

Type 3-specific Synthase of *Streptococcus pneumoniae* (Cap3B) Directs Type 3 Polysaccharide Biosynthesis in *Escherichia coli* and in Pneumococcal Strains of Different Serotypes

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Summary

The *cap3B* gene, which is involved in the formation of the capsule of *Streptococcus pneumoniae* type 3, encodes a 49-kD protein that has been identified as a polysaccharide synthase. *Escherichia coli* cells harboring the recombinant plasmid pTBP3 (*cap3B*) produced pneumococcal type 3 polysaccharide, as demonstrated by immunological tests. Biochemical and cell fractionation analyses revealed that this polysaccharide had a high molecular mass and was localized in substantial amounts in the periplasmic space of *E. coli*. Unencapsulated (S2⁻), laboratory pneumococcal strains synthesized type 3 polysaccharide by transformation with plasmid pLSE3B harboring *cap3B*. In addition, encapsulated pneumococci of types 1, 2, 5, or 8 transformed with pLSE3B can direct the synthesis of pneumococcal type 3 polysaccharide, leading to the formation of strains that display binary type of capsule.

Capsular polysaccharide protects bacteria from phagocytosis by polymorphonuclear leukocytes, and the virulence of *Streptococcus pneumoniae* is determined by the size and the chemical composition of the capsule (1). 90 different capsular types of *S. pneumoniae* have been described (2) and variations in capsular serotype by in vivo and in vitro experiments led to the identification of DNA as the "transforming principle" (3, 4). The importance of these types of genetic exchanges in nature has recently been suggested as a source of variation of the different pneumococcal strains to expand their capacity of colonizing the host organism (5).

Type 3 pneumococci produce a capsule composed of cellobiuronic acid units connected in a $\beta(1\rightarrow3)$ linkage (6). Cellobiuronic acid is a disaccharide consisting of D-glucuronic acid (GlcA)¹ $\beta(1\rightarrow4)$ linked to D-glucose (Glc). Recently, the genes implicated in the biosynthesis of type 3 capsular polysaccharide have been cloned and some of them have been partially characterized (7–12). At least three type 3-specific genes have been found, namely, *cap3A*, *B*, and *C* (also designated as *cps3D*, *S* and *U*, respectively). Genetic and biochemical analyses showed that Cap3A is a UDP-Glc dehydrogenase that converts UDP-Glc into UDP-GlcA (8, 12), whereas *cap3C* complements the *galU* defect of *Escherichia coli* and, therefore, codes for a UDP-Glc pyrophosphorylase (11). On the other hand, sequence comparison suggested that *cap3B* might code for the type-specific polysaccharide synthase, although a precise settlement could not be reached

at that moment. The genes *cap3A*, *B*, and *C* are transcribed together, and insertion-duplication mutagenesis revealed that *cap3C* is not essential for capsule formation (11). Classical genetic studies of pneumococcal capsular type 3 strains suggested that all but one of the spontaneous mutations that alter the formation of type 3 polysaccharide were located in *cap3A* (13). Several *cap3A* point mutations have now been characterized, and some of them completely blocked the synthesis of type 3 polysaccharide (8, 10), whereas only a deletion affecting simultaneously *cap3A* and *cap3B* (13) or insertion mutations (10) affecting *cap3B* have been reported so far. Since polar effects are likely to occur as a consequence of plasmid or transposon insertion, a definite conclusion on the role of Cap3B on capsular biosynthesis still awaited the isolation and characterization of *cap3B* point mutants.

In this communication, we report the isolation and characterization of a *cap3B* mutant. We also show that Cap3B is a synthase that led to the synthesis of type 3 pneumococcal polysaccharide of high molecular mass in *E. coli*, and that was sufficient to produce binary capsulated strains when cloned into several *S. pneumoniae* encapsulated strains belonging to several serotypes.

Materials and Methods

Bacterial Strains, Plasmids and Growth Conditions. The type 3 *S. pneumoniae* strain 406 has been described elsewhere (7). The pneumococcal strain M25, a type 1 encapsulated derivative of the laboratory strain M24 (7), was constructed by M. Mollerach in our laboratory by transformation with DNA from a type 1 clini-

¹Abbreviations used in this paper: Glc, glucose; GlcA, glucuronic acid; IPTG, isopropyl- β -D-thiogalactopyranoside.

cal isolate of *S. pneumoniae*. Other pneumococcal strains were clinical isolates belonging to serotypes 2 (15783/94), 5 (8595/95), and 8 (6028), and were provided by A. Fenoll from the Spanish Reference Laboratory for Pneumococci (Instituto de Salud Carlos III, Majadahonda, Madrid). The unencapsulated S3⁻ strain NR3-10 is a spontaneous mutant of the pneumococcal strain NS3-1 (8), isolated as described elsewhere in detail (7). Two non-encapsulated (S2⁻) mutants used in some experiments were the laboratory strains M11 and M31 (14). These strains were late descendants of the strain R36A (4). *S. pneumoniae* strains were grown in liquid C+Y medium (7) or on reconstituted tryptose blood agar base plates (Difco Laboratories, Detroit, MI) supplemented with 5% (vol/vol) defibrinated sheep blood. Serotyping was carried out by the "Quellung" reaction with type-specific antisera purchased from the Staten Seruminstitut (Copenhagen, Denmark) (15). The same antisera were used for other immunological techniques (see below). *E. coli* strains BL21(DE3) (16), C600 (17), and DH10B (F' *mcrA* Δ (*mrr hsdRMS-mcrBC*) ϕ 80*dlacZ* Δ M15 Δ *lacX74 deoR recA1 araD139* Δ (*ara-leu*)7697 *galU galK* λ^- *rpsL endA1 nupG*) (Life Technologies) were used as hosts for recombinant plasmids and grown in LB medium (17). Plasmids pLysS (16), pT7-7 (18), pLSE1 (19), and pKER2, pKER22, and pKER23 (8) have been already described.

DNA Manipulation, Plasmid Construction, and Genetic Transformation. Preparation of plasmid DNA, digestion with restriction endonucleases, ligation reactions, and agarose gel electrophoresis followed standard procedures (17). PCR amplification of *cap3B* was performed by using 2 U of *Taq* polymerase (Perkin-Elmer Cetus Corp., Norwalk, CT), 10 ng of plasmid pKER2, 1 μ M of each synthetic oligonucleotide primer, 250 μ M of each dNTP, and 5 mM MgCl₂ in the buffer recommended by the manufacturers. Amplification was achieved with a cycle of 3 min denaturation at 95°C, 2 min annealing at 69°C, and 5 min polymerase extension at 72°C, repeated 40 times with a final 6-min extension at 72°C and slow cooling to 4°C with a Gene ATAQ controller (Pharmacia LKB, Piscataway, NJ). The following synthetic oligonucleotides were used: PCAP3B (5'-CTATACACGCGATT-CATATGGCAGAG-3') and PCAP21 (5'-GTGTGCCAGCC-CTGCAGC-3'); the underlined sequence indicates restriction site for *Nde*I. Transformation of competent cells of *S. pneumoniae* (19) and *E. coli* (17) have been described. Before addition of transforming DNA, clinical isolates of *S. pneumoniae* were incubated with a crude preparation of competence factor, as previously reported (20). Transformants of *S. pneumoniae* and *E. coli* obtained with plasmids derived from pLSE1 were selected by plating with lincomycin (1 μ g/ml), or erythromycin (500 μ g/ml), respectively. When pT7-7 or its derivative pTBP3 (see below) were used as donor DNAs, *E. coli* BL21(DE3)pLysS transformants were scored on LB agar plates containing chloramphenicol (20 μ g/ml) and ampicillin (75 μ g/ml). DNA sequencing was carried out with appropriate oligonucleotides with an Abi Prism 377 DNA Sequencer (Perkin-Elmer Cetus).

Characterization of Capsular Polysaccharide. Highly purified pneumococcal polysaccharides were prepared essentially as described elsewhere (15). Briefly, exponentially growing cultures of *S. pneumoniae* in C+Y medium were lysed by the addition of 1% sodium deoxycholate (final concentration) and incubation at 37°C for 30 min. When *lytA* mutants were used, cells were incubated with 5 μ g/ml of *LytA* pneumococcal autolysin (6 \times 10⁵ U/mg) for 30 min at 37°C before the addition of sodium deoxycholate. The lysate was acidified with acetic acid (0.5% final concentration) and heated to boiling point. After cooling, the mixture was neutralized with NaOH and deproteinized with chloroform/isoamyl al-

cohol (24:1). The polysaccharide was precipitated by adding 2 vol of cold absolute ethanol. After standing overnight at 4°C, the precipitate was recovered by centrifugation (10,000 *g* for 30 min), washed once with 96% ethanol, twice with diethyl ether, and dried at 65°C. The polysaccharide was dissolved in distilled water and deproteinized again twice with phenol/chloroform/isoamyl alcohol (25:24:1) and once more with chloroform/isoamyl alcohol. Afterwards, the aqueous phase received sodium acetate (final concentration = 5%), and the polysaccharide was precipitated with half its volume of isopropanol. After standing overnight at 4°C, the polysaccharide was washed as described above, dried, and dissolved in distilled water. For rapid, crude preparations of pneumococcal polysaccharides, 1.5 ml of late exponentially growing cultures of *S. pneumoniae* were treated as described above, except that the deproteinization step was excluded, and the polysaccharide was precipitated only once with absolute ethanol and dissolved in 100 μ l of distilled water.

The uronic acid content of the purified type 3 polysaccharide was measured by the carbazole assay (21) with glucuronic acid as the standard. The mass of the polymers was determined by gel filtration (22) with Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden). Double-diffusion experiments were performed on 0.7% agarose-coated microscope slides as previously described (23). To estimate the amount of type 3 polysaccharide present in a sample, 5 μ l of serial dilutions of the extract and known amounts of purified polysaccharide were analyzed by immunodiffusion against 5 μ l of the same batch of type 3 antiserum. In these experimental conditions, 0.25 μ g of type 3 polysaccharide is enough to give a tiny but still visible precipitation band after overnight incubation.

Miscellaneous Techniques. SDS-PAGE was carried out as described by Laemmli (24). After SDS-PAGE, proteins were blotted onto a polyvinylidene difluoride membrane (Immobilon-P^{8Q}; Millipore Corp., Bedford, MA), and stained briefly with amido black (Sigma Immunochemicals, St. Louis, MO). Subsequently, the desired band was cut and the NH₂-terminal amino acid sequence was determined as described elsewhere (25). Preparation of spheroplasts and fractionation of *E. coli* cells were carried out as previously described (26).

Results

***cap3B* Is Involved in Capsular Biosynthesis.** We reported previously the isolation of 12 spontaneous S3⁻ strains that were characterized as *cap3A* mutants on the basis of transformation to the smooth phenotype with the wild-type *cap3A* gene (8). In a subsequent round of isolation of rough mutants, however, one of them (strain NR3-10) was not transformed to the S3⁺ phenotype by the *cap3A*⁺ allele, and was studied in detail. When the *cap3B* gene of the wild-type strain 406 was amplified by PCR and used to transform competent NR3-10 cells, fully capsulated transformants were obtained (Fig. 1). The mutation could be mapped more accurately by transformation experiments with different restriction fragments of *cap3B*. Sequence analysis of the PCR-amplified *cap3B* allele of NR3-10 revealed the insertion of an additional single base (T) in a preexistent run of six T's and close to the 5' end of the wild-type gene, causing the appearance of a termination codon (TAA) some 50 nucleotides downstream of the insertion. To exclude the possibility of an artifact introduced during the PCR amplification, several PCR rounds were carried out,

resistant clone and used to transform *E. coli* BL21(DE3) pLysS. The presence of pLysS was an absolute requirement for successful transformation with pTBP3, since preliminary experiments using the *E. coli* strain BL21(DE3) did not yield any transformants (not shown). To clone *cap3B* into pLSE1, a plasmid able to replicate both in *E. coli* and *S. pneumoniae* (19), the 2-kb PvuII/HindIII fragment of pKER2 was ligated to pLSE1 previously digested with EcoRV and HindIII (Fig. 2). The ligation mixture was used to transform *E. coli* C600, and pLSE3B was selected by screening among the erythromycin-resistant transformants. It was expected that expression of *cap3B* would be under the control of the tetracycline promoter of the vector plasmid, as it has been reported for other genes (28 and references therein).

For T7 expression, *E. coli* cells harboring pTBP3 were grown in LB medium up to an OD₆₀₀ of 0.6 and induced with isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM). A 49-kD protein, corresponding to the expected size of Cap3B (11), was observed after a 90-min induction, as shown by SDS-PAGE of cellular crude extracts (Fig. 3 A). This band was cut from the gel, and determination of the NH₂-terminal amino acid sequence yielded Met-Tyr-Thr-Phe-Ile-Leu, thus confirming the sequence deduced from the nucleotide analysis of the *cap3B* gene (11). Control cells containing pT7-7 did not show any additional protein band over a 3-h time course experiment.

Synthesis and Analysis of the Pneumococcal Type 3 Polysaccharide in *E. coli*. Sonicated extracts prepared from both induced and uninduced cultures of *E. coli* BL21 (DE3)pLysS harboring pTBP3 contained type 3 polysaccharide, as demonstrated by immunoprecipitation with a type-specific an-

tiserum (Fig. 3 B). The amount of type 3 polysaccharide found in uninduced *E. coli* cells was 10–25% of that of induced cultures. This result is in agreement with an incomplete repression of the T7 promoter in the host strain used here, as it has been reported previously (16). On the other hand, IPTG-induced *E. coli* cultures synthesized as much as 1–5% of the amount of type 3 polysaccharide synthesized by *S. pneumoniae* (between 10 and 50 μg per 10⁸ cells; our unpublished results). Uninduced *E. coli* cultures harboring pTBP3 were used to determine the subcellular localization of the pneumococcal polysaccharide. It was found that a substantial amount (~40%) of the type 3 polysaccharide was localized in the periplasmic space of *E. coli*, 40–50% was in the cytoplasm, and the rest were in the membrane fraction (not shown). This polysaccharide was further characterized by gel filtration chromatography, showing it to be highly polymerized as it eluted in the void volume of a Sepharose CL-4B column (not shown). These results indicated that Cap3B is actually the enzyme that synthesizes the type 3 pneumococcal polysaccharide.

Formation of Binary Cells of *Pneumococcus* by Transformation with the *cap3B* Gene. Pneumococcal strains that produce simultaneously two distinct capsular polysaccharides have been described previously (see reference 29 for a review). These strains arise by transformation reactions between pneumococci-expressing capsules that contain hexuronic acids, such as those belonging to serotypes 1 and 3. When the plasmid pLSE3B was introduced by transformation into the S1 pneumococcal strain M25, all the lincomycin-resistant transformants expressed both type 1 and type 3 capsular polysaccharides, as determined by the Quellung reaction (data not shown), as well as by immunodiffusion analysis of

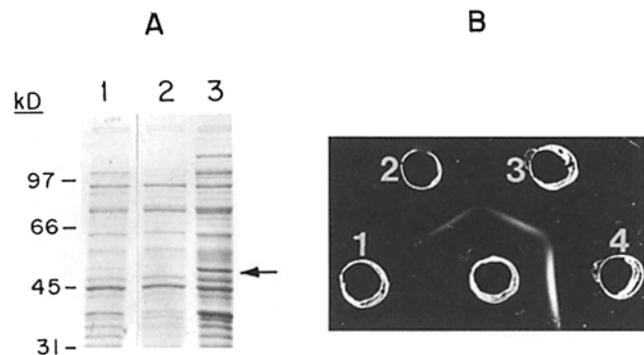


Figure 3. Expression of the Cap3B polysaccharide synthase and synthesis of type 3 pneumococcal polysaccharide by *E. coli* containing the recombinant plasmid pTBP3. (A), BL21(DE3)pLysS cells containing either pTBP3 or pT7-7 vector alone were grown to an OD₆₀₀ of 0.6, induced with 1 mM IPTG, and the crude sonicated extracts were analyzed by 10% SDS-PAGE. Lane 1, extracts from bacteria containing pT7-7. Lanes 2 and 3, extracts from bacteria harboring pTBP3 and removed before induction with IPTG and after 90 min induction, respectively. The location of the overexpressed Cap3B is indicated by an arrow. The molecular mass of the standards are indicated on the left. (B), Double immunodiffusion test. Wells labeled 1–3 contain extracts corresponding to samples 1–3 of A, respectively. Well 4 contains purified type 3 pneumococcal polysaccharide. The center well received type 3 antiserum. Each well received 10 μl of the corresponding sample.

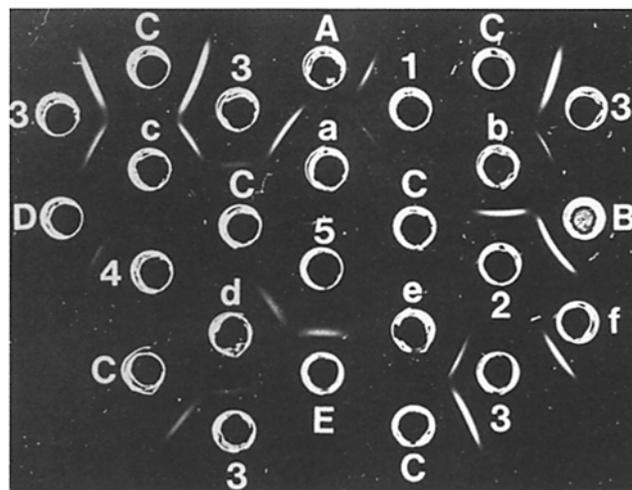


Figure 4. Double immunodiffusion in agarose of capsulated pneumococci. 1–5 indicate antisera against pneumococcal polysaccharides of types 1–3, 5, and 8, respectively. A–E designate capsular polysaccharides purified from the *S. pneumoniae* strains of serotypes 1–3, 5, and 8, respectively. a–d correspond to binary transformants of the following serotypes: 1/3, 2/3, 5/3, and 8/3, respectively. e and f contains polysaccharide prepared from S3⁺ transformants of M11 and M31, respectively. Each well received 10 μl of the corresponding sample.

cell extracts with specific antisera (Fig. 4). This result was not restricted to serotypes 1 and 3 because pneumococcal cells belonging to serotypes 2, 5, and 8, all of them producing capsules that contain glucuronic acid (30), also gave rise to binary transformants when transformed with pLSE3B. Furthermore, strains M11 and M31, two S²⁻ laboratory derivatives of the rough strain R36A, also synthesized type 3 capsule (but not type 2) when transformed with pLSE3B (Fig. 4). In every case, the type 3 polysaccharide produced was of high M_r , as demonstrated by gel filtration (not shown).

Discussion

The molecular organization of the genes implicated in capsulation of *S. pneumoniae* is currently being studied. As in Gram-negative bacteria (31), genes involved in the synthesis of the sugar precursors and the polymerization reactions appear to be specific for any given capsular type also in *S. pneumoniae* (7–12, 32). Type 3-specific genes are arranged as an operon, and the promoter is located just upstream of the first specific gene (*cap3A*) (11). In type 19F, however, Guidolin and co-workers (32) suggested that upstream of the type-specific genes, the transcript included several open reading frames common to all pneumococci. Thus, it appears that diverse functional organizations of the capsular genes may exist in different pneumococcal serotypes.

Very few gene products thought to be implicated in capsule formation in Gram-positive bacteria have been identified biochemically so far. Among these, the *S. pyogenes* *hasA* codes for an enzyme that directs hyaluronic acid biosynthesis, the component of the capsule of this microorganism (33–35). It has been shown that HasA is necessary and sufficient to synthesize hyaluronic acid both in *Enterococcus faecalis* and in *E. coli*, provided that the host bacterium produces the corresponding sugar nucleotides (UDP-GlcA and UDP-N-acetyl glucosamine) (36). This relationship appears also to be the case for the pneumococcal Cap3B synthase (Fig. 3 B). In addition to the omnipresent UDP-Glc, which plays a central role in the biosynthesis of most cellular polysaccharides, UDP-GlcA has been found both in *E. coli* (37–39) and in some *S. pneumoniae* serotypes (40). Since type 1 capsule contains galacturonic acid, a specific epimerase reaction is required to convert UDP-GlcA to UDP-GalA (41). Furthermore, since pTBP3 and pLSE3B contain exclusively the *cap3B* gene as an insert (Fig. 2), it is clear that the Cap3B synthase has both UDP-Glc and UDP-GlcA glycosyltransferase activities and possibly synthesizes the type 3 polysaccharide in a processive manner by monomer addition. In *S. pyogenes*, Dougherty and van de Rijn (34) speculated that HasA synthase may catalyze transfer of only one of the two sugars, N-acetyl glucosamine, to the nascent hyaluronic acid chain. Nevertheless, DeAngelis and Weigel (35) concluded that HasA is a synthase that possesses both glycosyl transferase activities that are required to polymerize hyaluronic acid.

In *E. coli* and in other capsulated Gram-negative bacteria, the capsular polysaccharide determinants were shown to contain three phenotypically distinct regions associated with polysaccharide expression. Region 1 is necessary for the transport of mature, lipid-linked polysaccharide across the outer membrane and its assembly into a capsule. Region 2 is serotype specific and codes for the enzymes involved in synthesis and polymerization. Region 3 is responsible for the translocation of the polysaccharide across the cytoplasmic membrane (42). The KpsMT proteins coded by genes located in region 3 and belonging to the family of ABC transporters (43) are capable of transporting a variety of acidic polysaccharides to the periplasm. Of particular interest is the finding reported here that ~50% of the pneumococcal type 3 polysaccharide synthesized in *E. coli* appears in the periplasmic space, although we can not determine whether the mechanism for this transport is the same as that for the homologous polysaccharide.

In Gram-positive bacteria, the mechanism of polysaccharide transport has not been characterized so far. In type 19F pneumococci, it has been suggested that some of the genes located upstream of the specific capsular cluster (namely, *cps19fA-D*) might be responsible for the transport of the intracellularly synthesized capsular polysaccharide (32). However, we have recently shown that the genes corresponding to those described by Guidolin et al. are not functional in pneumococci belonging to the type 3 serotype (11). The results reported here strongly suggest that another still-unknown mechanism may be responsible for the export of the capsular polysaccharide through the cytoplasmic membrane.

We have shown that the presence of the type 3 pneumococcal polysaccharide synthase is sufficient to direct the synthesis of a type 3 capsule in *S. pneumoniae* strains belonging to serotypes 1, 2, 5, and 8 (Fig. 4). It can be assumed that the mechanism of polysaccharide transport of the recipient cell is used for the formation of a type 3 capsule in the binary transformants. Early experimental evidence showed that when an unencapsulated mutant of type 3 is transformed with DNA from a capsulated strain of type 1, a minority (2–5%) of the transformants were of the binary type, i.e., they reacted with both types 1 and 3 antisera and synthesized two distinct polysaccharides (44). Transformation experiments with these stable binary strains suggested that a supernumerary gene cluster (i.e., type 1) had been inserted, presumably by homologous recombination, at a repeat element unlinked to the capsule locus (reviewed in 29). Recently, we have shown that copies of the DNA sequences located upstream of the *cap3ABC* operon are present in different locations in the genome (8). In contrast with this situation, the transformants described here resulted from the expression of the plasmid-borne *cap3B* gene. In conclusion, provided that the corresponding sugar nucleotide precursors (UDP-Glc and UDP-GlcA) are present, *cap3B* appears to be the only gene required to direct type 3 polysaccharide biosynthesis in the pneumococcal strains or in *E. coli*.

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