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) 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.

1A 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 diphosphoglucone.
quite frequent between the most virulent clinical isolates of pneumococci (15, 16). The strategy used to carry out inter-type 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-1,3-Glc-(1→2)-β-1,3-Glc) interlinked through β-1,3 bonds (18). The tts gene responsible for the formation of this capsule was located outside of the caps 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 (ΔlytA; 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 unencapsulated pneumococci used in this work. The number after the shift indicates the year of isolation of the corresponding strain. When working with Esherichia 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 Harstein 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 emc; 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 pUC19 previously digested with Sphi and XbaI. Plasmid pDLP40 contains a 1.7-kb SphI–KpnI DNA fragment of pDLP37 containing the tet gene, inserted into a EcoRI-deficient pUC18 previously digested 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 emc gene from plasmid pLSE1 using oligonucleotide primers OLB2 and OLB3. This promoterless gene was digested with SmaI and cloned into EcoRI-digested pDLP40. Before ligation, the EcorI site located in the Sphi gene had been filled in as described above. Plasmid pDLP41 was isolated among the erythromycin-resistant transformants of E. coli DH5α. Plasmid pLSE3, 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 CiaI, and ligated to pLSE1 previously treated with EcoRI and MspI. N E Blo t™ 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.

Nudeotide Sequences and Data Analysis. The sequence data were deposited in GenBank/EMBL/GenBank (DDBJ) 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 plasmid pLSE4-derived plasmid were scored on L-norleucine-containing plates using a filter technique to distinguish the LytA phenotype (28). Immunoprecipitation using anti-R serum (29) or coagglutination assays with type antiserum purchased from the Statens Serum Institut were carried out as previously described (31). Typing by the Quellung technique was carried out by L. Vicioso (Spanish Pneumococcal Reference Laboratory, Majadahonda, Spain).

Nudeotide Sequences. A cession 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.

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 ap 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 (S37-) 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 S37- 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 (S37-) 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 emC 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, MuniI, or SphI, digested 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
positions 1 to 1,479, the sequence matched part of contig sp_14. The apparent anomalous structure of the sequence of the ends of the PstI restriction fragment containing the gene(s) responsible for type 37 capsular polysaccharide biosynthesis (signal peptide coincides with transmembrane helix A). On the other hand, another copy of the 105-bp repeat element characteristic of S. pneumoniae (31) was also observed. On the other hand, another copy of the IS element IS1167 (30) was found (Fig. 3 A). The $\text{trp}$ gene should encode a defective transposase because it contains a frameshift mutation. Determination of the nucleotide sequence of the ends of the PstI fragment obtained from strain 1235/89 DNA, a copy of the IS element IS1167 (30) was found (Fig. 3 A). The $\text{trp}$ gene should encode a defective transposase because it contains a frameshift mutation.

**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. $\text{Ln}^R$, $\text{Ln}$-resistant; $\text{P}$, PstI; $X$, any restriction site not present in pUCE191.

From nucleotide 3,834 to 5,297 of the PstI fragment obtained from strain 1235/89 DNA, a copy of the IS element IS1167 (30) was found (Fig. 3 A). The $\text{trp}$ gene should encode a defective transposase because it contains a frameshift mutation. Determination of the nucleotide sequence of the ends of the PstI fragment obtained from strain 1235/89 DNA, a copy of the IS element IS1167 (30) was found (Fig. 3 A). The $\text{trp}$ gene should encode a defective transposase because it contains a frameshift mutation.

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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 that has been recently reported (54) was available from EMBL/GenBank/DDBJ under accession number AF068901. The PstI sites flanking the tts gene and relevant to this study are circled.

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 IS1167 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 is available from EMBL/GenBank/DDBJ under accession number AF068901. The PstI sites flanking the tts gene and relevant to this study are circled.
To demonstrate that tss 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 tss gene initiates 9 nucleotides after the –10 consensus sequence.

Figure 5. Primer-extension mapping of the transcription initiation site for the tss 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 tss is also shown. Note that the indicated sequence corresponds to the coding strand.

The deduced aa sequence of the tss 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 BLAST 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 glycosyltransferases (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).

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 RXKRXW 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 as substitutions are shown in gray. The accession numbers of the selected proteins are also indicated: U58283 (G. osypium hisutum CelA); U58284 (G. hirsutum CelA2); D48636 (O. rya sativa CelA); U15857 (A. xylinum AcsA1); P19449 (A. xylinum BcsA); L38689 (A. tumefaciens CelA); AE000738 (A. quillaoelus BcsA); AL031004 (A. bari- diposis thalaina ATF28M 20); AF047687 (Bradyrhizobium japoni- um NdvB); AJ009933 (L. ladis O rif); D90912 (Synechocystis subsp. S1137); and U42580 (Parachromatium Eriocellula vi- rus A473).

Genomic Reorganization Caused by Intertype Transformation in Type 37 Pneumococcal Strains. The tss 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 the tss gene (open arrow) inserted into the DN2 ts gene (solid arrow). The wild-type and mutant genes were PCR amplified using oligonucleotides D90 and D91 and digested with M unil (M) or EcoRI (E).

Interestingly, restriction enzyme analysis showed that the amplified DN2 DNA fragment was identical to that of the M24 strain, and the same oligonucleotide primers only rendered a PCR product in the case of DN2 DNA. 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.

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

To cause only the physical map of the Avery’s R6 strain (36), as repeated attempts to amplify M24 DNA using the same oligonucleotide primer (solid arrow). The wild-type and mutant genes were PCR amplified using oligonucleotides D90 and D91 and digested with M unil (M) or EcoRI (E).
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 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

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>gpmA</th>
<th>psaA</th>
<th>tts</th>
<th>pyrDA</th>
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<tr>
<td>ApaI M24</td>
<td>9</td>
<td>2</td>
<td>8*</td>
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<td>DN2</td>
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<td>DN5</td>
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<td>SacII</td>
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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 M 24 strain were obtained by using, as donor DNA, a 4.1-kb SacI–ClaI fragment containing the tts gene, the IS1167 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 IS1167 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 Lm-resistant transformants were scored in blood agar plates. Selected clones were then analyzed for capsule 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, 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 and IS1167 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 HaaA 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 its mutants lacking the 1,2-glucosyltransferase activity that would produce a callose-containing capsular polysaccharide (β1,3-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 A. baylyi bacter 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. xylulinum, or whether it might catalyze the synthesis of a lipid-Glcp precursor as suggested for the CelA protein of Agrobacterium tumefaciens (34).

Transformation of a laboratory strain (M 24) with type 37 chromosomal DNA produced at least two categories of strains. In one of them, the DN 2 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 IS1167, 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/
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 indistinguishable 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 capsule 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 (cap38) 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 IS1167. 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 cap3F gene cluster would restore to the wild-type genotype those genes mutated in cap37. On the other hand, its 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.

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