Tts, a Processive β-Glucosyltransferase of *Streptococcus pneumoniae*, Directs the Synthesis of the Branched Type 37 Capsular Polysaccharide in Pneumococcus and Other Gram-positive Species*

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Daniel Liull‡, Ernesto García§, and Rubens López
From the Centro de Investigaciones Biológicas, CSIC, Velázquez 144, 28006 Madrid, Spain

The type 37 capsule of *Streptococcus pneumoniae* is a homopolysaccharide built up from repeating units of \([β-D-\text{Glc}-(1\rightarrow 2)]\)-\(β-D-\text{Glc}-(1\rightarrow 3)\). The elements governing the expression of the *tts* gene, coding for the glucosyltransferase involved in the synthesis of the type 37 pneumococcal capsular polysaccharide, have been studied. Primer extension analysis and functional tests demonstrated the presence of four new transcriptional start points upstream of the previously reported *tts* promoter (tssp). Most interesting, three of these transcriptional start points are located in a RUP element thought to be involved in recombinational events (Oggioni, M. R., and Claverys, J. P. (1999) *Microbiology* 145, 2647–2653). Transformation experiments using either a recombinant plasmid containing the whole transcriptional unit of *tts* or chromosomal DNA from a type 37 pneumococcus showed that *tts* is the only gene required to drive the biosynthesis of a type 37 capsule in *S. pneumoniae* and other Gram-positive bacteria, namely *Streptococcus oralis*, *Streptococcus gordonii*, and *Bacillus subtilis*. The Tts synthase was overproduced in *S. pneumoniae* and purified as a membrane-associated enzyme. These membrane preparations used UDP-Glc as substrate to catalyze the synthesis of a high molecular weight polysaccharide immunologically identical to the type 37 capsule. In addition, UDP-Gal was also a substrate to produce type 37 polysaccharide since a strong UDP-Glc-4’-epimerase activity is associated to the membrane fraction of *S. pneumoniae*. These results indicated that Tts has a dual biochemical activity that leads to the synthesis of the branched type 37 polysaccharide.

*Streptococcus pneumoniae* (pneumococcus) is an important human pathogen and a common etiological agent of community-acquired pneumonia and meningitis in adults and of acute otitis media in children. The capsular polysaccharide has been identified as the main virulence factor of *S. pneumoniae* (1). The capsule confers to pneumococcus the advantage to resist phagocytosis and survive in the host. Pneumococcus has evolved by diversifying its capsule, and up to 90 different capsular types synthesizing polysaccharides with different immunological properties and chemical structures have been described (2). Capsular polysaccharide biosynthesis in *S. pneumoniae* is usually driven by genes located in the cap/eps locus, and the capsular cluster of 13 pneumococcal types has been sequenced recently (3). In remarkable contrast, only a single gene (*tts*) located far apart from the cap cluster, directs the synthesis of the type 37 capsule (4). Type 37 capsule polysaccharide is the only homopolysaccharide reported in pneumococcus. Clinical isolates belonging to this serotype synthesize a conspicuous capsular envelope that is a branched polysaccharide that has a linear backbone of \([β-D-\text{Glc}-(1\rightarrow 3)]\)-\(β-D-\text{Glc}-(1\rightarrow 3)\) linked to C2 of each Glc residue (sophorosyl subunits) (5). Several experimental approaches demonstrated that *tts* is the only gene required for the synthesis of the type 37-specific capsular polysaccharide in *S. pneumoniae*. The *tts* gene encodes a putative glucosyltransferase (Tts) that exhibits significant sequence similarities with cellulose synthases of bacteria and higher plants and other β-glycosyltransferases (4).

Only few gene products involved in pneumococcal capsular formation have been biochemically characterized, and almost nothing is known about mechanisms as important as regulation, transport, and assembly of the polysaccharide chain subunits (3). It is generally thought that these polysaccharides are synthesized via lipid-linked repeat unit intermediates because of the biochemical complexity of the repeating oligosaccharide subunit. In types 14 and 19F, the first step of this process involves the activity of the protein coded by *cps14/cps19fE* gene (6, 7). This protein catalyzes the selective incorporation of Glc from UDP-Glc to a membrane lipid-linked acceptor leading to the formation of a complex where other glycosyltransferases would transfer the sugars present in the polysaccharide repeating subunit (7). However, in type 3 pneumococci, sugars are transferred directly to the growing polysaccharide chain without intervention of an anchoring lipid molecule. We have demonstrated that Cap3B, the type 3 polysaccharide synthase, is the only protein required to synthesize high molecular weight type 3 capsular polysaccharide in *S. pneumoniae* or *Escherichia coli* strains provided that UDP-Glc and UDP-GlcUA, the precursors of type 3 capsular monosaccharides, were available (8). It has also been shown that Cap3B (also designated as Cps3S) is a processive enzyme able to transfer alternated residues of Glc and GlcUA from their respective UDP-sugars to the non-reducing end of the nascent polysaccharide chain (9). Cap3B possesses a double \(β-1,3\)- and \(β-1,4\)-glycosyltransferase activity in contrast to the other glycosyltransferases characterized so far among the enzymes implicated in synthesis of the pneumococcal capsule that only catalyze the transfer of a single glyco-
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sy residue (8). There is increasing evidence showing that this property is not so unusual as envisaged previously. Thus, the family of bacterial hyaluronan synthases (HAS) like those of Streptococcus pyogenes (10), Streptococcus equisimilis (11), or Pasteurella multocida (12), and the KflC enzyme of E. coli responsible for the synthesis of the E. coli K5 capsule (13), also provide examples for a dual enzymatic activity. It should be noted, however, that this enzymatic activity has only been demonstrated for enzymes that catalyze the formation of linear polysaccharides, whereas type 37 polysaccharide is a branched polymer. We report here the subcellular localization and biochemical characterization of the type 37 synthase in S. pneumoniae strains expressing the tts gene. We also show the ability of Tts to produce a type 37-specific capsule even when expressed in Gram-positive bacteria other than pneumococcus.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—The unencapsulated laboratory S. pneumoniae strains used are as follows: M24 (35), DN1 (lytA) (36, 37) (type 37) (1235/89, kindly provided by A. Fenoll (Spanish Pneumococcal Reference Laboratory, Majadahonda, Spain), and the type 37 laboratory transformants DN2 and DN5 have been described previously (4). Strain C2 is a type 37 linomycin-resistant (Ln R) transformant of the pneumococcal type 37-encapsulated laboratory transformants DN2 and DN5 have been described previously (4). Methods for transformation of Streptococcus oralis NCTC 11427 (type strain) (17), Streptococcus gordonii V288 (Challis) (18), and Bacillus subtilis YB886 (19) have also been described elsewhere. Clones obtained by transformation with derivatives of pLSE1 (ter erm) (17) were scored on blood agar plates containing 0.1 μg of Ln/ml (for S. pneumoniae and S. oralis), or brain-heart infusion agar plates (Difco) supplemented with 10 μg of Linomycin (Ery)/ml (for S. gordonii), or on LB agar plates containing 5 μg of Ery/ml (for B. subtilis). Plasmid pLESA is a promoter-probe vector that contains a promoterless lytA autolytic amidase under the control of the tss promoter of the tts gene.

DNA Techniques and Plasmid Construction—DNA manipulations and other standard methods were as described in Sambrook et al. (22). Primer extension mapping of the transcription initiation site (4) and polymerase chain reaction amplifications (23) were carried out as described previously. Conditions for amplification were chosen according to the G + C content of the corresponding oligonucleotides. The oligonucleotide primers mentioned in the text are as follows: (D101) 5′-TCTGCACCAACGTTACATCTGCA-3′; (D102) 5′-TCCCTAAACGTCACTTACTACCA-3′; and (D138) 5′-TCCATCTCCTGCTGCTCCAC-3′. Lowercase letters indicate nucleotides introduced to construct appropriate restriction sites; these are shown underlined (see Fig. 1A).

To construct pDLP50, chromosomal DNA prepared from the 1235/89 strain was polymerase chain reaction-amplified with oligonucleotide primers D101 and D112 and made blunt-ended with the Klenow fragment of the E. coli DNA polymerase I (Pol{k}). Subsequently, the DNA fragment was digested with XbaI and ligated to pLESA that had previously been digested with SphI, filled in with Pol{k}, and then treated with XbaI. The ligation mixture was used to transform S. pneumoniae M31, and a clone harboring pDLP50 was isolated by scoring the Ln R activity. Conditions for amplification were chosen according to the G + C content of the corresponding oligonucleotides. The oligo-
amount of enzyme that catalyzed the hydrolysis (solubilization) of 1 µg of pneumococcal cell wall material in 10 min.

**RESULTS**

**Transcriptional Analysis of the tts Gene**—We have reported previously the identification of the tts promoter and its transcription start point (4). The tss promoter contains a −10 consensus sequence with an extended TtTG motif characteristic of the −16 region of S. pneumoniae (30) and transcription initiates 9 nucleotides after the −10 consensus sequence (4). However, we have now observed that unencapsulated pneumococcal cells transformed with a recombinant plasmid (pDLPG49) containing the region upstream of tss formed colonies noticeably more mucous than those from cells transformed with pDLPG48, an equivalent plasmid that only contains tss and the structural tts gene. To determine the promoter strength of both constructs, we compared the cell wall lytic activity (see “Experimental Procedures”) expressed in a pneumococcal ΔlytA strain (M31) transformed either with pDLPG6 (4) (Fig. 1A), which contains the reporter lytA gene under the control of tss, or with pDLPG50, a construct that also includes the upstream region of tss (Fig. 1A). Sonicated cell extracts prepared from M31 [pDLPG50] showed 6 times more LytA activity than those from M31 [pDLPG36] (Fig. 1B). In addition, M31 [pDLPG50] exhibited a faster autolysis at the end of exponential phase of growth than M31 [pDLPG36] (Fig. 1C). Furthermore, primer extension analysis using total RNA extracted from M31 [pDLPG50] revealed the presence of at least four additional transcription start points upstream of tss (Fig. 2). Interestingly, three of them lie in a RUP element present in this position in the clinical type 37 strains (4) (Fig. 3). RUP elements are thought to be insertion sequence derivatives that facilitate recombinational events (31), but a promoter activity had never been described in these elements.

**Expression of Tts in other Gram-positive Bacteria**—According to the results described above, we used pDLPG49 to transform competent cells of S. pneumoniae M24, S. oralis NCTC 11427, S. gordonii V288, and B. subtilis Y886. LnR (or EryR) transformants were isolated, and selected colonies were grown for 21055. We have reported previously the identification of the tts gene and its functional characterization of its promoter region. A, the upper part of the figure shows a schematic representation of the DNA fragments of the pneumococcal strain 1235/89 (type 37) cloned into pLSE1 (pDLPG48 and pDLPG49) and pLSE4 (pDLPG36 and pDLPG50) to study the expression of tts. Pertinent restriction sites and oligonucleotide primers were employed to construct the corresponding recombinant plasmids are described under “Experimental Procedures.” The black box corresponds to the location of tss. B, lytic activity of sonicated extracts prepared from the ΔlytA pneumococcal strain M31 harboring different plasmids assayed on [3H]choline-labeled pneumococcal cell walls. ND, not detectable. C, growth (and lysis) curves of the S. pneumoniae M31 strain harboring plasmids pLSE4 (●), pDLPG36 (○), or pDLPG50 (△). Cells were incubated in C+Y medium containing Ln (0.7 µg/ml), and growth was followed by nephelometry (N). One N unit corresponds to about 2 × 10⁸ colony-forming units/ml.

![Fig. 1. Analysis of the expression of the tts gene and functional characterization of its promoter region.](image)

**Subcellular Localization of the Tts Activity**—To prepare a homologous system for biochemical assays, we used the type 37 pneumococcal strain M24 [pDLPG49] described above. Subcellular fractions of M24 [pDLPG49] were tested for incorporation of radioactivity into a macromolecular product by using UDP-[14C]Glc, assuming that UDP-Glc was the natural substrate for Tts. The membrane fraction turned out to incorporate the label, whereas the soluble fraction did not (data not shown). SDS-PAGE analysis of a membrane preparation from M24 [pDLPG49] revealed the presence of an overproduced protein with a molecular mass of ~50 kDa (Fig. 5). This protein was absent in membranes prepared from M24 [pLSE1], a strain harboring

| Strain | Cell wall lytic activity (units/mg of protein) |
|--------|---------------------------------------------|
| M31 [pLSE4] | ND |
| M31 [pDLG36] | 13 |
| M31 [pDLG50] | 80 |

![Table 1](image)
activity was stimulated in the presence of 10 mM MgCl₂ or Mg²⁺ (data not shown). Furthermore, EGTA only produced a small inhibition of the reaction (Table I). Globally, this behavior is similar to that already described for several glycosyltransferases like cellulose synthases, HAS, or the pneumococcal type 37 specific synthase. In addition, 50 mM NaCl increased 2-fold the incorporation of [14C]Glc into a macromolecular product using different experimental conditions. Membranes prepared from S. pneumoniae type 37 clinical isolate C2; S. gordonii, type 37 laboratory strain M24; E, M24 [pLSE1]; F, M24 [pDP49]; G, S. oralis NCTC 11427 [pLSE1]; H, S. oralis [pDP49]; I, an Ln⁻ isolate of S. oralis obtained by transformation with chromosomal DNA from the pneumococcal type 37 Ln⁻ strain C2; J, S. gordonii V288; K, S. gordonii [pLSE1]; L, S. gordonii [pDP49]; M, B. subtilis YB886; N, B. subtilis [pLSE1]; N, B. subtilis [pDP49].

**Biochemical Properties of the Type 37 Synthase**—Membranes of the pneumococcal M24 [pDP49] strain were used to evaluate the incorporation of [14C]Glc from its precursor UDP-[14C]Glc into a macromolecular product using different experimental conditions. Membranes prepared from S. pneumoniae M24 [pLSE1] cells were employed as a negative control. Tts activity was stimulated in the presence of 10 mM MgCl₂ or MnCl₂. Moreover, 10 mM EDTA completely inhibited the reaction (Table I). However, Ca²⁺ ions stimulated only slightly Tts activity when added at low concentration (1 mM) in the absence of Mg²⁺ (data not shown). Furthermore, EGTA only produced a small inhibition of the reaction (Table I). Globally, this behavior is similar to that already described for several glycosyltransferases like cellulose synthases, HAS, or the pneumococcal type 3-specific synthase. In addition, 50 mM NaCl increased 2-fold the incorporation of [14C]Glc into a macromolecular product (data not shown). Other important properties of Tts are reported in the composite Fig. 6. The Tts synthase exhibited a noticeable pH dependence, and the optimal activity was
achieved between 6.8 and 7.5 (Fig. 6A). Formation of the radiolabeled macromolecular product of Tts was proportional to protein concentration and proceeded linearly with time for up to 15 min and then slowed down (Fig. 6B and C). The enzymatic activity reached a maximum when the reaction was carried out at 30 °C in the presence of the substrate UDP-[14C]Glc. Tts was relatively stable when incubated at 0 °C for up to 60 min, but its activity drastically decreased when preincubation was carried out at 25 °C or higher temperatures (Fig. 6D).

We have also assayed the effect of sugars or sugar nucleotides on the Tts synthase activity (Table I). A strong inhibition occurred when membranes were preincubated in the presence of UMP, UDP, UTP, GMP, or CMP. The following buffers were used: 70 mM Tris/maleic NaOH (pH 7.0), 70 mM sodium phosphate (pH 7.0), 70 mM Tris-HCl (pH 7.0), 70 mM glycyglycine-NaOH (pH 7.0). Incorporation assays were carried out as described under “Experimental Procedures.” Effect of protein concentration (A) and incubation time (B) on Tts activity. Thermal stability (D) was studied by preincubating the membranes at the indicated temperatures before adding the substrate. Aliquots were withdrawn at different times, and Tts activity was assayed as described under “Experimental Procedures.” The data represent the amount of product synthesized during the assay period.

![Table I](http://www.jbc.org/)

| Addition | Enzymatic activity units | % |
|---------|--------------------------|---|
| Experiment A | None | 90.7 | 100 |
| | EDTA (10 mM) | ND | ND |
| | EGTA (1 mM) | 85.3 | 94 |
| | EGTA (2 mM) | 83.1 | 91.6 |
| | pHMB (5 μM) | ND | ND |
| | MB (2 mM) | 76.2 | 84 |
| | pHMB (5 μM) + ME (2 mM) | 72.4 | 79.8 |
| | Bacitracin (1 μg/ml) | 89.5 | 98.7 |
| | Bacitracin (100 μg/ml) | 90.1 | 99.3 |
| Experiment B | None | 67.5 | 100 |
| | TMP | 8.7 | 12.9 |
| | UMP | 8.4 | 12.2 |
| | UDP | ND | ND |
| | UTP | ND | ND |
| | GMP | 44.1 | 65.3 |
| | CMP | 51.6 | 76.4 |
| | UDP-Gal | 1.4 | 2.1 |
| | UDP-Xyl | ND | ND |
| | UDP-Man | 3.4 | 5.0 |
| | CDP-Glc | 36.1 | 53.5 |
| | GDP-Glc | 43.8 | 64.9 |
| | Glc | 60.8 | 90.1 |
| | Gal | 61.4 | 91.0 |
| | GalUA | 53.6 | 79.4 |
| | GlcUA | 51.9 | 78.9 |
| | Ara | 61.6 | 91.3 |
| Experiment C | None | 58.5 | 100 |
| | Brij 58 (1%) | 16.1 | 27.5 |
| | Brij 58 (0.1%) | 51.9 | 88.7 |
| | DOC (1%) | 1.8 | 3.1 |
| | DOC (0.1%) | 6.4 | 10.9 |
| | Triton X-100 (1%) | 12.8 | 21.9 |
| | Triton X-100 (0.1%) | 15.3 | 26.1 |
activity (Table I) suggesting a close association between Tts and the cell membrane. Interestingly, titration of the Tts synthase with p-hydroxymercuribenzoate (pHMB) resulted in a complete loss of enzymatic activity that could be partially prevented by addition of 2-mercaptoethanol (ME) (Table I) indicating that there might be thiol groups implicated in the folding of the protein, in its enzymatic activity, or both. Finally, bacitracin added at concentrations of 1 or 100 \( \mu \text{g/ml} \) to the reaction mixture did not inhibit the reaction (Table I), strongly suggesting that a lipid intermediate is not involved in the biosynthesis of the type 37 capsular polysaccharide of \( S. \) pneumoniae.

**Effect of UDP-Gal on the Enzymatic Activity of Tts**—As reported above (Table I) UDP-Gal is a potent inhibitor of Tts synthase. Moreover, we have shown that Tts shares conserved motifs with cellulose synthases and other \( \beta \)-glucosyltransferases (4) that are presumably implicated in substrate binding (UDP-Glc) (32, 33). These motifs might be specific for UDP-Glc, although we cannot rule out the possibility that they only recognize the nucleotide part of the molecule, as already suggested for the mechanism of action of this family of enzymes (34, 35). If this were the case, it might account for the inhibitory effect found when adding UTP, UDP, or UMP to the reaction mixture (Table I). It is also conceivable that UDP-Gal (and perhaps any other UDP-sugar showing an inhibitory effect) may serve as substrate of the Tts synthase for polysaccharide biosynthesis. Interestingly, we were able to detect the formation of a radiolabeled high molecular weight product by gel filtration through a Sepharose CL-4B column in experiments where either UDP-[\( ^{14} \text{C} \)]Glc or UDP-[\( ^{14} \text{C} \)]Gal was used as substrate (Fig. 7A). The macromolecular product(s) of these reactions that eluted in the \( V_0 \) of the column was immunoprecipitated with an anti-type 37 polysaccharide serum. In this case, a polymer immunologically indistinguishable from type 37 polysaccharide using either UDP-[\( ^{14} \text{C} \)]Glc or UDP-[\( ^{14} \text{C} \)]Gal as substrate.

**Characterization of the Polysaccharide Product of Tts Synthase**—As shown above, the polymer(s) synthesized by using either UDP-[\( ^{14} \text{C} \)]Glc or UDP-[\( ^{14} \text{C} \)]Gal as substrate eluted in the void volume of a Sepharose CL-4B column, whereas non-incorporated radioactive UDP-sugars appeared in the \( V_T \) (Fig. 7A). The excluded fractions were pooled and hydrolyzed with 2.5 \( \text{m} \) trifluoroacetic acid as described under “Experimental Procedures,” and the samples were analyzed by HPLC. In addition, fractions containing the non-incorporated UDP-[\( ^{14} \text{C} \)]sugars were hydrolyzed with 10 \( \text{mM} \) HCl, neutralized, and also subjected to HPLC analysis. The radioactivity found in the excluded, hydrolyzed fractions co-eluted with a Glc standard solution irrespectively of the labeled precursor used in the reaction (Fig. 7B). Identical results were obtained when the same fractions were analyzed by TLC; that is, radioactivity was detected only in the spot corresponding to Glc irrespective of the labeled precursor used in the reaction (Fig. 7B). These results confirmed that, in both cases, Tts synthesized a polymer composed exclusively of Glc. These findings imply that UDP-[\( ^{14} \text{C} \)]Gal must be epimerized to UDP-[\( ^{14} \text{C} \)]Glc before incorporation into the nascent polysaccharide chain. Some authors (7, 36, 37) had suggested the presence of a strong UDP-Glc-4'-epimerase activity associated with the membrane fraction of \( S. \) pneumoniae belonging to various capsular types that did not include type 37. Here we show that this is also the case for type 37 pneumococcal membranes as fully confirmed by HPLC analysis of the hydrolyzed UDP-sugars obtained from the fractions eluted at the \( V_0 \) of the Sepharose CL-4B column (Fig. 7C). Independently of the radiolabeled precursor used in the assay, the presence of the pneumococcal membranes promoted the appearance of both epimers, UDP-[\( ^{14} \text{C} \)]Glc and UDP-[\( ^{14} \text{C} \)]Gal.

**DISCUSSION**

We have recently reported that a single gene (\( \text{tts} \)) located outside of the \( \text{caplcp} \) locus drives the synthesis of the capsular...
polysaccharide in type 37 pneumococci (4). We have now found that transcription of the \textit{tts} gene also initiates at four different points located upstream of the previously reported promoter \textit{ttsP} (Figs. 1 and 2). It is important to point out that three of the additional transcription start points are located inside a RUP element (Fig. 3). Several features of RUPs led to the proposal that these small (107 base pairs long) intergenic elements could be trans-mobilized by the transposase of IS630-\textit{Spn} insertion sequence (31) and possibly promote sequence rearrangements (4, 38). If this were the case, the presence of a RUP element upstream of the structural \textit{tts} gene might represent a regulatory mechanism for capsule expression since transposition (or inversion) of the RUP element should lead to a variable expression of the capsular polysaccharide in type 37 pneumococci during infection. In addition, the finding that promoter activity is associated with RUP elements may have other potentially interesting implications in the physiology of this microorganism. Since up to 108 copies of this intergenic element are distributed all along the pneumococcal genome (31), it is conceivable that they could contribute to the regulation of virulence (and non-virulence) genes. Interestingly, besides the type 37 \textit{tts} locus, RUP elements have been found close to genes coding for several important pathogenicity factors of \textit{S. pneumoniae} such as capsular polysaccharides, neuraminidases, the hyaluronidase, etc. (31).

A type 37 capsule was immunologically detected when several Gram-positive species were transformed with a recombinant plasmid (pDLP49) harboring the type 37 \textit{S. pneumoniae} \textit{tts} gene (Fig. 4). This finding demonstrates that \textit{tts} is sufficient for capsular synthesis in heterologous systems. Furthermore, a single copy of the \textit{tts} gene inserted into the chromosome of \textit{S. oralis} also led to capsule formation providing the first example where a polysaccharide capsule has been described in this species. This result illustrates how the commensal \textit{S. oralis} might acquire the capacity to synthesize this important virulence factor in the nasopharynx, the natural habitat where many streptococci live. Similar DNA interchanges have already been reported for other pneumococcal genes, e.g., the spread of resistance to \textit{\beta}-lactam antibiotics has been attributed to horizontal transfer events involving fragments of the genes coding for penicillin-binding protein(s) of pneumococcus and other related streptococcal species (39). Moreover, compelling evidence for recombination events between the \textit{galU} gene of \textit{S. pneumoniae} and that of several streptococcal species has also been provided recently (40).

Hydropathy analysis of \textit{Tts} predicted six potential transmembrane domains and a central cytoplasmic region presumably containing the catalytic site(s) (residues 64–346) (4). We show here that when the \textit{tts} gene was overexpressed in \textit{S. pneumoniae}, an \textit{$\sim$}50-kDa active protein was found to be associated with the membrane fraction (Fig. 5). Furthermore, both ionic and non-ionic detergents drastically affect the \textit{Tts} synthase activity associated with these membranes (Table I). The \textit{M}\textsubscript{r} of the overproduced \textit{Tts} deduced from SDS-PAGE analysis was smaller than that predicted from sequence analysis (\textit{$\sim$}59 kDa), which might be due to an anomalous migration of the protein as it has been already reported for two streptococcal HAS (10, 11). \textit{Tts} contains five Cys residues presumably located in the cytoplasmic loop (residues at positions 105, 114, 262, 278, and 299), and one more (Cys-470) between the potential transmembrane regions V and VI. Since ME did not noticeably affect the enzymatic activity of \textit{Tts} (Table I), it can be assumed that these Cys residues are not forming disulfide bonds. However, Cys residues appear to be necessary or important for \textit{Tts} activity since a complete inhibition of the enzyme was obtained upon titration with the sulfhydryl-reactive agent \textit{pHMB} (Table I).

The pneumococcal membranes containing \textit{Tts} incorporate \textit{$^{14}$C}Glc from UDP-\textit{$^{14}$C}Glc into a polymer immunologically indistinguishable from that of type 37 clinical strains (Table II). It should be emphasized that, although immunological cross-reactions have been reported among several anti-pneumococcal diagnostic sera (41), the type 37 antiserum appears to be very specific since it only recognizes the homologous polysaccharide. The only cross-reactivity reported for the type 37 capsule is a slight precipitin reaction between this polysaccharide and an antiserum raised against pneumococci of serogroup 12 (41). Types 12F and 12A contains branches of kojibiosyl residues (42). More recently, the sophorosyl unit has been demonstrated to be the main immunological determinant of type 37 capsule polysaccharide by quantitative hapten inhibition studies (43). Other disaccharides of the isomeric series of \textit{\beta}- and \textit{\alpha}-(1\textendash 2), -(1\textendash 3), -(1\textendash 4), and -(1\textendash 6) were poorly active as competitive inhibitors of antibody precipitation (43). Here we have shown (Table II) that when a linear (1\textendash 3)-\textit{\beta}-D-glucan (curdlan) was employed, no inhibition of the immunoprecipitation reaction was observed (Table II), which fully confirmed that the anti-type 37 serum preferentially recognizes the branched part of the type 37 polysaccharide. Since the type 37 polysaccharide contains two different \textit{\beta}-glucosidic bonds (\textit{\beta}-1,3 and \textit{\beta}-1,2), \textit{Tts} should be responsible for the formation of both linkages according to our findings that \textit{tts} is the only gene required for a type 37 capsule synthesis. The polysaccharide synthesized by \textit{Tts} was composed exclusively by Glc, as revealed by HPLC analysis (Fig. 7) and TLC (not shown). Combined similar HPLC and TLC analyses and immunological tests revealed that when UDP-Gal was used \textit{in vitro} as substrate, the polymer synthesized was indistinguishable from that formed by using UDP-Glc. This finding implies the presence of an epimerase that converts UDP-Gal to UDP-Glc (Fig. 7C).

Computer analyses have revealed that \textit{\beta}-glycosyltransferases share conserved sequences and structural features (34). The processive transferases contain a \textit{DX(X\textendash\textit{\alpha}D)\textit{DX}X\textendash\textit{\alpha}D\textit{DX}X\textendash\textit{\alpha}D\textit{DX}X\textendash\textit{\alpha}D\textit{DX}X\textendash\textit{\alpha}D\textit{DX}X\textendash\textit{\alpha}D} motif distributed over two domains, named “A” and “B,” whereas nonprocessive enzymes lack domain B, and so have only the first two Asp residues of the motif (34, 44). Both domains have also been identified in \textit{Tts} since this enzyme contains the conserved motif \textit{DX(X\textendash\textit{\alpha}D)\textit{DX}X\textendash\textit{\alpha}D\textit{DX}X\textendash\textit{\alpha}D\textit{DX}X\textendash\textit{\alpha}D\textit{DX}X\textendash\textit{\alpha}D\textit{DX}X\textendash\textit{\alpha}D\textit{DX}X\textendash\textit{\alpha}D\textit{DX}X\textendash\textit{\alpha}D\textit{DX}X\textendash\textit{\alpha}D\textit{DX}X\textendash\textit{\alpha}D\textit{DX}X\textendash\textit{\alpha}D\textit{DX}X\textendash\textit{\alpha}D\textit{DX}X\textendash\textit{\alpha}D\textit{DX}X\textendash\textit{\alpha}D\textit{DX}X\textendash\textit{\alpha}D}(44). A classification of glycosyltransferases using nucleotide diphospho-sugars, nucleotide monophospho-sugars, and sugar phosphates (EC 2.4.1.x), and related proteins into 48 distinct sequence-based families has been proposed (45). \textit{Tts} belongs to family 2 that includes, among other inverting glycosyltransferases, cellulose synthases, HAS, and \textit{\beta}-1,3-glucan synthases. Although the HAS from \textit{P. multocida} is currently a member of this family, it appears to be structurally

### Table II

**Immunological characterization of the product of \textit{Tts} synthase**

Standard reactions were carried out as described under “Experimental Procedures.” The reaction mixtures were filtered through a Sephacryl CL-4B column, and the excluded fractions (0.5 ml each) containing about 10 mg of \textit{$^{14}$C}Glc-labeled type 37 polysaccharide were incubated for 2–3 h with protein \textit{A} from \textit{Staphylococcus aureus} (Sigma) sensitized either with type 3- or type 37-specific antiserum. When curdlan was employed, this polysaccharide was preincubated with protein \textit{A} from \textit{S. aureus} at room temperature for 1 h before addition of the type 37 polysaccharide. Mixtures were centrifuged (10,000 \textit{g}, 15 min) to separate the immunoprecipitated material, and radioactivity was determined. Values are the mean of three independent experiments.

| Antiserum          | Total cpm | Supernatant | Pellet |
|--------------------|-----------|-------------|--------|
| Anti-S3            | 2376      | 268         |        |
| Anti-S37           | 214       | 3261        |        |
| Anti-S3 + curdlan  | 208       | 3350        |        |
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distinct from other HAS (46). Experimental evidence for the role of carboxyl residues in β-glycan synthases comes from site-directed mutagenesis of chitin synthase 2 from Saccharomyces cerevisiae (47) and of the AcsAB cellulose synthase from Acetobacter xylinum (44) as well as from the use of amino acid-modifying reagents on a β-(1,3)-glucan synthase from ryegrass (48). Based on these and other results it was assumed that Asp residues are involved in the acid-base catalytic mechanism of this kind of glycosyltransferases (49). Nevertheless, the recent elucidation of the three-dimensional crystal structure of SpA, a member of family 2 of glycosyltransferases implicated in the synthesis of the mature spore coat of B. subtilis, has allowed us to shed light on the mechanisms of this ubiquitous family of inverting glycosyltransferases (50). It has been found that the invariant Asp residues of domain A are intimately involved with UDP binding, whereas a candidate for the general base has not been identified with certainty. It should be noted, however, that the glycosyltransferase specificity of SpA has not been characterized as yet and that this enzyme lacks the domain B characteristic of the processive transferases. Nevertheless, the observed inhibitory effects of UDP, UTP, UMP, or UDP-sugars on Tts activity (Table I) are in agreement with the involvement of the conserved Asp residues in binding to the nucleotide rather than to the sugar moiety of the UDP-sugar substrate.

To the best of our knowledge, the Tts synthase, which catalyzes both β-1,2 and β-1,3 linkages, is the first inverting glucosyltransferase able to synthesize a branched polysaccharide. Perhaps the most intriguing characteristic of Tts is that in most of these transporters only four to eight transmembrane regions at the C-terminal half and two more at the N-terminus of Tts (4) might suggest that the formation of Tts synthase of a membrane pore to facilitate the extrusion of the polymer is unlikely since the presence of at least 12 transmembrane helices are apparently required to build a channel in other sugar transporters (54). However, it should be mentioned that in most of these transporters only four to eight transmembrane helices are usually involved in sugar transport since there are actually two pores, a sugar and a cation pore (55). Evidence suggesting that in the HAS from S. pyogenes only four transmembrane domains and two membrane-associated regions that, however, do not appear to traverse the cell membrane are required to create a pore-like structure through which a nascent HA chain can be extruded to the cell exterior has been reported recently (56). However, several alternative mechanisms might allow the transport of the hydrophilic type 37 polysaccharide across the membrane both in pneumococcus and in other Gram-positive species. These include the use of unspecific transporters, association of several Tts monomers in the membrane to conform a pore, or interaction of the synthase with membrane phospholipids, as recently proposed for HA transport (57). Additional efforts using the experimental tools developed in this work are required to determine if the current models for polymerization and transport of linear polysaccharides can be applied to the synthesis of the branched structure of type 37 polysaccharide that represents the most simplified strategy developed by pneumococcus to synthesize its main virulence factor.

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