The complete *cps* gene cluster from *Streptococcus thermophilus* NCFB 2393 involved in the biosynthesis of a new exopolysaccharide

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The *cpsFGHIJKL* genes from the *cps* cluster of *Streptococcus thermophilus* NCFB 2393 involved in the biosynthesis of EPS were identified, cloned and nucleotide sequenced. The complete *cps* cluster is contained on an ~ 11·2 kb chromosomal region which contains 12 ORFs, including the previously cloned *cpsABCDE* genes. Functions were assigned to some of the predicted gene products on the basis of homology to known sequences as follows: *cpsK* encodes a protein thought to be involved in the polymerization and export of the polysaccharide; *cpsE*, *cpsF*, *cpsG*, *cpsH*, *cpsI* and *cpsJ* encode putative sugar transferases. Two insertion sequences, IS1193 and ISS1, were identified within and flanking the 3' end of the *cps* cluster respectively. Analysis of the expression of the *cpsE* gene in *Escherichia coli* demonstrated that it encodes a glucose-1-phosphate transferase; the enzyme which catalyses the first step in EPS biosynthesis in *S. thermophilus* NCFB 2393.

Keywords: capsule polysaccharide, exopolysaccharide, *Streptococcus thermophilus*, glycosyltransferase, IS1193

INTRODUCTION

Streptococcus thermophilus is a thermophilic lactic acid bacterium used as a starter culture in the production of yoghurt and Swiss or Italian-type cheeses. In yoghurt, the presence of exopolysaccharide (EPS) produced by strains of *Str. thermophilus* leads to an improvement in the viscosity and texture, an increased resistance to mechanical handling and a decreased susceptibility to 'whey-off'. The chemical structures of five different EPSs produced by different strains of *Str. thermophilus* have been determined (Bubb *et al.*, 1997; Doco *et al.*, 1990; Lemoine *et al.*, 1997; Low *et al.*, 1998). These were all found to be heteropolymers containing differing amounts of four sugars: glucose, galactose, rhamnose and *N*-acetylgalactosamine. In one case, fucose was also part of the repeat unit. The pathway for EPS biosynthesis in *Str. thermophilus* is postulated to resemble the *wzy*-

dependent systems reported for O-antigen biosynthesis in *Salmonella typhimurium* and *Escherichia coli* (Griffin *et al.*, 1996; Stingle *et al.*, 1996; Whitfield, 1995). These pathways involve the synthesis of individual repeat units on a lipid carrier (undecaprenyl phosphate) by the sequential activities of glycosyltransferase enzymes. Lipid-linked repeat units are then transported across the plasma membrane and exported from the bacterial cell (Whitfield, 1995). Recently, an operon directing the biosynthesis of EPS in the Sfi6 strain of *Str. thermophilus* has been identified (Stingle *et al.*, 1996), while four complete genes involved in EPS biosynthesis in strain NCFB 2393 have been cloned and sequenced (Griffin *et al.*, 1996). Interestingly, the predicted glycosyltransferase CpsE from NCFB 2393 differed from its homologue in the Sfi6 strain, indicating that these strains may produce EPSs with different chemical structures. We report here the cloning and nucleotide sequence analysis of an ~ 11 kb DNA fragment containing the remaining *cps* genes and genes flanking this cluster in *Str. thermophilus* NCFB 2393. We compare the sugar composition of the EPS produced by the NCFB 2393 strain to that reported for Sfi6 and several other strains

Abbreviations: CPS, capsule polysaccharide; EPS, exopolysaccharide.

The GenBank accession number for the sequence reported in this paper is Y17900.

of *Str. thermophilus*. Expression in *E. coli* was used to identify the glycosyltransferase activity encoded by the NCFB 2393 *cpsE* gene.

METHODS

Strains, culture conditions and fermentation. The bacterial strains and vectors used in this work are listed in Table 1. *E. coli* was grown in LB broth, Terrific broth or LB agar (Sambrook *et al.*, 1989) supplemented with 200 µg ampicillin ml⁻¹ when applicable and incubated at 37 °C aerobically for 18 h. *Str. thermophilus* was grown in M17 medium (Terzaghi & Sandine, 1975) supplemented with 1% sucrose (SM17) and incubated at 42 °C without shaking. For plates, agar was added at 15 g l⁻¹. For the production of EPS, *Str. thermophilus* was grown in MMSt (Cerning *et al.*, 1988) using a 1 l fermenter (419 Modular Fermenter; Wood Enterprises), connected to a fermenter meter unit (H. R. Flow Inducer MHRE200; Watson-Marlow). Incubation was at 40 °C for 24 h with constant agitation (300 r.p.m.). The pH was maintained at 5.5 with 5 M NaOH.

EPS isolation and characterization. EPS was isolated as described by Stingle *et al.* (1996) except that 3 vols acetone were used instead of one and precipitation of EPS was carried out for 18 h at room temperature. EPS was further purified by passage through a Sephacryl S-400 column (M. Meldgaard, Chr. Hansen's Laboratories). The molecular mass of the EPS was estimated by high-performance size-exclusion chromatography as follows: 1 mg freeze-dried EPS was added to 1 ml 10 mM ammonium bicarbonate (Sigma) and the solution filtered through a 0.2 µm pore size filter (Millipore). The solution was loaded immediately onto a Polysep column (Phenomenex) for Hewlett Packard HPLC, pre-standardized with a series of pullulan synthetic polysaccharide samples of known molecular mass (Shodex). Neutral sugar analysis of the EPS was performed by GC as described by Fox *et al.* (1989). The aminosugar content of the EPS was analysed by the method of Ludowieg & Benmaman (1967).

General DNA manipulations. DNA digestion and ligation was performed as recommended by the enzyme manufacturer. Electroporation into *E. coli* TG1 cells was carried out as described previously (Dower *et al.*, 1988). Plasmid DNA from *E. coli* was extracted using SV-Plus miniprep columns from Promega or by centrifugation through caesium chloride/ethidium bromide gradients (Sambrook *et al.*, 1989). Total DNA was extracted from *Str. thermophilus* as described by Lewington *et al.* (1987). PCRs were carried out on Hybaid Omnigene thermocyclers. Template concentration was 600 ng per 50 µl reaction for chromosomal DNA; each primer was added to 0.1 µM. AmpliTaq polymerase enzyme (Perkin Elmer; 5 U µl⁻¹) was used at 0.2 µl per 50 µl reaction. Annealing and extension conditions were dependent on the nature of primers and the expected product; these were as predicted by Oligo 4.0 for Macintosh.

DNA sequencing and sequence analysis. A primer-walking strategy was used to obtain the DNA sequence from the lambda inserts containing the *cps* genes. For the sequencing reactions, the Dye Terminator DNA Sequencing Kit (Applied Biosystems) was used and primer was added at 3.2 pmol per 20 µl reaction. Lambda DNA template for sequencing was used at 2–3 µg per 20 µl reaction. Sequencing reactions were electrophoresed and analysed using a Perkin Elmer 373A Fluorescence DNA sequencer. Primer design was checked in Oligo 4.0 for Macintosh. Sequence data were analysed using

the Wisconsin GCG package Version 10, Laser Gene Package for the Macintosh version 1.58 (DNASTar), TFASTA (Pearson *et al.*, 1997), BLASTP (Altschul *et al.*, 1990), CLUSTAL W (Higgins *et al.*, 1994) and PHDsec (Columbia University; <http://www2.ebi.ac.uk>). Hydrophobicity domains were determined using DNA STRIDER (version 1.1). Prediction of transmembrane domains and cytoplasmic/extracytoplasmic loops was also carried out using six different programs available from the internet at the ExPASy site (PHDhtm, 'DAS', HMMTOP, TopPred2, TMpred and TMHMM).

Cloning of the *Str. thermophilus cps* genes. A Lambda DASH II (Stratagene) library was constructed according to the manufacturer's instructions. This was screened for the presence of the *cpsE* gene, using PCR primers designed from the 3' end of a 4 kb insert in pAG14 as described previously (Griffin *et al.*, 1993). Lambda DNA from positive clones was extracted as described by Sambrook *et al.* (1989) or using the λ-DNA extraction kit (Qiagen), and their inserts sequenced and analysed.

Cloning and expression of *cpsE* in *E. coli*. Primers for the amplification of the *cpsE* gene designed from positions 4707–4726 and 6188–6206, were as follows: E60 5'-GGGATGATGCGGTTCTTAT-3' and E61 5'-CCCTTAGAACCAACGATAT-3', respectively. These were used to amplify a 1.48 kb fragment from lambda clone 9L3, using a proof-reading polymerase mixture (Boehringer). This fragment, which contained the entire *cpsE* gene and the upstream RBS (but no promoter), was cloned into the vector pCR2.1 (Stratagene), in both the sense and antisense orientations in respect of the promoter for the β-galactosidase gene (*P_{lac}*) (confirmed by nucleotide sequencing). The resulting plasmids were called p13 and p34 respectively. *E. coli* TG1 cells were transformed with plasmids p13 and p34 by electroporation. Diluted (1/10) cultures of the clones containing the *cpsE* gene constructs were grown in 100 ml Terrific Broth until mid-exponential phase (*OD*₆₀₀ = 0.475). At this stage induction was carried out with 1 mM IPTG. Cultures of TG1 containing the pCR2.1 vector or no vector at all were treated in the same way and used as negative controls. Membrane extracts of all the *E. coli* clones were prepared as described by Kolkman *et al.* (1996). The efficiency of the membrane extraction was checked by loading 12 µl aliquots on a denaturing 10% polyacrylamide gel as described by Laemmli (1970) and gels stained with Coomassie brilliant blue (Gibco-BRL). The protein concentration was determined using a simplification of the Lowry method (Peterson, 1977).

Glucosyltransferase assay and TLC. Membrane extracts (40 µl of 5 mg ml⁻¹) from clones containing the *cpsE* gene construct as well as negative controls were incubated with UDP-[¹⁴C]glucose as described by Kolkman *et al.* (1996). One-third of the lipid fraction was used for scintillation counting and the remainder was vacuum dried and analysed by TLC. For TLC, the dried reacted samples were resuspended in 100 µl *N*-butanol and mild hydrolysis was carried out by adding 100 µl 50 mM trifluoroacetic acid and incubating at 95 °C for 20 min. The hydrolysed samples were vacuum dried and resuspended in 10 µl 40% 2-propanol, 8 µl of which was run on a silica gel plate (Merck HPTLC Alufolien Kieselgel 60). The developer used was a mixture of butanol/ethanol/water (5:3:2 by vol.). Standards of monosaccharides (40 µg each) were run simultaneously. Detection of radioactivity was done by exposing an X-ray sensitive film directly to the TLC plate, previously sprayed with a β-enhancer spray (En³Hance, New England Nuclear), for 5 d at -80 °C. The standards were visualized by spraying with 5% sulfuric acid in ethanol and heating at

Table 1. Bacterial strains and vectors used in this study

Strain/vector	Description	Source
Strains		
<i>E. coli</i> strains		
FI9070	<i>E. coli</i> TG1/pAG14	A. Griffin
FI9298	<i>E. coli</i> TG1/p13	This study
FI9299	<i>E. coli</i> TG1/p34	This study
TG1	$\Delta(lac-proAB)$, <i>supE</i> , <i>thi</i> , <i>hsd</i> Δ 5, F'[<i>tra</i> Δ 36 <i>proA</i> ⁺ <i>B</i> ⁺ <i>lacI</i> ^a <i>lacZ</i> Δ M15]	Gibson (1984)
XL1-Blue	$\Delta(mcrCB-hsdMR-mrr)$ 173, <i>endA</i> 1, <i>supE</i> 44, <i>thi</i> -1,	Stratagene
MRA (P2)	<i>gyrA</i> 96, <i>relA</i> 1, <i>lac</i> (P2 lysogen)	
<i>Str. thermophilus</i> strain		
NCFB 2393	EPS-producing strain from dairy starter	IFR culture collection
Vectors		
p13 (pFI2172)	5.3 kb; pCR2.1 containing <i>cpsE</i> from NCFB 2393 under P _{lac}	This study
p34 (pFI2173)	5.3 kb; as p13 but the insert is in the opposite orientation to P _{lac}	This study
pAG14	6.7 kb; pUC18 containing 4 kb <i>EcoRI</i> fragment from NCFB 2393 for EPS biosynthesis	Griffin <i>et al.</i> (1996)
pCR2.1	3.9 kb; Amp and Km resistance, TA cloning vector	Invitrogen
Lambda DASH II	λ replacement vector. Accepts fragments of 9–23 kb	Stratagene

110 °C for 10 min. The assays described here were repeated and identical results were obtained.

RESULTS AND DISCUSSION

Analysis of EPS produced by NCFB 2393

Using the fermentation conditions outlined in the Methods, a maximum of 300 mg EPS l⁻¹ was obtained from NCFB 2393 cultures. Prior passage of NCFB 2393 (on a daily basis) through MMSt medium, at least five times, was required to achieve this level of EPS. Chemical analysis of the EPS showed that it consists of heptasaccharide repeating units containing the sugars GalNAc, Gal, Rha and Glc in a ratio of 1:1:2:3. Such a repeat unit has not been reported before for any of the EPSs produced by the Sfi6, Sfi39, Sfi12, OR901 and MR-1C strains of *Str. thermophilus* (Doco *et al.*, 1990; Lmoine *et al.*, 1997; Bubb *et al.*, 1997; Low *et al.*, 1998). Indeed all of these *Str. thermophilus* strains contain variation in the sugar composition, ratio of sugars, presence/absence of branches and type of linkages between sugar units of the EPS repeat unit. The molecular mass of the sample was estimated by high-performance size-exclusion chromatography to be $\sim 2 \times 10^5$ Da. This was lower than the published 1×10^6 Da reported for the Sfi6 strain (Doco *et al.*, 1990) and the 2×10^8 Da reported by Lmoine *et al.* (1997) for the Sfi12 and Sfi39 strains. Since the functional properties of a polysaccharide are influenced both by the chemical composition and the molecular mass (Sutherland, 1996), the NCFB 2393 polymer identified here is likely to display novel functional properties. Additional experiments are under way to demonstrate this.

Nucleotide sequence analysis of the *cps* cluster

In a previous study, we identified, cloned and nucleotide sequenced a 4074 bp chromosomal fragment containing part of the *cps* gene cluster (Griffin *et al.*, 1996). To identify the remaining genes, primers designed from the 3' end of the NCFB 2393 *cpsE* gene were used to screen a lambda library of *Str. thermophilus* genomic DNA. This was used to identify three positive lambda clones; λ 9L3, λ 3K1 and λ 8FJ1 (see Fig. 1). Nucleotide sequencing of the inserts from the lambda clones was performed to obtain the complete sequence data for a 14.62 kb region. Analysis of these sequence data revealed the presence of 16 ORFs including the 3' end of *cpsA* and the *cpsBCDE* genes previously reported by Griffin *et al.* (1996) with strong conformity with the streptococcal codon-usage table (not presented) (Fig. 1). Potential ribosome-binding sites were identified upstream of all genes (see accession no. Y17900) and putative promoters were identified upstream of six genes (see Fig. 1 and accession no. Y17900). An AT rich region, 5'-AAA-ACGTTTTTTTGTGTTTTTTTGAAGAAAAA-3', similar to the consensus sequence of an upstream promoter (UP) element, 5'-NNAAA(AT)(AT)T(AT)T-TTTNNAAAANN-3' (Estrem *et al.*, 1998) was identified upstream of the -35 box of the *cpsA* gene between positions -72 and -42. UP elements have been reported to stimulate promoter activity up to 30-fold, and are found both in Gram-negative and Gram-positive bacteria (Estrem *et al.*, 1998). Thus strong transcription may occur from the *cpsA* promoter, resulting in the generation of a single long transcript. The short intergenic spacing between *cps* genes suggestive of translational coupling, supports this view. In *Staphylococcus*

Fig. 1. Genetic organization of the EPS gene cluster in *Str. thermophilus* NCFB 2393. Lambda clones which contain the *cps* genes are shown below. Vertically hatched arrows represent flanking genes outside the *cps* cluster. Flags represent putative promoters. Drumsticks represent putative transcription terminators for *deoD* and *orf14.9*.

aureus, *Streptococcus pneumoniae* type 1 and *Str. thermophilus* Sfi6, it has been shown that expression of the *eps* or *cps* gene loci results in a single transcriptional unit despite the presence of putative promoter sequences within the operons (Muñoz *et al.*, 1997; Sau *et al.*, 1997; Stingle *et al.*, 1999). No hairpin loops resembling putative *rho*-independent transcription terminators were found within the *cps* genes; however one was identified downstream of *orf14.9*.

Sequence analysis of the *cps* genes

The *cpsE*, *F*, *G*, *H* and *I* genes encode proteins with predicted molecular masses of 51.8, 44.6, 42.6, 21.3 and 37.8 kDa respectively. When the amino acid sequences of these were compared to the databases, all showed homology to sugar transferases from a variety of different organisms (see Table 2). Given that the NCFB 2393 EPS contains the sugars GalNAc, Gal, Rha and Glc in a ratio of 1:1:2:3, we expected to find one GalNAc transferase, one galactosyltransferase, two rhamnosyltransferases and three glucosyltransferases.

Analysis of CpsE. CpsE is homologous at the amino acid level, to a large number of undecaprenyl-glucosyl-1-phosphate transferases from a large number of different species (Table 2). These proteins catalyse the first step in polysaccharide biosynthesis in these organisms. Comparison of hydrophobicity plots of the amino acid sequence of NCFB 2393 CpsE with those from homologous glycosyltransferases, showed that CpsE from NCFB 2393, CpsE from *Str. pneumoniae* type 23F, CpsE from *Str. pneumoniae* type 14 and WbaP from *Salmonella enterica* are highly related along their entire length (Fig. 2). By homology to WbaP, which was shown to be bifunctional (Wang *et al.*, 1996), CpsE from NCFB 2393 could have the transferase activity assigned to the C-terminal domain and a role in the release of the repeat unit from the lipid carrier assigned to the N-terminal domain. EpsD from *Lactococcus lactis* NIZO B40, ExoY from *Rhizobium meliloti* and EpsE from *Str. thermophilus* Sfi6 probably are not bifunctional since these contain only the C-terminal region with the glycosyltransferase activity.

The C-terminal region of NCFB 2393 CpsE also contained a conserved catalytic motif DXD (residues 342–344) preceded by three hydrophobic amino acids (MFXM). This domain was found to be conserved by a diverse range of glycosyltransferase families, both prokaryotic and eukaryotic (Wiggins & Munro, 1998). A second putative catalytic region containing a pair of charged residues, Glu and Asp (residues 386 and 438 respectively) previously reported to be within a catalytic site in β -glycosyltransferases (Saxena *et al.*, 1995; Van Kranenburg *et al.*, 1999) was also identified.

The topological model of CpsE (Fig. 3), depicts CpsE spanning the cytoplasmic membrane five times (segments TM I, II, III, IV and V) and having two large loops, the first one extracytoplasmic, the second one intracytoplasmic (C-terminal region). The transmembrane domains are composed mainly of hydrophobic residues, which are predicted to adopt an α -helix conformation. The C-terminal domain, containing the active site, is in the cytoplasm. This contains three domains, A, B and C, reported to be common to glycosyltransferases by Wang *et al.* (1996). Domains A and B are reported to be associated with the interaction of the glycosyltransferase with the membrane lipid, while block C seems to be specific for the transferred sugar. The model presented here is in agreement with reports that EPS biosynthesis occurs at the internal side of the cytoplasmic membrane, with subsequent translocation of the repeat unit across the membrane (Kolkman *et al.*, 1997; Stingle *et al.*, 1996).

Analysis of CpsF–CpsJ. CpsF and CpsG displayed greatest similarity to a rhamnosyltransferase and an *N*-acetyl-galactosyltransferase respectively (Table 2), therefore it is reasonable to assign these enzyme activities to these proteins. Biochemical analysis will be required to confirm these predictions. CpsH displayed high similarity to a number of different glucosyltransferases (Table 2), therefore it seemed logical to predict that this represents the second of the three expected glucosyltransferases. CpsI had highest homology to galactosyltransferases (Table 2), therefore it may be the single galactosyltransferase expected in this system. However,

Table 2. Amino acid homologies for the gene products of the *Str. thermophilus* NCFB 2393 *cps* cluster and flanking regions

Und-1P-Glc transferase, undecaprenyl-P-glucosyl-1-phosphate transferase; Cyt/ubiquit oxidase, cytochrome/ubiquinol oxidase; NADH-ubiquinone OR, NADH-ubiquinone oxidoreductase.

Product amino acid highest homologies				Species	Function of homologue	Accession no.
Protein from strain 2393	Highest homology to	Similarity (%)	Identity (%)			
DeoD	DeoD	99	98	<i>Str. thermophilus</i> Sfi6	Nucleotide metabolism	Q56037
	DeoD/Pnp	89	74	<i>L. lactis</i> subsp. <i>cremoris</i>	Nucleotide metabolism	O32810
CpsE	Cps23E	75	57	<i>Str. pneumoniae</i> 23F	Und-1P-Glc transferase	AF030373
	Cps14E	74	55	<i>Str. pneumoniae</i> S14	Und-1P-Glc transferase	P72513
	EpsE	63	43	<i>Str. thermophilus</i> Sfi6	Und-1P-Glc transferase	U40830
	GumD	42	30	<i>X. campestris</i>	Und-1P-Glc transferase	U22511
CpsF	CpsT	85	57	<i>Str. pneumoniae</i> 23F	Rhamnosyltransferase	AF030373
	OrfY	63	47	<i>Str. mutans</i> AB631	Unknown	AB000631
	EpsF	53	23	<i>Str. thermophilus</i> Sfi6	Glucosyltransferase	U40830
	Cps23F	47	17	<i>Str. pneumoniae</i> 23F	Mannosyltransferase	AF030373
CpsG	OrfX	45	26	<i>Bacillus subtilis</i> 60015	Spore coat biosynthesis	P46915
	EpsG	51	25	<i>Str. thermophilus</i> Sfi6	α -GalNAc transferase	U40830
	VipC	46	24	<i>Sal. typhimurium</i>	LPS biosynthesis	Q04975
	RfaK (WaaK)	48	23	<i>Sal. typhimurium</i>	1,2-GlcNAc transferase	P26470
CpsH	Hi1244	64	38	<i>Haemophilus influenza</i>	Unknown	P44134
	Cps19K	50	25	<i>Str. pneumoniae</i> 19F	Epimerase	U09239
	Cps19E	51	24	<i>Str. pneumoniae</i> 19F	Glucosyltransferase	U09239
	Cps14E	50	22	<i>Str. pneumoniae</i> S14	Glucosyltransferase	P72513
CpsI	Cps14J	54	36	<i>Str. pneumoniae</i> S14	Galactosyltransferase	X85787
	EpsI	55	33	<i>Str. thermophilus</i> Sfi6	Glucosyltransferase	U40830
	EpsG	57	33	<i>L. lactis</i> NIZO B40	Glucosyltransferase	U93364
CpsJ	Gtf-s	47	21	<i>Streptococcus sobrinus</i>	Dextranucrase precursor	P29336
	91 kDa protein	49	20	<i>Saccharomyces cerevisiae</i>	Unknown	P53243
	RfbG (WbbG)	48	18	<i>Shi. flexneri</i> 2a	Di-rhamnosyltransferase	X71970
CpsK	Nup133	54	29	<i>Sac. cerevisiae</i>	Pore protein	P36161
	CaaX	54	26	<i>Sac. cerevisiae</i>	Prenyl endoprotease	P47154
	RfbX (Wzx)	47	20	<i>Shi. flexneri</i> 2a	LPS transport	X71970
	Yeast protein	46	17	<i>Debaryomyces occidentalis</i>	K ⁺ -affinity transporter	P50505
CpsL	RfaS (WaaS)	52	27	<i>E. coli</i> K-12	LPS outer core assembly	P27126
OrfM	Undescribed ORF	95	91	<i>Str. thermophilus</i> CNRZ 368	Unknown	Y13713
	CYOB	53	29	<i>E. coli</i> K-12	Cyt/ubiquit oxidase	P18401
	GR78	41	24	<i>Kluyveromyces lactis</i>	Glc-regulated precursor	P22010
	HSP70	40	23	<i>Glycine max</i> (soybean)	Heat Shock protein 70	P26413
Orf14.9	Orf14.9	97	96	<i>Str. thermophilus</i> Sfi6	Unknown	U40830
	VanZ	59	33	<i>Enterococcus faecium</i> BM4147	Antibiotic resistance	Q06242
	Nu5M	57	26	<i>Caenorhabditis elegans</i>	NADH-ubiquinone OR	P24896
OrfN	Ypud	51	27	<i>B. subtilis</i>	Unknown	P17616

it also contained an AX₇LXD motif reported to be found in the β -glucosyltransferase and cellulose synthase superfamilies (Becker *et al.*, 1997; Wiggins & Munro, 1998). This could imply that CpsI could be the third glucosyltransferase, thus a detailed biochemical analysis will be required to establish which sugar CpsI transfers. While CpsE–CpsI clearly encode five of the seven sugar transferases, the identity of the remaining two transferases is not so clear. Good candidate sugar transferases

are CpsJ and CpsL. CpsJ had homology to dextranucrase, a processive glucosyltransferase enzyme. This could indicate that CpsJ may transfer more than one sugar unit (and so account for the two remaining transferases). Transfer of more than one sugar unit has been reported for glycosyltransferases from other systems, for example RfbG (WbbG) from *Shigella flexneri* transfers two consecutive rhamnosyl units (Morona *et al.*, 1995).

CpsE *Str. thermophilus* NCFB 2393 WbaP (RfbP) *Sal. enterica* LT-2 CpsE *Str. pneumoniae* type 23F EpsD *L. lactis*
NIZO B40 ExoY
R. meliloti CpsE *Str. pneumoniae* serotype 14 EpsE
Str. thermophilus Sfi6

Fig. 2. Hydrophobicity plots of CpsE and CpsE proteins from *Str. pneumoniae* type 23F (Coffey *et al.*, 1998), *Str. pneumoniae* serotype 14 (Kolkman *et al.*, 1997), and WbaP from *Sal. enterica* (Wang *et al.*, 1996), EpsD from *L. lactis* NIZO B40 (Van Kranenburg *et al.*, 1997), ExoY from *R. meliloti* (Mueller *et al.*, 1993) and EpsE from *Str. thermophilus* Sfi6 (Stingele *et al.*, 1996).

Fig. 3. Putative topology of the CpsE glycosyltransferase from *Str. thermophilus* NCFB 2393. The protein is predicted to span the membrane five times (transmembrane fragments I–V), with an extracytoplasmic loop plus a cytosolic loop containing the catalytic domain. Domains A and B represent regions of predicted interaction with membrane lipid; domain C represents a region of predicted glucosyltransferase activity (Wang *et al.*, 1996). The dotted line represents a region with a high number of conserved residues.

Analysis of *cpsK* and *cpsL*. The *cpsK* and *cpsL* genes encode proteins with predicted molecular masses of 14.0 and 35.0 kDa respectively. When the amino acid se-

quence of *cpsK* was compared to the databases (see Table 2), similarity to several proteins involved in transport was detected. CpsL displayed significant

Fig. 4. Expression and functional analysis of *cpsE* from *Str. thermophilus* in *E. coli*. (a) SDS-PAGE (10% acrylamide) of *E. coli* TG1 cell extracts containing *cpsE* gene constructs and *E. coli* negative controls. (b) TLC analysis of sugar-lipid intermediates from the same *E. coli* clones. p13, NCFB 2393 *cpsE* gene in the same orientation as P_{lac} ; p34, *cpsE* gene in the opposite orientation to P_{lac} ; pCR, clone containing the pCR2.1 plasmid with no insert; TG1, *E. coli* TG1 cells with no plasmid; HM, high molecular mass protein marker (in kDa); i indicates that the cultures were induced with 1 mM IPTG. Expressed CpsE is marked with an arrow in (a).

homology to only one protein: WaaS from the LPS biosynthetic pathway of *E. coli*. A specific function has not been assigned to WaaS, however it has been suggested that it may act as an accessory protein required for the assembly of outer-core oligosaccharide, interacting either with glycosyltransferases or with the inner core LPS moiety (Pradel *et al.*, 1992). Alternatively, Heinrichs *et al.* (1998) speculate that WaaS may be a rhamnosyltransferase, thus CpsL may be a rhamnosyltransferase. The position of a glycosyltransferase downstream of a putative export gene has been reported before for the *cps/eps* clusters of *L. lactis* and *Str. pneumoniae* (Van Kranenburg *et al.*, 1999; Morona *et al.*, 1999). More experiments are required to resolve the function of *cpsL*.

Expression of *cpsE* in *E. coli*

The NCFB 2393 *cpsE* gene was cloned into a high copy number vector and expressed in *E. coli* TG1. Expression from plasmid p13 (containing *cpsE* under the control of P_{lac}) resulted in a protein of approximately 42 kDa, which was smaller than the expected molecular mass of 51.8 kDa (Fig. 4a). The reason for this is unclear. A

Table 3. Scintillation counts for the *cpsE* clones after 90% efficiency was reached (maximum of 10 min)

cpsE(+) and *cpsE*(-) indicate insert cloned in the same orientation or opposite orientation to P_{lac} respectively.

Clone	c.p.m.
TG1/p13 <i>cpsE</i> (+)	10034
TG1/p13 induced <i>cpsE</i> (+)	9078
TG1/p34 <i>cpsE</i> (-)	5663
TG1/p34 induced <i>cpsE</i> (-)	5551
TG1/pCR2.1	72
TG1/pCR2.1 induced	218*
TG1	32
TG1 induced	37

* Probably unincorporated radiolabelled glucose.

glycosyltransferase assay using membrane extracts from *E. coli* cells containing either p13, p34 (*cpsE* cloned in the opposite direction to P_{lac}) or pCR2.1 (vector alone) revealed enzymic activity, detected as the incorporation of UDP-[14 C]Glc in extracts from TG1/p13 and TG1/p34 (Table 3). No protein band was detected in extracts from p34, therefore we were surprised to detect glycosyltransferase activity in these. This activity may have resulted from expression from a non-specific promoter within the vector, yielding a level of protein too low to be detected by SDS-PAGE. Use of extracts from *E. coli* containing the vector alone confirmed that the glycosyltransferase activity detected was genuinely due to expression of the NCFB 2393 *cpsE* gene. No significant difference in glucosyltransferase activity was detected between the induced and uninduced clones, suggesting that the chromosomal *lacI^a* gene in the background *E. coli* TG1 is insufficient to completely repress expression from P_{lac} in pCR2.1. TLC analysis demonstrated that CpsE transferred only 14 C-labelled glucose to the lipid fraction (Fig. 4b), demonstrating that the conversion of glucose to galactose by the activity of an epimerase that was reported to occur in some strains of *E. coli* by Kolkman *et al.* (1997), did not occur here. In addition, only labelled monosaccharide was detected in this study, demonstrating that CpsE transfers only a single glucose residue and is not a processive glucosyltransferase. Results from these assays combined with the data for the homology of NCFB 2393 CpsE to *Xanthomonas campestris* GumD (Ielpi *et al.*, 1993), imply that NCFB 2393 CpsE transfers a glucose 1-phosphate from UDP-glucose to undecaprenyl phosphate.

Analysis of genes outside the *cps* region

Comparison of the predicted amino acid sequences to the databases revealed that those at the 5' and 3' end of the region sequenced were not involved in EPS biosynthesis (Fig. 1 and Table 2). The positions of *deoD*

and *orf14.9* are conserved when compared to *Str. thermophilus* strain Sf6 (Stingle *et al.*, 1996); also in the CNRZ 368 strain these genes are associated with the EPS locus (Bourgoin *et al.*, 1999).

A partial copy of the ISS1 transposase was identified next to *orf14.9* in *Str. thermophilus* NCFB 2393. This contains only one long terminal repeat (at the 3' end) identical to that found in the CNRZ 368 ISS1. The NCFB 2393 ISS1 has 64% identity (78% similarity) to its homologue from *Str. thermophilus* CNRZ 368 (Bourgoin *et al.*, 1999); 32% identity (58% similarity) to the IS257 transposase from *Sta. aureus* Tn4003 (accession no. P14506) and 28% identity (54% similarity) to the ISS1 transposase from *L. lactis* IL946 (accession no. L35176). Copies of ISS1 members are widespread among industrial strains of *Str. thermophilus* and *L. lactis*, where they have been found in different positions on the chromosome including the EPS gene clusters (Bourgoin *et al.*, 1999; Duwat *et al.*, 1997; Mercenier & Lemoin, 1989; Stingle *et al.*, 1996).

A second IS element, IS1193, was also identified; this contained two 19 bp imperfect inverted repeats and two 8 bp perfect direct repeats, flanking a transposase gene (1246 bp). The NCFB 2393 IS1193 displayed 88% identity (91% similarity) at the amino acid level to IS1193 from *Str. thermophilus* CNRZ 368 (Bourgoin *et al.*, 1999), 62% identity (75% similarity) to TnpA from *Str. pneumoniae* WU2 (accession no. U66845), and 57% identity (72% similarity) to IS1167 from *Str. pneumoniae* RX1 (accession no. M31680). A short ORF (*orfM*, 234 bp) has been identified within IS1193 in the opposite orientation to the transposase gene. *orfM*, including its putative RBS, is also present in *Str. thermophilus* CNRZ 368, although this was not reported by the authors. Recently, a new IS element from strain CNRZ 368, IS1194, was identified in the *eps* cluster (Bourgoin *et al.*, 1998). IS1194 and other members of the IS5 subfamily all contain two ORFs, the shorter one (of unknown function) in the opposite direction to and overlapping the larger ORF which encodes the transposase gene. *orfM* might be a remaining part of some element needed for the transposition of all IS5 members, or might be the vestige of a gene picked up during transposition between organisms.

Copies of ISS1 and IS1193 are widely present in strains of *Str. thermophilus* and *L. lactis* and it has been suggested that these could allow horizontal transfer to occur between these two organisms during co-culture in dairy manufacture (Bourgoin *et al.*, 1996, 1999). Horizontal transfer and generation of new capsular polysaccharides or LPS as a consequence of homologous recombination between insertion elements has also been reported to occur in the CPS and LPS loci of strains of *Str. pneumoniae* and *Sal. enterica* (Morona *et al.*, 1997; Muñoz *et al.*, 1997; Xiang *et al.*, 1994). The G + C content of the NCFB 2393 *cps* cluster is 35.6 mol%; this is close to the average G + C content reported for *Str. thermophilus* of 37–40 mol% (Farrow & Collins, 1984). Interestingly, the G + C content of four genes at the 3'

end of the cluster, *cpsIJKL*, is only of 30 mol%. These genes are located next to IS1193, suggesting that foreign genes could have been introduced into the NCFB 2393 *cps* locus by horizontal transfer. Recombination could have been facilitated by the presence of mobile elements such as IS1193 and ISS1, or by the presence of conserved genes within these clusters. Additionally, given the similarity of some of our genes to the *cps* genes from *Str. pneumoniae*, horizontal transfer between different strains of streptococci might also have occurred at some time during the evolution of these two species.

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