The Capsule Polysaccharide Synthesis Locus of *Streptococcus pneumoniae* Serotype 14: Identification of the Glycosyl Transferase Gene *cps14E*

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To identify a chromosomal region of *Streptococcus pneumoniae* serotype 14 involved in capsule polysaccharide synthesis, two strategies were used: (i) Tn916 mutagenesis, followed by the characterization of four unencapsulated mutants, and (ii) cross-hybridization with a capsule polysaccharide synthesis gene (*cps*) probe from *S. agalactiae*, which has a structurally similar capsule. The two approaches detected the same chromosomal region consisting of two adjacent *EcoRI* fragments. One of these *EcoRI* fragments was cloned and hybridized with a cosmid library. This resulted in clone cMK02. A similar cosmid clone was obtained from an unencapsulated Tn916 mutant, Spn14.H. Sequence analysis of the two cosmid clones revealed that in the Tn916 mutant, a gene, *cps14E*, which is homologous to other bacterial genes encoding glycosyl transferases, had been inactivated. An open reading frame immediately downstream of *cps14E*, designated *cps14F*, shows no significant homology with any known genes or proteins. A functional assay showed that *cps14E* encodes a glycosyl transferase and that a gene-specific knockout mutant lacks this enzyme activity, whereas inactivation of *cps14F* does not have this effect.

Many bacteria contain surface polysaccharides which act as a protective layer against the environment. Surface polysaccharides of pathogenic bacteria usually make the bacteria resistant to the defense mechanisms of the host, e.g., the lytic action of serum or phagocytosis (7). In this respect, the serotype-specific capsular polysaccharide (CP) of *Streptococcus pneumoniae*, a human pathogen causing pneumonia, meningitis, and otitis media, is an important virulence factor (27). Unencapsulated strains are avirulent (34), and antibodies directed against the CP are protective (5, 17). Protection is serotype specific; each serotype has its own, specific CP structure. Ninety different capsular serotypes have been identified (16). Currently, CPs of 23 serotypes are included in a vaccine (20).

The CP structures of most pneumococcal serotypes have been determined (8). They contain polymeric repeats of identical oligosaccharide subunits. Figure 1 shows the subunit structure of the serotype 14 CP. Little is known about the molecular biology and biochemistry of pneumococcal CP biosynthesis. The classic genetic experiments of McCarty et al. (3, 25) indicated that the CP synthesis (*cps*) genes are localized in one cluster. Recent studies by Dillard et al. (9) and Guidolin et al. (15) confirm this observation. In analogy with other systems, it may be assumed that CP biosynthesis in serotypes with a more or less complex oligosaccharide subunit structure starts with the production of activated monosaccharides, which is followed by the formation of complete oligosaccharide sub-

units on a lipid carrier molecule by the subsequent addition of monosaccharides by transferases and, finally, polymerization of completed oligosaccharide subunits (4, 19, 28, 33).

CP synthesis and the involved genes have been studied in only a few members of the genus *Streptococcus*: *S. agalactiae* type III (23, 31) and *S. pneumoniae* serotype 3 (1, 9, 12) and serotype 19F (15). The CP structure of *S. pneumoniae* serotype 14 is very similar to that of *S. agalactiae* type III (Fig. 1). The only difference is an extra sialic acid residue linked to the galactose side group of *S. agalactiae* polysaccharide. Since the *cps* locus of type III of *S. agalactiae* has been characterized recently (23, 30, 31), we used clones from this *cps* locus for cross-hybridizations. In addition, Tn916 transposon mutagenesis was used to identify the *cps* locus of *S. pneumoniae* type 14.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. pneumoniae* serotype 6B, 14, and 23F strains were NCTC 11888, NCTC 11902, and NCTC 11910, respectively. The pneumococcal strain CP1200 (*malM511 str-1*) was described previously (26). *Enterococcus faecalis* CG110 (13), containing Tn916, and *Escherichia coli* CG120 (13), containing pAM120, were a gift from C. E. Rubens (University of Seattle, Seattle, Wash.). *S. pneumoniae* and *E. faecalis* were grown aerobically at 37°C in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY medium) in a static culture or in an anaerobic atmosphere on THY medium with 1.5% agar (unless stated otherwise). A spontaneous, streptomycin-resistant mutant of *S. pneumoniae* serotype 14 was obtained by growing bacteria (aerobically) overnight on blood agar plates containing 100 µg of streptomycin per ml. Then cells were collected and replated on the same medium containing 1,000 µg of streptomycin per ml. After 2 days, one of the colonies was chosen and used for further experiments. Confirmation typing of pneumococci was done by growth on blood agar plates under aerobic conditions and determination of alpha hemolysis and optochine sensitivity. *E. coli* NM554 (Stratagene, La Jolla, Calif.) was used as a host for pPR691 (21), and *E. coli* DH5α (32) was used as a host for pBluescript II KS (Stratagene) and pPR691. All *E. coli* strains and clones were grown in or on LB or TB medium (32) containing the appropriate antibiotic concentration.

Plasmids and antibodies. pPR691 (obtained from P. Reeves, University of Sydney, Sydney, New South Wales, Australia) is a low-copy-number cosmid vector (21) bearing antibiotic resistance to kanamycin (50 µg/ml), streptomycin

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FIG. 1. Subunit structure of pneumococcal serotype 14 and *S. agalactiae* type III capsule polysaccharides. Glc, glucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; NeuNAc, *N*-acetylneuraminic acid.

(10 µg/ml), and spectinomycin (20 µg/ml). pBluescript II KS was used for subcloning of fragments from the cosmid clones. pAM120 is a pGIII01 (13) derivative containing a copy of *Tn916* (tetracycline [10 µg/µl] and ampicillin [50 µg/ml]). Clones pCER101, -107, -108, -113, and -114, covering the *cps* locus of *S. agalactiae* type III, were kindly provided by C. E. Rubens. Monoclonal antibodies HASP14.1 (specific for serotype 14 polysaccharide) and HASP8 (reacting to pneumococcal C-polysaccharide) were obtained from U. B. Sørensen (The Statens Serum Institute, Copenhagen, Denmark).

Transposon mutagenesis of type 14 and screening for capsule-negative mutants. The streptomycin-resistant strain of *S. pneumoniae* serotype 14 and *E. faecalis* CG110 were grown to the stationary phase in the presence of antibiotics, washed once with THY medium without antibiotics, mixed at a ratio of 10:1 (pneumococci/enterococci), and incubated for 18 h on THY agar plates. Then the bacteria were collected, plated at several dilutions on THY plates containing tetracycline (10 µg/ml) and streptomycin (1 mg/ml), and incubated for 2 days. Streptomycin- and tetracycline-resistant cells were stored at -80°C in 15% glycerol-THY medium. Twenty microliters of the Tn916 library (approximately 10⁷ cells), stored at -80°C, was added to 970 µl of THY medium and 10 µl of HASP14.1. This suspension was rotated at room temperature for 2 h. A visible precipitate was removed by centrifugation at low speed (500 × g for 3 min). The supernatant was plated on THY plates containing tetracycline and incubated at 37°C for 2 days. Pneumococcal colonies had a flat, transparent appearance, and 400 were subcultured and screened for capsule production as described by Rubens et al. (30).

Construction of a serotype 14 cosmid library. Chromosomal DNA of *S. pneumoniae* serotype 14 was partially digested with *Sau3A* and run on a 0.5% agarose gel. Fragments of 30 to 40 kb were isolated with Gene-Clean (Bio 101, La Jolla, Calif.) and ligated with *Bam*HI-*Pvu*II arms of pPR691 as described before (21). DNA was packaged into phage particles by using Gigapack II Gold packaging extract (Stratagene), and transformants were selected on LB agar medium containing kanamycin, streptomycin, and spectinomycin.

Other DNA techniques. Most DNA techniques were performed as described before (32). Chromosomal DNA was isolated as described by Ausubel et al. (2). Probes for hybridization experiments were labeled with ^{32}P by using a random priming labeling kit (Amersham). Hybridizations were carried out at 60 or 65°C without formamide. Low-stringency washes were done twice with 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4 , and 1 mM EDTA [pH 7.7]) at 42°C for 10 to 20 min. Restriction enzymes were purchased from Pharmacia-LKB.

DNA sequencing and analysis. Plasmid and cosmid DNA was sequenced by using an A.L.F. DNA sequencer (Pharmacia-LKB). Primers Tn-R-O and TnLO-2 (6) were used for sequencing the flanking regions of Tn9/6. Sequencing data were assembled by using PC/GENE (IntelliGenetics) and compared with databases available at the National Center for Biotechnology Information, Bethesda, Md., using the programs TFASTA and BLAST. Multiple alignments of amino acid sequences were carried out by using MACAW (24).

Construction of *cps14E* and *cps14F* mutant strains. Insertion mutations of *cps14E* and *cps14F* were constructed in vitro by blunt ligation of a 4.9-kb *HincII* fragment of *Tn916* containing the tetracycline resistance gene into the unique *EcoRI* site of the insert of pMK100 and in the unique *PvuII* site of the insert of pGV100, respectively. After transformation of *E. coli* DH5 α , clones in which the direction of transcription of the tetracycline resistance gene was similar to that of the adjacent *cps* genes were selected. This yielded plasmids pKOM3 and pKOM9, respectively. The *cps14E* and *cps14F* insertion mutations were introduced into the chromosome of the wild type by natural transformation as described by Yother et al. (35), using the constructs pKOM3 and pKOM9. Pneumococcal strain CP1200 was used as a donor strain for competence factor. Transformants were selected for tetracycline resistance, and mutations were confirmed by Southern hybridization analysis.

Incorporation of ^{14}C -labeled glucose into capsule-specific intermediates. Pneumococcal membranes containing capsule-specific glycosyl transferase activity were prepared by the method of Osborn et al. (28), with some modifications. Cultures were grown overnight and then diluted 10-fold in 250 ml of medium and incubated at 37°C . Cells were harvested at mid-log phase (optical density at 600 nm of 0.3 to 0.4) by centrifugation and resuspended in 10 ml of 0.7 M sucrose–50 mM Tris-Cl (pH 8.0)–1 mM EDTA. Approximately 100 U of mutanolysin (Sigma) and 20 mg of lysozyme were added, and this suspension was incubated for 2 to 4 h at 4°C . Protoplasts were collected by centrifugation, lysed in 20 ml of 20

mM Tris-Cl (pH 8.0)–1 mM EDTA–200 μ M phenylmethylsulfonyl fluoride, and sonicated three times for 20 s each. Debris was removed by low-speed centrifugation, and then membranes were spun down at 30,000 $\times$ g for 1 h at 0°C. Membranes were washed once in 20 ml of buffer A (50 mM Tris-acetate [pH 8.3], 1 mM EDTA, 200 μ M phenylmethylsulfonyl fluoride), centrifuged again, and finally resuspended in 0.5 ml of buffer A. Glycosyl transferase activity was measured by adding 10 mM $MgCl_2$ and 30,000 to 50,000 cpm of uridine diphospho-[U - ^{14}C]glucose (300 mCi/mmol; Amersham) to membrane preparations in a final volume of 50 μ l. The protein concentration was approximately 0.8 mg/ml. After 1 h of incubation at 10°C, the reaction was stopped by addition of 1 ml of chloroform-methanol (2:1). This solution was extracted three times with 0.2 ml of PSUP (1.5 ml of chloroform, 25 ml of methanol, 23.5 ml of water, and 0.183 g of KCl). The incorporation of ^{14}C into the glycolipid fraction in the organic phase was measured in a scintillation counter (Beckman).

Nucleotide sequence accession number. The GenBank accession number of *cps14E* and *cps14F* is X85787.

RESULTS

Construction of a Tn916 transposon library and screening for mutants deficient in capsule production. Conjugational

transposon mutagenesis between *E. faecalis* and *S. pneumoniae* serotype 14 yielded 8,000 to 10,000 tetracycline-resistant pneumococcal colonies. Ten randomly chosen mutants were analyzed by Southern hybridization; *Hind*III digests were hybridized to a Tn916 probe (pAM120 [data not shown]). We observed one to three Tn916 insertions per genome. As the hybridization patterns among these mutants were different, Tn916 insertion had taken place randomly in the chromosome. To isolate capsule-deficient mutants, we used monoclonal antibody HASP14.1 to agglutinate the encapsulated bacteria that were present in the Tn916 library. Nonagglutinating bacteria were grown on THY-agar plates containing tetracycline. Among 400 colonies replica plated and screened for reactivity with HASP14.1, 27 colonies showed no or very weak reactions with HASP14.1. These bacteria were checked once more for agglutination with HASP14.1 and grown on blood agar plates to confirm alpha-hemolysis and optochine sensitivity. Four unencapsulated *S. pneumoniae* Tn916 mutants, Spn14.H, Spn14.8, Spn14.D, and Spn14.F, remained after this procedure. Bacteria of all four mutants showed agglutination with HASP8, a C-polysaccharide-specific monoclonal antibody, while wild-type serotype 14 bacteria were not agglutinated by this antibody.

Characterization of the four unencapsulated mutants. To count the Tn916 insertions in each mutant, total cellular DNA of the wild-type strain and the four mutants was digested with *Sau3A* and probed with ³²P-labeled pAM120 (Fig. 2). No hybridizing fragments were detected in the chromosome of the wild-type strain. Since Tn916 contains eight internal *Sau3A* sites (11), a single insertion would result in nine hybridizing fragments: seven internal fragments of 4,093, 3,091, 2,890, 2,870, 997, 717, and 262 bp, as indicated in Fig. 2, and two additional end-specific fragments. In Spn14.H, two additional end-specific fragments hybridized with the probe, indicating a single insertion of Tn916. Mutant Spn14.8 showed four additional bands, indicating two Tn916 insertions. Mutants

FIG. 2. Southern blot analysis of chromosomal DNA of serotype 14 (wild type [wt]) and mutants Spn14.H (lane H), Spn14.8 (lane 8), Spn14.D (lane D), and Spn14.F (lane F) digested with *Sau3A* and probed with 32 P-labeled pAM120. The internal *Sau3A* fragments and their sizes are indicated on the right in base pairs; the smallest internal fragment of 262 bp is not visible.

Spn14.D and Spn14.F demonstrated identical hybridization patterns with two *Tn916* copies in the chromosome. We were unable to obtain single-copy *Tn916* mutants via natural transformation of wild-type serotype 14 bacteria with chromosomal DNA of the mutants. We were able, however, to clone the *Tn916* copy of mutant Spn14.H and sequence its flanking regions (see below).

Cross-hybridization between *cps* genes of *S. pneumoniae* serotype 14 and *S. agalactiae* type III. In a complementary approach, *cps* clones of *S. agalactiae*, containing genes involved in the biosynthesis of a CP with a structure similar to that of type 14 (Fig. 1), were used as probes in cross-hybridization experiments. The 3.6-kb *EcoRI* insert of clone pCER108, which contains *cpsD* (31), showed a specific signal at low-stringency hybridization conditions with DNA of serotype 14 but not with DNA of serotypes 6B and 23F (data not shown). The latter serotypes were used as negative controls since their CP structure differs significantly from that of type 14 and *S. agalactiae*. Two *EcoRI* fragments of 2.4 and 8 kb hybridized with the pCER108 probe. The 2.4-kb *EcoRI* fragment was cloned in the pBluescript, resulting in pGV100 (see Fig. 4). The insert of pGV100 hybridized with both the 2.4- and 8-kb *EcoRI* fragments of serotype 14 chromosomal DNA (Fig. 3). As we will show below, the 8-kb band was found to be the result of an incomplete digestion of type 14 chromosomal DNA.

All four *Tn916* mutants have an insertion in a DNA region homologous to the insert of pGV100. Chromosomal DNA of the four mutants and wild-type bacteria was digested with *EcoRI*, which does not cut within *Tn916* (18 kb). As mentioned, the pGV100 probe reacts positively with 2.4- and 8-kb

FIG. 3. Southern blot analysis of chromosomal DNA of serotype 14 and mutants Spn14.H, Spn14.8, Spn14.D, and Spn14.F digested with *EcoRI* and probed with the 32 P-labeled insert of pGV100. Lanes are labeled as in Fig. 2.

FIG. 4. Restriction enzyme map of 8 kb of the *S. pneumoniae* serotype 14 genome involved in CP production. The positions of the clones pGV100, pMK100, pMK102, and pMK103 and of the *Tn916* insertion in mutant Spn14.H are indicated. The locations of *cps14E* and *cps14F* and the direction of transcription are shown by the arrows. E, *EcoRI*; X, *XbaI*; S, *SpeI*; P, *PvuII*; E*, *EcoRI* site partially digested in chromosomal DNA of serotype 14.

EcoRI bands of serotype 14 DNA. In the lanes with DNA of all four mutants, the 8-kb *EcoRI* band has disappeared and a band migrating at about 25 kb is present instead. No mutants having a transposon insertion in the 2.4-kb *EcoRI* fragment were observed.

Cloning of *Tn916* and its flanking regions from the capsule-deficient mutant Spn14.H. Chromosomal DNA of Spn14.H was digested with *Bam*HI, which does not cut within the transposon, and ligated into the *Bam*HI site of the low-copy-number cosmid vector pPR691. Two tetracycline-resistant cosmid clones, cMK10 and cMK11, were isolated. The DNA inserts of these two cosmid clones have identical restriction patterns. *EcoRI* digests of the two cosmid clones were hybridized with the insert of pGV100; in both cases, only a 2.4-kb *EcoRI* fragment reacted with the probe (data not shown). This finding indicated that the previously observed hybridization of pGV100 with an 8-kb *EcoRI* fragment in wild-type *S. pneumoniae* serotype 14 was due to a partial digestion of chromosomal DNA at the *EcoRI* site linking the 2.4- and 5.6-kb *EcoRI* fragments (Fig. 4) and that insertion of *Tn916* into the 5.6-kb *EcoRI* fragment led to abolishment of capsule synthesis.

Construction of a cosmid library of wild-type *S. pneumoniae* serotype 14. A genomic cosmid library of *S. pneumoniae* serotype 14 in pPR691 was made, and the 400 independent clones were screened with probe pGV100. Two colonies (cMK01 and cMK02) hybridized positively. Both clones contained the 2.4- and 5.6-kb *EcoRI* fragments. *SpeI* and *XbaI* fragments of cMK02 were subcloned in pBluescript and mapped (Fig. 4).

Sequencing of the *cps14E* and *cps14F* genes and their homology. To characterize the gene inactivated by transposon insertion in Spn14.H, the flanking regions of *Tn916* cloned in cMK10 were sequenced from both ends of *Tn916*. The sequence data indicated that *Tn916* had inserted into a region of serotype 14 that showed 98% identity to the *cps19fE* gene of *S. pneumoniae* serotype 19F (15). The complete nucleotide sequence of the *cps14E* gene was obtained after sequencing of pMK100, pMK102, and pMK103. *cps14E* is also homologous to several other bacterial glycosyl transferase genes: *cpsD* of *S. agalactiae* (31), *rfbP* of *Salmonella typhimurium* (22), *exoY* of two *Rhizobium* species (14, 29), and *xps2A* of *Xanthomonas campestris* (18). Figure 5 shows the alignment of the potential 52.6-kDa polypeptide encoded by *cps14E* with the amino acid sequence of *cps19fE* (identity 95.8%) and these other bacterial glycosyl transferases. The homology is most pronounced in the C termini of the transferases. There are two blocks of highly conserved amino acids in this region (Fig. 5).

An open reading frame of 447 bp (designated *cps14F*) is

FIG. 5. Schematic alignment of Cps14E with homologous proteins. The homologies detected with the program BLASTX are as follows (GenBank accession numbers are given in parentheses): *S. pneumoniae* serotype 19F Cps19fE (U09239), 95.8% identity over 455 amino acids; *Rhizobium meliloti* ExoY (Q02731), 37.0% identity over 200 amino acids at the C-terminal end; *Rhizobium* species ExoY (P14186), 36.5% identity over 200 amino acids at the C-terminal end; *Salmonella enteritica* serovar Typhimurium LT2 RfbP (P26406), 31.3% identity over 469 amino acids; *X. campestris* Xps2a (S31721), 24.7% identity over 466 amino acids; and *S. agalactiae* CpsD (Q04664), 43.7% identity over 279 amino acids. The black bars, made with the program MACAW (24), indicate amino acid positions that are conserved in all sequences.

situated immediately downstream *cps14E*. *cps14E* and *cps14F* are in the same reading frame, being separated by six nucleotides. *cps14F* showed no homology to any known genes or proteins.

A *cps14E* gene-specific knockout mutant lacks glycosyl transferase activity. Since *cps14E* showed homology to several glycosyl transferase genes, this step in polysaccharide biosynthesis was analyzed by incorporation of [¹⁴C]glucose into polysaccharide-specific glycolipid intermediates (10, 28, 33). Membranes of the four Tn916 mutants were, in contrast to wild-type membranes, not able to incorporate radioactively labeled UDP-glucose into a glycolipid fraction (data not shown). However, the lack of glycosyl transferase activity of these mutants also may be caused by polar effects of the Tn916 insertion on genes located downstream *cps14E*. The *cps* genes may form a single transcriptional unit, as in serotype 19F (15) and other bacterial species. Therefore, we created two gene-specific knockout mutants, Spn14#E and Spn14#F, in which *cps14E* and *cps14F*, respectively, are disturbed. A 4.9-kb *HincII* fragment containing the tetracycline resistance gene of Tn916 was cloned into the unique *EcoRI* site of the insert of pMK100. A clone in which the directions of transcription of the tetracycline resistance gene and *cps14E* were similar was selected and designated pKOM03. Plasmid pKOM09 contains the tetracycline resistance cassette in the same direction of transcription as the adjacent *cps14F* sequences in the *PvuII* site of pGV100. Both plasmids were transformed into wild-type serotype 14, and cells were selected for tetracycline resistance. Since the pBluescript replicon is unable to function in *S. pneumoniae*, tetracycline-resistant transformants are the result of a homologous recombination event. This leads to integration of the tetracycline resistance block into the host chromosome and consequent disruption of *cps14E* and *cps14F* with plasmids

pKOM03 and pKOM09, respectively. Disruption of *cps14E* in mutant Spn14#E and *cps14F* in Spn14#F at the correct location in the chromosome was confirmed by Southern hybridization analysis (data not shown). The two mutants showed no agglutination with HASP14.1, indicating that both *cps14E* and *cps14F* are involved in CP synthesis. Membranes of wild-type bacteria and the two mutants were prepared and incubated with radioactively labeled UDP-glucose (Table 1). Membranes of wild-type bacteria and mutant Spn14#F showed the same level of incorporation of [¹⁴C]glucose. Mutant Spn14#E demonstrated a reduced transferase activity of approximately 10% of the activity of the wild-type strain, even lower than that of serotype 4 (which contains no glucose in its polysaccharide structure).

DISCUSSION

Transposon mutagenesis in combination with a cross-hybridizing *cps* probe of *S. agalactiae* type III has allowed us to identify and clone the locus responsible for serotype-specific

TABLE 1. Incorporation of [¹⁴C]glucose by pneumococcal membranes into glycolipids

Membrane prepn	cpm incorporated/ μ g of membrane protein
Serotype 14	478
Serotype 4	100
Spn14#E	53
Spn14#F	463

polysaccharide production of *S. pneumoniae* serotype 14. Both approaches revealed the same region on the chromosome and the same gene, *cps14E*, encoding a polypeptide homologous to bacterial glycosyl transferases.

We observed that membranes of serotype 14 were able to direct incorporation of [¹⁴C]glucose into a glycolipid fraction. In 1964, Distler and Roseman (10) performed similar experiments using serotype 14 and observed incorporation of not only [¹⁴C]glucose but also of [¹⁴C]galactose. However, the third component of the type 14 repeating subunit, [¹⁴C]N-acetylglucosamine (Fig. 1), could not be recovered from the glycolipid fraction. The authors then speculated on the presence of an epimerase activity which converts UDP-glucose to UDP-galactose and vice versa. This means that either glucose or galactose may be the first monosaccharide of the subunit structure. Furthermore, since labeled sugars were recovered from a glycolipid fraction, subunit synthesis takes place on a membrane-bound lipid carrier molecule, as in the biosynthesis of polysaccharides of other bacteria (4, 18, 29, 31). Interestingly, the deduced amino acid sequence of the gene in which the transposon was inserted, *cps14E*, shows homology to polypeptides (CpsD, RfbP, ExoY, and Xps2A) known to catalyze the linkage of the first sugar to the lipid carrier. The observation that the membranes of a *cps14E*-specific knockout mutant lacked any glycosyl transferase activity is in good agreement with the hypothesis that Cps14E is the enzyme that catalyzes this first step in polysaccharide biosynthesis. Reduced transferase activity of this mutant is not caused by polar effects on genes downstream *cps14E*, since a *cps14F* mutant (*cps14F* is located immediately downstream *cps14E*) showed the same level of [¹⁴C]glucose incorporation as the wild-type strain. The amino acid alignment of the glycosyl transferase proteins shows a conserved domain at the C terminus. This, however, does not allow deductions as to the function for this region, nor does it predict substrate specificity, e.g., UDP-glucose or UDP-galactose. *cps14E* is almost identical to *cps19fE*, but both pneumococcal *cpsE* genes are only partially identical to the *cpsD* gene of *S. agalactiae*. The *cpsD* gene product transfers galactose to the lipid carrier (31). Comparison of the subunit structures of serotype 14 and 19F [-4)-β-D-ManNAc-(1-4)-α-D-Glc-(1-2)-α-L-Rha-(1-)] shows that the only sugar that they have in common is glucose, which may indicate that the *cpsE* genes of serotypes 14 and 19F are glucosyl transferases. Preliminary data confirm this suggestion, since serotype 19F membranes also incorporate [¹⁴C]-labeled glucose (data not shown). In this case, incorporation of [¹⁴C]galactose synthesized by an epimerase activity can be excluded, because the CP of type 19F contains no galactose. *cps14F* is also involved in CP synthesis, since a *cps14F*-specific knockout mutant appeared to be unencapsulated. However, the function of *cps14F* is not clear.

Capsule synthesis and C polysaccharide synthesis are not linked, since the four capsule-deficient mutants still produce C polysaccharide. The C polysaccharide is a constitutive component of the pneumococcal cell wall, while the level of capsule expression is regulated by growth rate and growth conditions. Therefore, it seems plausible that the two polysaccharides are encoded by two separate, independently regulated loci. The fact that glucose is a component of C polysaccharide may account for the low background level of [¹⁴C]glucose incorporation in serotype 4 and the *cps14E* mutant. Possibly, C polysaccharide production proceeds via a lipid-linked intermediate but at a much lower synthesis level than capsule production.

Guidolin et al. (15) have cloned 6,322 bp of the *cps* locus of *S. pneumoniae* serotype 19F. So far, they have identified six open reading frames in addition to *cps19fE*. Four of these

reading frames are homologous to sequences of serotype 14. Our sequencing of several reading frames upstream *cps14E* showed them to be almost identical to the serotype 19F genes (data not shown). These data, together with the functional assay for the *cps14E*-encoded transferase, suggest that we have identified the first, and so far only, cosmid clone containing at least a large part of the locus responsible for the synthesis of an *S. pneumoniae* CP.

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