i.e., the genes *gpmA* and *pyrDA*) lie close together after transformation, as evidenced by PFGE experiments (Fig. 7 and Table I). This situation reconstructed that found in the clinical type 37 pneumococcal isolates. In the other class of transformants (strain DN5), the *tts* gene is integrated immediately downstream of *gpmA* without any major chromosomal rearrangement. In addition, by using transforming DNA exclusively containing the *tts* gene and IS*1167*, it appears that the IS element plays a secondary role in the integration events. The observation that pneumococcal strains isolated almost 60 years apart at different geographic locations contain not only an identical *tts* gene inserted at the same site but also a cryptic *cap33f* locus, together with the finding on the potential capacity of *tts* to integrate and express in all of the pneumococcal strains tested, strongly supports the hypothesis of the clonal origin of capsular genes in *S. pneumoniae*, as has already been proposed for the *cap1* cluster involved in the synthesis of type 1 polysaccharide (4). In fact, in the two cases where complete sequence data of the *cap* genes of two different strains of the same serotype are available, types 3 (5, 6) and 23F (unpublished sequence available from EMBL/

GenBank/DDBJ under accession number AF030373; reference 10), >95% identical nucleotides were found among the *cap* genes of different pneumococcal strains.

During the last few years, several researchers have reported that some clinically relevant (multiresistant) pneumococcal strains are essentially identical in overall genotype but differ in capsular type (15, 43–48). This finding has been interpreted as evidence that the new strains were the result of intertype transformation. Very recently, Coffey et al. (16) studied in detail eight type 19F variants that were otherwise identical to the major Spanish multiresistant 23F clone and confirmed that recombination at the *cap* locus had taken place on at least four occasions. In all of the cases reported so far, in vivo intertype transformation implies that the recipient *cap* locus is substituted by that of the donor strain, that is, the transformant gains new capsular genes but loses its own *cap* cluster. In the case reported here, however, the capsular *tts* gene of the donor strain does not replace the recipient *cap33f* cluster but integrates in a different, distant place and originates a genetically binary strain, a strain containing two capsular loci. Binary encapsulated strains, i.e., those synthesizing two chemically and immunologically distinguishable capsules, were constructed in the laboratory many years ago, and it was observed that one type of capsule predominates (for a comprehensive review see reference 14). Moreover, transformation experiments using DNA prepared from binary cells showed that the supernumerary capsular cluster was inserted in a region different from the usual capsular polysaccharide–determining one (49). Binary transformants appear to be stably maintained, except in some rare cases where unstable binary strains were obtained (50). In the latter case, linkage between the donor and recipient capsular genes could be demonstrated. More recently, binary strains were constructed by cloning the type 3 polysaccharide synthase gene (*cap3B*) into *S. pneumoniae* strains belonging to several types (40). In addition, genetically binary type 3 strains were prepared by transformation of unencapsulated *cap3A* mutants impaired in the synthesis of UDP-Glc dehydrogenase with the homologous *cap1K* gene from type 1 pneumococci (4). In this case, the introduction of the *cap1K* gene in the recipient chromosome was facilitated by the presence of a closely linked copy of the IS*1167*. Nevertheless, with the only exception of Griffith (51), who reported a pneumococcal strain that agglutinated specifically with the sera of two different types, natural isolates of *S. pneumoniae* having two capsules have not been described so far. In addition, the possibility that Griffith's observation was caused by some kind of immunologic cross-reactivity between capsular polysaccharides cannot be ruled out (52, 53).

The type 37 pneumococci reported here are binary strains from the genetic viewpoint. This status might provide a potential advantage against the immunological host defenses. Although currently silent, the recipient *cap37* locus might eventually recover its capacity to synthesize type 33F capsular polysaccharide, e.g., we can envisage that transformation events involving DNA fragments of the *cap33f* gene cluster would restore to the wild-type genotype those genes mutated in *cap37*. On the other hand, *tts* cryptic homologues might be also present in some clinical isolates of pneumococcus. Although preliminary searches for these putative mutants have been unsuccessful, these variants should be good candidates for the rapid acquisition of a type 37 capsule. Regardless of these possibilities, from the results presented here, de novo acquisition by *S. pneumoniae* of a *tts* gene via genetic transformation appears to be a rather likely event.

We thank P. García, J.L. García, and E. Díaz for their critical reading of the manuscript.

R. Muñoz was the recipient of a Contrato Temporal de Investigadores from the CSIC, and D. Llull was the recipient of a fellowship from Programa Sectorial de Formación de Profesorado Universitario y Personal Investigador (Ministerio de Educación y Cultura). This study was supported by grant PB96-0809 from the Dirección General de Investigación Científica y Técnica.

Preliminary sequence data were obtained from The Institute for Genomic Research (TIGR) through the website at http://www.tigr.org. Sequencing of the *S. pneumoniae* genome was accomplished with support from TIGR, the National Institute of Allergy and Infectious Diseases (NIAID), and the Merck Genome Research Institute (MGRI).

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Submitted: 15 March 1999 Accepted: 18 May 1999

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