Molecular and genetic characterization of the capsule biosynthesis locus of *Streptococcus pneumoniae* type 23F

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The authors have previously reported the nucleotide sequence of the 5' and 3' portions of the *Streptococcus pneumoniae* type 23F capsular polysaccharide biosynthesis locus (*cps23f*) (from *dexB* to *cps23fB* and from *cps23fL* to *aliA*). These regions of *cps23f* were very similar to the sequence reported for *cps19f*, the capsule locus of *S. pneumoniae* type 19F. However, Southern hybridization analysis indicated that no other genes closely related to *cps19f* are present in the *cps23f* locus. In this study long-range PCR was used to amplify and clone the section of the *S. pneumoniae* type 23F capsule locus between *cps23fB* and *cps23fL*. This region is 13 kb in size and contains 12 new ORFs, designated *cps23fC-E*, *I*, *J*, and *T-Z*. Functions are proposed for all of the protein products, including functional homologues of Cps19fC-E, Cps19fI and Cps19fJ. A biosynthetic pathway for type 23F capsular polysaccharide is also proposed.

Keywords: *Streptococcus pneumoniae*, capsule locus, capsular polysaccharide biosynthesis

INTRODUCTION

Streptococcus pneumoniae (the pneumococcus) is an important cause of invasive disease in human populations throughout the world, resulting in high morbidity and mortality. Control of pneumococcal disease is being complicated by the increasing prevalence of antibiotic-resistant strains and the suboptimal clinical efficacy of existing vaccines. *S. pneumoniae* produces a polysaccharide capsule, which is essential for virulence, because it protects the pneumococcus from the non-specific immune defences of the host during an infection (Austrian, 1981). All fresh isolates from patients with pneumococcal infection are encapsulated, and spontaneous non-encapsulated (rough) derivatives of such strains are almost completely avirulent.

There are now 90 recognized serotypes of *S. pneumoniae* (Henrichsen, 1995), each of which produces a structurally distinct capsular polysaccharide (CPS). CPS

production requires a complex pathway including synthesis of the component monosaccharides, activation of each to a nucleotide precursor, co-ordinated transfer of each sugar, in sequence, to the repeating oligosaccharide, and subsequent polymerization, export and attachment to the cell surface. The exception to this is type 3, which has a very simple CPS repeat unit consisting of glucose and glucuronic acid, and appears to be synthesized by a processive transferase (Arrecubieta *et al.*, 1995; Dillard *et al.*, 1995). Classical genetic studies by Austrian *et al.* (1959) demonstrated that the genes encoding CPS biosynthesis are closely linked on the *S. pneumoniae* chromosome. Recently, this has been confirmed by sequence analysis of complete capsule loci (*cps*) from *S. pneumoniae* serotypes 1, 3, 14, 19F and 19B (Arrecubieta *et al.*, 1995; Dillard *et al.*, 1995; Guidolin *et al.*, 1994; Kolkman *et al.*, 1997; Morona *et al.*, 1997a, b; Muñoz *et al.*, 1997). These studies demonstrated that, in each case, the *cps* loci are located between *dexB* and *aliA* in the pneumococcal chromosome. Moreover, with the exception of type 3, they have a common organization, with type-specific genes located in the central portion of the locus flanked by genes common to multiple serotypes. Thus, transformation from one capsular serotype

Abbreviation: CPS, capsular polysaccharide.

The GenBank accession number for the nucleotide sequence reported in this paper is AF030373.

Long-range PCR. The Expand Long Template PCR System (Boehringer Mannheim) was used for long-range PCR according to the manufacturer's instructions and was performed in a Hybaid Touchdown Thermal Cycler. The long-range PCR programme used consisted of 25 cycles, after an initial incubation at 92 °C for 2 min to allow complete denaturation of the DNA template. The first 10 cycles consisted of denaturation at 92 °C for 30 sec, annealing initially at 55 °C for 1 min, decreasing by 1 °C per cycle to 45 °C at cycle 10, and elongation at 68 °C for 15 min. Cycles 11–25 consisted of denaturation at 92 °C for 30 s, annealing at 55 °C for 1 min at cycle 11, decreasing to 45 °C at cycle 25, and elongation at 68 °C for 15 min at cycle 11, increasing by 1 min per cycle to 35 min at cycle 25. The sequences of the two primers used are as follows: J39, homologous to the 3' end of *cps19fB* (TAGTTCATGTAGTTGCAAGTGACATGCACAA, bases 2190–2220 of the *cps19f* sequence); and J27, complementary to *cps19fL* (GGCTGGATCCAAACCATCTGGACTT, bases 11787–11811 of the *cps19f* sequence).

Southern hybridization analysis. Chromosomal DNA (2.5 µg) was digested with appropriate restriction enzymes and the digests electrophoresed on agarose gels in TBE buffer. DNA was then transferred to a positively charged nylon membrane (Hybond-N⁺, Amersham) as described by Southern (1975), hybridized to digoxigenin (DIG)-labelled probe DNA, washed, and then developed using anti-DIG-alkaline phosphatase conjugate (Boehringer Mannheim) and 4-nitro blue tetrazolium/X-phosphate substrate according to the manufacturer's instructions. DIG-labelled lambda DNA, restricted with *Hind*III, was used as a DNA molecular size marker.

DNA sequencing and analysis. Nested deletions of pneumococcal DNA cloned into pBluescript KS were constructed by the method of Henikoff (1984) using the Erase-a-Base kit (Promega). This DNA was transformed into *E. coli* DH5 α and the resulting plasmid DNA was characterized by restriction analysis. Double-stranded template DNA for sequencing was prepared as recommended in the Applied Biosystems sequencing manual. The sequence of both strands was then determined using dye-labelled primers on an Applied Biosystems model 373A automated DNA sequencer. The sequence was analysed using DNASIS and PROSIS Version 7.0 software (Hitachi Software Engineering). The program BLASTX (Altschul *et al.*, 1990) was used to translate DNA sequences and to conduct homology searches of the protein databases available at the National Center for Biotechnology Information, Bethesda, MD. Amino acid sequence alignments were performed with the program CLUSTAL W (Thompson *et al.*, 1994). Hydropathy plots were generated according to the method of Kyte & Doolittle (1982), and were aligned using PROFILEGRAPH (Hofmann & Stöffel, 1989).

Isolation of the type 23F *cps* genes

We have previously reported the nucleotide sequence for both the 5' and the 3' portions of the *S. pneumoniae* type 23F CPS biosynthesis locus (*cps23f*) (Coffey *et al.*, 1998a). These regions of *cps23f* (from *dexB* to *cps23fB* and from *cps23fL* to *aliA*) were highly similar to the sequence reported for *cps19f*. However, Southern hybridization analysis indicated that homologues of the

Bacterial strains and plasmids. The multiresistant *S. pneumoniae* type 23F Spanish strain SP-264 has been described previously (Coffey *et al.*, 1998a). Pneumococci were routinely grown in Todd-Hewitt broth with 0.5% (w/v) yeast extract or on blood agar. *Escherichia coli* K-12 DH5 α (Bethesda Research Laboratories) was grown in Luria-Bertani broth (Maniatis *et al.*, 1982) with or without 1.5% (w/v) Bacto-agar (Difco). Where appropriate, ampicillin was added to the growth medium at a concentration of 50 μ g ml⁻¹. The vector pBluescript KS was obtained from Stratagene.

Bacterial transformation. Transformation of *E. coli* with plasmid DNA was carried out as described by Brown *et al.* (1979).

DNA manipulations. *S. pneumoniae* chromosomal DNA used in Southern hybridization experiments was extracted and purified using the Wizard Genomic DNA purification kit from Promega. Chromosomal DNA was purified according to the manufacturer's instructions except that cell lysis was induced by the addition of 0.1% (w/v) deoxycholate followed by incubation at 37 °C for 10 min. Plasmid DNA was isolated from *E. coli* by the alkaline lysis method (Morelle, 1989). Analysis of recombinant plasmids was carried out by digestion of DNA with one or more restriction enzymes under the conditions recommended by the supplier. Restricted DNA

Fig. 2. Physical map of the *S. pneumoniae* type 23F CPS biosynthesis locus (*cps23f*). Black arrows represent previously described ORFs which are homologous to *cps19f* genes (Coffey *et al.*, 1998a). Vertically hatched arrows represent *cps23f* ORFs with lesser, but nevertheless significant (> 70 %) similarity to *cps19f* genes. Oblique-hatched arrows represent *cps23f* ORFs with negligible similarity to *cps19f* genes. Gene designations are indicated below the map; *cps23fB–O* are abbreviated to *B–O*, respectively. Restriction sites are as follows: B, *Bam*HI; C, *Cl*al; E, *Eco*RI; H, *Hind*III; P, *Pst*I. The long-range PCR product and the regions of DNA subcloned into various recombinant plasmids are shown below the map.

remaining *cps19f* genes (*cps19fC–K*) were not present in the *cps23f* locus (Morona *et al.*, 1997a). This central portion of *cps19f* would be replaced in the *cps23f* locus by the genes required specifically for type 23F CPS biosynthesis. Long-range PCR was used to amplify this region of *cps23f*. The resultant PCR product was approximately 13 kb in size, approximately 4 kb larger than the equivalent region of *cps19f*. A restriction map of the 13 kb PCR product was generated, using *Bam*HI, *Cl*al, *Hind*III, *Pst*I and *Eco*RI, and the PCR product was then subcloned, generating five plasmids with inserts as shown in Fig. 2.

Both strands of the pneumococcal DNA inserts of each of the above plasmids (or nested derivatives thereof) were subjected to sequence analysis in order to compile the sequence of the *cps23f* locus. The sequence across non-overlapping restriction sites and for both ends of the PCR product was obtained by direct dye-terminator sequencing of long-range PCR product using specifically designed primers. The compiled sequence was then linked to the existing *cps23f* data (*dexB–cps23fB* and *cps23fL–aliA*). As already reported (Coffey *et al.*, 1998a), the first 4.6 kb at the 5' end of the complete sequence has a high degree of similarity to the *cps19f* sequence. This region contained *dexB*, the 5' intergenic region (but it did not contain IS1202 as found in type 19F), and the homologues of *cps19fA* and *cps19fB* (*cps23fA* and *cps23fB*, exhibiting 96.5 % and 87.4 % identity, respectively). A consensus σ^{70} promoter sequence is located 5' to *cps23fA*. The promoter sequence is identical to that identified in *cps* loci from *S. pneumoniae* types 1, 14 and

19F (Muñoz *et al.*, 1997; Kolkman *et al.*, 1997; Morona *et al.*, 1997a), except for a single base difference in the –35 region (AAGACA for *cps23f*, compared with TAGACA for the others). The next three genes in the *cps23f* locus (*cps23fC*, *D* and *E*) were also very similar to *cps19fC–E* (70.3 %, 74.3 % and 72.1 % identity, respectively). The sequence then diverges suddenly at nucleotide 7460 of the *cps23f* sequence, which corresponds to the stop codon of *cps23fE*. There are nine new potential open reading frames (ORFs) between *cps23fE* and *cps23fL*; we have designated these *cps23fT*, *I*, *U*, *V*, *J*, *W*, *X*, *Y* and *Z*. Each ORF is preceded by a ribosome-binding site, and most ORFs are closely linked together, suggesting that they are transcriptionally coupled. The only significant intergenic gaps are 300 nucleotides between *cps23fT* and *cps23fI*, and 160 nucleotides between *cps23fZ* and *cps23fL*. However, no potential stemmed-loop structures were found in these regions. The 3' end of the *cps23f* sequence again shows a high degree of similarity to the *cps19f* sequence, starting from nucleotide 17711. This is the first nucleotide of the *cps23fL* gene, which has 91.3 % identity to *cps19fL*. The remaining three genes of the *cps23f* locus (*cps23fM*, *N* and *O*) all exhibit greater than 97 % identity to their respective *cps19f* homologues.

Characterization of the *cps23f* genes

The locations and several properties of *cps23fC*, *D*, *E*, *T*, *I*, *U*, *V*, *J*, *W*, *X*, *Y* and *Z* are summarized in Table 1. Significant similarities with other known proteins,

Table 1. Summary of ORFs *cps23fA-O*

ORF	Location in sequence	Predicted M_r	No. of amino acids	Hydrophobicity index*	Predicted pI	G + C content (mol %) [†]	Putative function of predicted protein product
<i>cps23fA</i>	2500–3945	53 516	482	0.01	8.24	38.2	Regulatory protein
<i>cps23fB</i>	3947–4678	28 163	244	−0.43	5.72	40.7	Unknown
<i>cps23fC</i>	4687–5379	25 450	230	0.02	9.75	42	Chain length regulation
<i>cps23fD</i>	5389–6078	24 997	229	−0.14	8.36	38.4	Chain length regulation
<i>cps23fE</i>	6093–7460	52 192	445	0.16	9.42	37.4	Glucose-1-phosphate transferase
<i>cps23fT</i>	7491–8669	45 137	393	−0.45	5.9	36.6	Rhamnosyltransferase
<i>cps23fI</i>	8969–10162	45 281	397	0.86	9.99	26.7	Polysaccharide polymerase
<i>cps23fU</i>	10166–11 137	37 476	323	−0.21	5.08	32	Galactosyltransferase
<i>cps23fV</i>	11 144–12 376	47 656	411	−0.32	9.43	30.8	Rhamnosyltransferase
<i>cps23fJ</i>	12 378–13 763	51 864	461	1.00	9.41	28.4	Repeat unit transporter
<i>cps23fW</i>	13 771–14 919	44 881	382	−0.27	6.76	31.2	Glycerophosphotransferase
<i>cps23fX</i>	14 955–15 962	36 772	335	−0.02	5.74	36.6	Glycerol-2-phosphate dehydrogenase
<i>cps23fY</i>	15 999–16 703	26 110	234	0.02	4.56	31.9	Glycerol-2-phosphate cytidyltransferase
<i>cps23fZ</i>	16 718–17 551	31 171	277	−0.10	4.64	32.4	Glyceraldehyde-2-phosphotransferase
<i>cps23fL</i>	17 711–18 580	32 221	289	−0.21	4.7	41	Glucose-1-phosphate thymidyltransferase
<i>cps23fM</i>	18 581–19 174	22 273	197	−0.37	5.05	41.2	dTDP-4-keto-6-deoxy-glucose-3,5-epimerase
<i>cps23fN</i>	19 187–20 236	39 041	349	−0.46	5.16	41.8	dTDP-glucose-4,6-dehydratase
<i>cps23fO</i>	20 302–21 153	32 387	283	−0.52	4.72	41.1	dTDP-L-rhamnose synthase

* According to Kyte & Doolittle (1982), as implemented in PROSIS.

[†] Of coding region.

revealed by comparison with sequence databases, are described below.

The first five genes (ORFs) of the *cps23f* locus, *cps23fA–E*, have a high degree of identity to *cps19fA–E*; therefore the predicted functions of the gene products would be the same as described previously (Guidolin *et al.*, 1994). Cps23fA (96% identity to Cps19fA) may have a regulatory role, based on its similarity to the transcriptional regulator, LytR, from *Bacillus subtilis* (Lazarevic *et al.*, 1992). The function of Cps23fB (88.5% identity to Cps19fB) is unknown; however, homologues of this protein have, so far, only been identified in other Gram-positive CPS loci. Cps23fC and D (69.6% and 79.3% identity to Cps19fC and D, respectively) are both predicted to be involved in chain length regulation and export of the CPS, based on their similarity to the first and last thirds of ExoP from *Rhizobium* (*Sinorhizobium*) *meliloti* (Glucksmann *et al.*, 1993), respectively. Cps23fE (71.2% identity to Cps19fE) is likely to have the same function as the closely related Cps14E (95.8% identity to Cps19fE), which transfers glucose to the lipid carrier (Kolkman *et al.*, 1996).

The *cps23fT* gene encodes a putative protein which has limited similarity to putative mannosyltransferases from *Aquifex aeolicus* (18.9% identity) (Deckert *et al.*, 1998),

Synechocystis sp. (22.3% identity) (Kaneko *et al.*, 1995) and *Archaeoglobus fulgidus* (25.5% identity) (GenBank accession number AE001104). Therefore, Cps23fT is probably a glycosyltransferase involved in the synthesis of the type 23F CPS repeat unit. However, mannose is not present in the type 23F capsule, and when the predicted specificities for the other glycosyltransferases present in the *cps23f* locus are considered, it seems most likely that Cps23fT is a rhamnosyltransferase.

Database searches with Cps23fI failed to identify any significant similarity to any other proteins. However, Cps23fI is a very hydrophobic protein and its hydropathy profile indicates that it has at least 12 hydrophobic, potentially membrane-spanning domains. This is a typical hydropathy profile for Rfc-like proteins, which polymerize polysaccharide repeat units. Fig. 3 shows an alignment of the hydropathy profile for Cps23fI with those for putative polysaccharide polymerases from *S. pneumoniae* types 1, 14, 19B and 19F. In spite of negligible sequence similarities amongst these proteins, the profiles are very similar and on this basis, we suggest that Cps23fI is likely to be the polysaccharide polymerase for type 23F CPS.

The *cps23fU* gene encodes a protein with similarity to Cps14J from *S. pneumoniae* type 14 (33.5% identity), an

Fig. 3. Alignment of hydropathy plots of putative pneumococcal polysaccharide polymerases. Hydropathy plots for Cps23fl, Cps19fl from type 19F (Morona *et al.*, 1997a), Cps19bl from type 19B (Morona *et al.*, 1997b), Cap1H from type 1 (Muñoz *et al.*, 1997) and Cps14H from type 14 (Kolkman *et al.*, 1997) were generated by the method of Kyte & Doolittle (1982) and aligned using PROFILEGRAPH (Hofmann & Stöffel, 1989). Positive numbers on the y-axis indicate hydrophobic regions. The position of every 10th amino acid is marked on each x-axis.

Fig. 4. Alignment of the N-terminal portion (114 amino acids) of Cps23fU with Cps14J from *S. pneumoniae* type 14 (Kolkman *et al.*, 1997), EpsI from *Streptococcus thermophilus* (Stingle *et al.*, 1996), EpsG from *Lactococcus lactis* subsp. *cremoris* (van Kranenburg *et al.*, 1997) and TrsB from *Yersinia enterocolitica* (Skurnik *et al.*, 1995) using the default settings of the program CLUSTAL W (Thompson *et al.*, 1994). Identical residues are boxed; a hyphen (-) indicates absence of a residue. The residues marked by asterisks correspond to a motif previously found in a variety of rhamnosyl- and 6-deoxyhexosyltransferases (Morona *et al.*, 1995, 1997a).

experimentally determined galactosyltransferase (Kolkman *et al.*, 1997). Cps23fU also has similarity to several other galactosyltransferases: EpsI from *Streptococcus thermophilus* (36% identity) (Stingle *et al.*, 1996), EpsG from *Lactococcus lactis* subsp. *cremoris* (29.6% identity) (van Kranenburg *et al.*, 1997) and TrsB from

Yersinia enterocolitica (29.7% identity) (Skurnik *et al.*, 1995). The similarity is greatest for the first 100 amino acids at the amino-terminal end, and an alignment of this region is shown in Fig. 4. Interestingly, the most conserved regions are similar to potential active sites identified in a variety of Gram-negative rhamnosyl-

and 6-deoxyhexosyltransferases (Morona *et al.*, 1995). Thus, Cps23fU is likely to be the galactosyltransferase which adds galactose to the type 23F CPS repeat unit.

The *cps23fV* gene encodes a putative protein which has no significant similarity to any other proteins on any database.

The *cps23fJ* gene encodes a highly hydrophobic protein (Table 1) with similarity to several putative oligosaccharide repeat unit transporters: EpsM from *Streptococcus thermophilus* (32.1% identity) (Stingle *et al.*, 1996), Cps14L from *S. pneumoniae* (16.7% identity) (Kolkman *et al.*, 1997), TrsA from *Yersinia enterocolitica* (17.9% identity) (Skurnik *et al.*, 1995), and Cap8K protein of *Staphylococcus aureus* (20.6% identity) (Sau *et al.*, 1997). Cps23fJ has a typical hydropathy profile for this type of protein (result not shown), with 10–12 hydrophobic, membrane-spanning domains (Macpherson *et al.*, 1995). Thus, Cps23fJ is likely to be the polysaccharide repeat unit transporter.

The *cps23fW* gene encodes a protein with 27.8% identity to the carboxy-terminal end of the TagF protein from *B. subtilis*, which is a glycerophosphotransferase (Pooley *et al.*, 1992). It also has 37% identity to ORF1, which is located at the 3' end of the *cps14* locus (Kolkman *et al.*, 1997). The type 23F CPS repeat unit has a glycerol phosphate side-chain attached to the Gal residue (Fig. 1), and so Cps23fW is probably the transferase which adds glycerol-2-phosphate to the repeat unit.

The next three genes, *cps23fX*, *Y* and *Z*, encode putative proteins that we predict would be involved in the biosynthesis of CDP-2-glycerol, the precursor required for the addition of the glycerol 2-phosphate side-chain in type 23F CPS. Biosynthesis of CDP-2-glycerol probably occurs via a three-step pathway. The first step of this involves the synthesis of glyceraldehyde 2-phosphate from glyceraldehyde, which is catalysed by the kinase glyceraldehyde-2-phosphotransferase. The most likely candidate for this protein is Cps23fZ, which has similarity to NagD from *E. coli* (32% identity) (Plumbridge, 1989) and AraL from *B. subtilis* (26.3% identity) (Sa-Nogueira *et al.*, 1997); however, the functions of both NagD and AraL are unknown. These proteins also have similarity (25–30% identity) to a group of proteins with 4-nitrophenylphosphatase activity; this reaction can proceed in either direction (depending on the physiological conditions), suggesting that these proteins may in fact function as kinases. Thus, Cps23fZ could be the enzyme glyceraldehyde-2-phosphotransferase. In the second step of the putative pathway, glycerol-2-phosphate dehydrogenase would catalyse the conversion of glyceraldehyde 2-phosphate to glycerol 2-phosphate. Cps23fX has similarity to glycerol-1-phosphate dehydrogenases from *Archaeoglobus fulgidus* (32.3% identity) (GenBank accession number AE000988), *Methanococcus jannaschii* (34.4% identity) (GenBank accession number U67518) and AraM from *B. subtilis* (28.6% identity) (Sa-Nogueira *et al.*, 1997). Thus, *cps23fX* probably encodes a glycerol-2-phosphate dehydrogenase. The final step involves the synthesis of

CDP-2-glycerol by the enzyme glycerol-2-phosphate cytidyltransferase. Cps23fY has 27.5% identity to RfbF from *Archaeoglobus fulgidus*, a predicted glucose-1-phosphate cytidyltransferase (GenBank accession number AE001025). Thus, Cps23fY is likely to be the glycerol-2-phosphate cytidyltransferase, required for synthesis of CDP-2-glycerol.

The final four genes, *cps23fL–O*, are almost identical to *cps19fL–O*, which have been shown to encode the four enzymes necessary for biosynthesis of dTDP-rhamnose, the precursor of rhamnose in the type 19F CPS repeat unit (Morona *et al.*, 1997a).

Specificity of the *cps23f* genes

To examine the relationship between *cps23f* and encapsulation loci of other *S. pneumoniae* serotypes, individual *cps23f* genes were labelled with digoxigenin and used in Southern blots to probe (at high stringency) *Clal*-restricted chromosomal DNA from representative pneumococci belonging to the following types/groups: 2, 3, 4, 6A, 6B, 7F, 8, 9N, 9V, 12, 14, 16, 17, 18C, 19F, 19A, 19B, 19C, 22, 24, and two other 23F isolates. The genes *cps23fA–E* and *cps23fL–O*, which have a high degree of identity to *cps19f* genes, were not used as probes; and the results can be inferred from those previously reported for *cps19f* (Morona *et al.*, 1997a). The probes for *cps23fI* (bases 8819–10198), *cps23fU* (bases 10199–11061), *cps23fV* (bases 11062–12324), *cps23fJ* (bases 11063–13496), *cps23fW* (bases 14028–15087), *cps23fX* (bases 15277–16063), *cps23fY* (bases 16064–16686) and *cps23fZ* (bases 16937–17572) hybridized only with the other type 23F isolates. Unfortunately, isolates belonging to other members of serogroup 23 (types 23A and 23B) were not available for testing, so it was not possible to determine which of these group-23-specific genes are unique to type 23F. The *cps23fT* probe (bases 7526–8600) hybridized with DNA from serotypes 2, 6A, 6B, 16, 17, 18C, 22 and 23F. Interestingly, types 2, 17 and 22 also contain $\beta(1 \rightarrow 4)$ -linked rhamnose in their CPS repeat units, and the other reactive types also contain rhamnose in their CPS repeat units. Thus, the hybridization properties are consistent with the proposed function of the *cps23fT* gene product as a rhamnosyltransferase.

Type 23F CPS biosynthetic pathway

Database searches have enabled us to identify candidate functions for most of the ORFs in the *cps23f* locus. Three of the five transferases required for the synthesis of the repeat units have been assigned (Cps23fE, Cps23fU and Cps23fW), as have the polysaccharide repeat unit transporter (Cps23fJ) and the polysaccharide polymerase (Cps23fI). The genes involved in the synthesis of both CDP-2-glycerol (Cps23fX–Z) and dTDP-rhamnose (Cps23fL–O) have also been assigned. Cps23fC and Cps23fD are both believed to be involved in chain length regulation and export of the CPS, although this remains to be confirmed experimentally. However, the exact functions of Cps23fA and B are

Fig. 5. Putative biosynthetic pathway for *S. pneumoniae* type 23F CPS.

unknown, although they are common to all pneumococcal CPS loci, except type 3. Only two ORFs remain whose functions have not been clarified by comparison with other proteins of known function. Cps23fT does have some similarity to other transferases and is likely to be the rhamnosyltransferase which adds rhamnose to glucose via a $\beta(1 \rightarrow 4)$ linkage in the repeat unit backbone. This predicted function for Cps23fT is strengthened by the observation that the *cps23fT*-specific probe hybridized to DNA from serotypes 2, 17 and 22, which also contain $\beta(1 \rightarrow 4)$ -linked rhamnose in their repeat units (van Dam *et al.*, 1990). A second rhamnosyltransferase is required to add the rhamnose side-chain to the galactose in the backbone of type 23F CPS, and the only ORF which could encode such a transferase is *cps23fV*. The $\alpha(1 \rightarrow 2)$ linkage of this rhamnose side-chain to the galactose is quite unique and thus it is not surprising that Cps23fV has no similarity to other known transferases whose sequences are available in GenBank. On the basis of the above information, we propose a biosynthetic pathway for *S. pneumoniae* type 23F CPS, as shown in Fig. 5.

Conclusions

In the present study we have characterized the type-specific portion of the *cps* locus of the *S. pneumoniae*

type 23F strain SP-264, a representative of the Spanish multidrug-resistant clone. This, combined with our previous study (Coffey *et al.*, 1998a), indicates that *cps23f* consists of 18 genes, located in the pneumococcal chromosome between *dexB* and *aliA*. The genes *cps23fA-E*, *I*, *J* and *L-O* have been named to indicate sequence similarity and/or functional homology with respective *cps19f* genes described previously (Guidolin *et al.*, 1994; Morona *et al.*, 1997a). The remaining genes (*cps23fT-Z*) have neither sequence similarity nor functional homology with *cps19f* genes. We have been able to assign putative functions to the majority of *cps23f* genes based on similarity with known proteins and with reference to the structure of the type 23F repeat unit (Richards & Perry, 1988). This enabled us to propose a biosynthetic pathway for type 23F CPS.

Whilst this manuscript was being revised, Ramirez & Tomasz (1998) published the sequence of the *cps* locus from a Mexican multidrug-resistant clone of *S. pneumoniae* type 23F (strain Him18). Comparison of the two *cps23f* sequences indicates a high degree of similarity for each of the ORFs (97–100% identity at DNA level). However, the nomenclature used by Ramirez & Tomasz (1998) for individual genes differs from that employed in the present study and in a previous description of the last four *cps23f* genes (Coffey *et al.*, 1998a). The only notable difference within the two loci occurs in the gene we have designated *cps23fX*; a single base change terminates the predicted product of this gene seven amino acids earlier in SP-264 than in Him18. Marked differences are also seen in the 5' intergenic region, including a small deletion in SP-264 compared with Him18 and several other pneumococcal *cps* loci, affecting the first nucleotide of the -35 sequence. Ramirez & Tomasz (1998) also found a truncated copy of IS1202 upstream of the *cps23f* promoter of Him18, which was absent in SP-264. Apart from the absence of IS1202, the 5' intergenic region in SP-264 was very similar to the corresponding region from our previously described type 19F strain Rx1-19F (Guidolin *et al.*, 1994; Morona *et al.*, 1994). Interestingly, the 5' intergenic region from Him18 is virtually identical to the corresponding region from *S. pneumoniae* isolates belonging to types 19A, 19B and 19C, sharing three separate DNA deletions between *dexB* and IS1202 (Morona, 1998). This is highly suggestive of a common clonal origin for these four strains. An additional difference between the two studies was noted in the pattern of hybridization using probes specific for *cps23fT*. Using a 1074 bp probe spanning 91% of the *cps23fT* ORF, we detected hybridization to several other serotypes, including types 2 and 6B. These serotypes did not hybridize with the homologous probe in the study of Ramirez & Tomasz (1998), presumably a consequence of differences in hybridization conditions or the length of the gene-specific probe employed.

The general organization of the *cps23f* locus is analogous to that reported for most other serotypes characterized to date (specifically types 1, 14, 19F and 19B) (Guidolin *et al.*, 1994; Kolkman *et al.*, 1997; Morona *et al.*, 1997a, b; Muñoz *et al.*, 1997). The 5' portion of these

loci contains four genes (common to the majority of serotypes) believed to be involved in regulation and export of CPS. This is followed by a serotype-specific region encoding glycosyltransferases, the polysaccharide polymerase and the repeat unit transporter. The 3' region encodes enzymes for synthesis of activated monosaccharide precursors, some of which are common to several serotypes (e.g. those encoding dTDP-rhamnose synthesis). In *cps23f* the genes involved in biosynthesis of CDP-2-glycerol (*cps23fX-Z*) are also clustered in the 3' portion immediately upstream of those for synthesis of dTDP-rhamnose. This conserved organization of *cps* loci undoubtedly facilitates genetic recombination which may result in switching of capsular type, or perhaps even generation of a novel functional *cps* locus encoding synthesis of a structurally distinct CPS.

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