Identification of lipopolysaccharide O antigen synthesis genes required for attachment of the S-layer of *Caulobacter crescentus*

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The outer surface of *Caulobacter crescentus* consists of a two-dimensional crystalline protein lattice layer (S-layer). A fraction of the LPS has an O antigen polymer attached to the core to form a 'smooth' LPS (S-LPS), which is required for attachment of the protein S-layer to the outer-membrane surface. A method to screen for strains defective in LPS production, based on loss of S-layer attachment, was developed and applied to libraries of transposon-generated mutants. Eighteen distinct insertions were found with transposon interruptions in genes affecting S-LPS production, 12 of which were located near the S-layer subunit protein gene, *rsaA*, and its transporter genes. Sequence adjacent to transposon insertion points was determined and used to search a *C. crescentus* genome database. Twelve ORFs likely to be involved in S-LPS synthesis were identified. Seven of the predicted ORFs were linked to *rsaA*. Six of the putative genes had identity with proteins involved in synthesis of sugar residues, including five predicted to make perosamine. The remaining six ORFs were similar to glycosyltransferases involved in forming linkages between sugar residues in the O antigen, while one may be a transcription repressor. Other chemical and preliminary proton NMR studies of the S-LPS O antigen indicate that it contains an *N*-acetylated 4,6-dideoxy-4-aminohexose, but is not assembled as a simple, uniform homopolymer, consisting of several different linkages between sugar residues. The ORFs described here include homologues of all the enzymes involved in the synthesis of *N*-acetylperosamine, a 4,6-dideoxy-4-aminohexose. Overall, the data are consistent with the hypothesis that the O antigen of *C. crescentus* S-LPS consists primarily of *N*-acetylperosamine residues polymerized with multiple anomeric linkages.

Keywords: perosamine, dideoxyaminohexose, outer membrane, glycosyltransferase

INTRODUCTION

Caulobacter crescentus is a Gram-negative, motile eubacterium found widely in soil and aquatic environments (Poindexter, 1981). The bacterium is covered by a surface layer (S-layer) consisting of a two-dimensional, self-assembled array of the protein RsaA that covers the entire surface of the bacterium (Smit *et al.*, 1981). Once

secreted by a Type I secretion mechanism (Awram & Smit, 1998), the RsaA subunits assemble into the S-layer and attach to the outer membrane (Walker *et al.*, 1994).

The outer membranes of Gram-negative bacteria contain phospholipids, proteins and LPS (Nikaido & Vaara, 1985). In many cases, including *C. crescentus* strains, there is also an extracellular polysaccharide (Ravenscroft *et al.*, 1991). The S-layer is external to all of these molecules (although the extracellular polysaccharide may pass through the S-layer). Smooth LPS (S-LPS) is a major component of the outer membrane of Gram-negative bacteria and consists of three regions. The lipid A moiety is anchored in the outer leaflet of the outer membrane. The core, a branched chain oligo-

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Abbreviations: S-layer, surface layer; S-LPS, smooth LPS.

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saccharide linked to ketodeoxyoctulosonic acid (KDO), is attached to the lipid A molecule. Extending from the core, when present, is the O antigen, which contains a repeating linkage of oligosaccharides (Schnaitman & Klena, 1993), forming S-LPS.

Although there is only a limited number of instances identified so far, it appears that in Gram-negative bacteria S-LPS is required for attachment of the S-layer (Boot & Pouwels, 1996; Yang *et al.*, 1992). This is the case in *C. crescentus* where the S-LPS anchors the S-layer to the cell surface (Walker *et al.*, 1994). A region near the N terminus of RsaA appears to be responsible (Bingle *et al.*, 1997b). Immunolabelling showed that the S-LPS is completely occluded by the S-layer (Walker *et al.*, 1994). Isolation and characterization of the S-LPS showed that the core sugars and fatty acids are identical to those of the rough LPS and that the O antigen is of a homogeneous length, unlike the variable length O antigen found in many enteric bacteria. If the S-LPS in *C. crescentus* is disrupted or absent, the S-layer detaches from the membrane (Walker *et al.*, 1994).

The precise nature of the interaction between the RsaA S-layer subunit protein and the O antigen is not known, in large part because the exact chemical composition of the oligosaccharide is not yet clarified. Reported here is a genetic approach toward that definition. A number of potential S-LPS synthesis genes were identified by transposon mutagenesis events that resulted in an S-layer shedding phenotype. These genes suggest that the *C. crescentus* O antigen is composed of the 4,6-dideoxyaminohexose perosamine. A number of glycosyl-transferases were also identified, suggesting that they are involved in polymerization of the O antigen.

METHODS

Strains, plasmids and growth conditions. The *Escherichia coli* K-12 strains DH5 α and JM109 were used for *E. coli* cloning manipulations. *E. coli* was grown at 37 °C in Luria broth (1% tryptone, 0.5% NaCl, 0.5% yeast extract), with 1.2% agar for plates. *C. crescentus* CB15 strains were grown at 30 °C in PYE medium (0.2% peptone, 0.1% yeast extract, 0.1% CaCl₂, 0.2% MgSO₄, with 1.2% agar for plates). Ampicillin was used at 100 μ g ml⁻¹, streptomycin at 50 μ g ml⁻¹ and kanamycin at 50 μ g ml⁻¹ in both *C. crescentus* and *E. coli*, and chloramphenicol was used at 2 and 20 μ g ml⁻¹ in *C. crescentus* and *E. coli*, respectively, when appropriate.

Recombinant DNA manipulations. Standard methods of DNA manipulation and isolation were used (Sambrook *et al.*, 1989). Electroporation of *C. crescentus* was performed as described previously (Gilchrist & Smit, 1991). For cloning of chromosomal DNA adjacent to Tn5 insertions, chromosomal DNA of a Tn5 mutant was digested with *Bam*HI, *Sal*I or *Xma*I. *Bam*HI fragments were cloned directly into the *Bam*HI site of pTZ18 vectors selecting for kanamycin resistance. The *Sal*I and *Xma*I fragments were cloned using the inverse PCR method described by Martin & Mohn (1999). The Tn5 library has been described previously (Awram & Smit, 1998). Aliquots of the library were stored at -70 °C.

Southern blot analysis of Tn5 insertion points. Southern blot hybridizations were done according to the membrane manu-

facturer's instructions (Hybond-N; Amersham). Radio-labelled probes were made by nick translation using the DNase/DNA Pol manufacturer's instructions (Gibco-BRL). Chromosomal DNA was isolated as described previously (Yun *et al.*, 1994). Identical Tn5 insertions were determined by cutting chromosomal DNA from the Tn5 mutants with restriction enzymes that cut Tn5 (*Bam*HI, *Bgl*II) and probing with Tn5. Identical banding patterns indicated that Tn5 had inserted in the same place in the chromosome. Linkage of Tn5 insertions was determined by cutting chromosomal DNA with enzymes that did not cut Tn5 (*Eco*RI, *Sst*I) and probing with a fragment of Tn5. Identical banding patterns indicated that the Tn5 insertions were linked. To ensure that the shedding phenotype was not a result of an altered *rsaA* gene, the chromosomal DNA was cut with *Hind*III and probed with the *rsaA* gene. If the banding pattern differed from the wild-type pattern, the Tn5 mutant was discarded.

SDS-PAGE and Western blot analysis. Proteins and S-LPS were isolated from *C. crescentus* as described previously (Walker *et al.*, 1992, 1994). SDS-PAGE and Western immunoblot analysis was performed as described previously (Walker *et al.*, 1992). After transfer of proteins to nitrocellulose, the blots were probed with polyclonal antibody and antibody binding was visualized using goat anti-rabbit serum coupled to horseradish peroxidase and colour-forming reagents (Smit & Agabian, 1984). Preparation of polyclonal primary antibodies against RsaA (Walker *et al.*, 1992) and S-LPS (Walker *et al.*, 1994) were described previously.

To detect *C. crescentus* whole cells synthesizing an S-layer, a colony immunoblot assay was used (Bingle *et al.*, 1997b). Briefly, cell material was transferred to nitrocellulose by pressing the membrane onto the surface of an agar plate containing bacterial colonies. The membrane was air-dried, washed in a blocking solution (3% skim milk powder, 20 mM Tris, pH 8.0, 0.9% NaCl) with vigorous agitation on a rotary shaker and then processed in the standard fashion for Western blots (Bingle *et al.*, 1997a).

Surface protein from *C. crescentus* cells was extracted using 100 mM HEPES at pH 2.0 as described previously (Walker *et al.*, 1992). To compare the amounts of surface protein extracted from different mutants, equal amounts of cells growing at exponential phase were harvested and equal amounts of the protein extract were loaded on the protein gel. SDS-PAGE and Western blotting were performed according to standard procedures (Sambrook *et al.*, 1989).

Nucleotide sequencing and sequence analysis. Sequencing was performed on a DNA sequencer (Applied Biosystems model 373). Sequencing of the DNA surrounding the RsaA transporter complex is described elsewhere (Awram & Smit, 1998; P. Awram & J. Smit, unpublished). The Tn5 insertion sites were determined by sequencing using the primer Tn5L32 (5'-AAACGGGAAAGGTTCCGTTTCAGGA-3') which hybridizes 32 bp from the end of Tn5. Nucleotide and amino acid sequence data were analysed using Geneworks and MacVector software (Oxford Molecular Group) and the NCBI BLAST e-mail and web server using the BLASTX algorithm (Altschul *et al.*, 1990). Primers were designed with the assistance of MacVector 6.0 software (Oxford Molecular Group). Protein alignments were generated using the CLUSTAL w algorithm as implemented by the MacVector software using the default settings. The position of putative promoters was predicted using the *C. crescentus* promoter consensus sequence (Malakooti *et al.*, 1995). Preliminary sequence data of the *C. crescentus* genome were obtained from The Institute

for Genomic Research through the website at <http://www.tigr.org> and were used to determine the ORFs interrupted by the Tn5 insertions.

RESULTS AND DISCUSSION

Several Tn5 mutants producing altered S-LPS were found

Previously, a colony immunoblot assay was used to screen a Tn5 library for mutants with defective transport of RsaA (Awram & Smit, 1998). This assay also had the advantage of identifying mutants that were defective in attachment of the S-layer as a 'halo' could be detected around colonies when the S-layer could not anchor to the cells (e.g. cells with a defective S-LPS). Using this method, it was possible to differentiate between cells secreting RsaA, cells secreting and shedding S-layer and cells without an S-layer. This method was used to isolate a total of 26 S-layer shedding mutants (F1–F26) from the NA1000 Tn5 library. In each case the S-LPS isolated from these mutants was altered, compared to wild-type (Fig. 1). In most cases the mutant completely lost the S-LPS band. In one case, F1, a consistently reproducible reduced amount of S-LPS is found. In addition, in some cases a minor band was seen in the Western blots using polyclonal antibody to the O antigen, but not in the silver-stained gels. These likely represent residual amounts of S-LPS because the mutation did not completely inhibit enzyme activity or because an alternate pathway allowed for some S-LPS production. These are

discussed below in relation to the individual Tn5 insertions.

Southern blot analysis of these mutants showed that the 26 mutants consisted of 18 different Tn5 insertions (Table I) and the majority of Tn5 insertions were linked to *rsaA*. Of the remaining mutants, F10, F22 and F26 were linked together, as were F9 and F23, while F3 and F24 were not linked to any of the others (data not shown). These data have been further confirmed by the release of the genome sequence for *C. crescentus*.

The Tn5 insertion points were determined for these mutants

A portion of Tn5 and the adjacent chromosomal DNA was cloned from a representative of each of these groups. Sequencing from the end of Tn5 revealed the insertion site of Tn5 and this sequence was used to search the *C. crescentus* genome library from The Institute for Genome Research (TIGR) for the DNA surrounding the Tn5 insertion site. All of the Tn5 insertion sites were found in the partial genome sequence. Several of the insertions were localized to DNA 3' of the S-layer protein subunit gene (*rsaA*) that was cloned previously (Awram & Smit, 1998; P. Awram & J. Smit, unpublished). The sequence was used to search the known protein databases for similar proteins using the BLASTX algorithm (Altschul *et al.*, 1990). Protein homology and codon preference were then used to determine ORFs for analysis.

Fig. 1. S-LPS extracts from representative NA1000 shedder Tn5 mutants. (a) Silver-stained polyacrylamide gel. (b) Western blots probed with antibody against S-LPS. NA1000 has wild-type S-LPS; JS1000 is a spontaneous shedder mutant with a defective S-LPS. The arrows denote where S-LPS runs. Lane M, molecular mass markers.

Fig. 2. Diagrammatic representation of the genome region containing the S-layer subunit gene (*rsaA*), the Type I secretion transporter genes (*rsaD-F*) and the putative S-LPS O antigen genes. The *wbq* genes are described in the text. Where appropriate the representative homologous gene is identified. F2–F20 indicate the approximate positions of insertions for the individual Tn5 mutants described in the text. RsaD, RsaE and RsaF are components of the type I secretion system that secretes RsaA. ABC transporter, MFP and OMP refer to the generic names for Type I secretion components, namely antigen-binding cassette transporter, membrane fusion protein and outer-membrane protein, respectively.

The S-LPS synthesis genes were genetically linked to the RsaA transport genes

Analysis of the DNA sequence around the *rsaA* transporter complex (Awram & Smit, 1998; P. Awram & J. Smit, unpublished) had previously revealed five ORFs with coding sequences having significant similarity to enzymes expected to be involved with perosamine or S-LPS synthesis. Most occurred between *rsaE* and *rsaF*; one ORF was found 3' of *rsaF* (Fig. 2). Immediately 3' of *rsaE* is an ORF which encoded a protein with similarity to GDP-D-mannose dehydratase (Currie *et al.*, 1995; Stroehrer *et al.*, 1995), the second ORF encoded a protein with similarity to UDP-N-acetylglucosamine acyltransferases (Canter Cremers *et al.*, 1989; Vuorio *et al.*, 1994) and the third protein had similarity to perosamine synthetase (Bik *et al.*, 1996; Stroehrer *et al.*, 1995). The fourth and fifth proteins had similarities to mannosyltransferases (Drummelsmith & Whitfield, 1999; Rocchetta *et al.*, 1998). These five ORFs have been designated *gmd*, *wbqR*, *per*, *wbqZ* and *wbqY*, respectively (Fig. 2). Immediately 3' of *wbqY*, the gene for outer-membrane component of the RsaA transporter, *rsaF*, was found (P. Awram & J. Smit, unpublished), but further sequencing revealed another ORF, designated *wbqA*, which had similarity to glycosyltransferases (Kido *et al.*, 1998). Further 3' of *wbqA* an ORF was found with no similarity to known genes and no other possible LPS-related genes were found in this 3' region.

The sequence 5' of the *rsaA* gene reveals a number of

ORFs transcribed in the opposite orientation to *rsaA*. Immediately 5' of *rsaA* is the ORF *sstI*, which has been described previously (Fisher *et al.*, 1988). Then there is an ORF (ORF B in Fig. 2) with an apparently unrelated function. Following ORF B is *wbqX*, with high similarity to mannosyltransferase A. This homology and the proximity of *wbqX* to the rest of the LPS mutants suggests that it may also be involved in the synthesis of LPS (Fig. 2).

Analysis and proposed function of individual proteins involved in S-LPS synthesis

A total of 14 ORFs associated with the formation of the S-LPS were found (Table 1). Another ORF interrupted by two Tn5s, F10 and F22, was found, but has no similarity to known genes. All of the putative genes start with an ATG codon except *manC* and *wbqL*, where sequence similarity and codon preference indicate that the GTG is the most probable start codon. Using the *C. crescentus* promoter consensus for biosynthetic genes (Malakooti *et al.*, 1995), promoters were predicted with transcript start sites 31 and 99 bp 5' of *manB*, 52 bp 5' of *manC*, 204 bp 5' of *lpsI*, 154 bp 5' of *wbqP* and 63 bp 5' of *wbqV*.

Gmd resembles GDP-mannose 4,6-dehydratases

The start codon for *gmd* is 143 bp 3' of *rsaE* (Fig. 2). No promoter matching the consensus sequence was found upstream of *gmd*, as would be expected if there is a

Table 1. Deduced proteins involved in O antigen synthesis and their homologues

BLASTX and FASTA alignments were used to determine identity and similarity.

<i>Caulobacter</i> protein	Length (aa)	Tn5 insertions*	Similar proteins	Organism	Function	Percentage identity/similarity (overlap)†	Accession no.
Gmd	325		GCA	<i>Pseudomonas aeruginosa</i>	GDP-mannose dehydratase	65.2/88.6 (325)	AAC44117
			RfbB	<i>Synechocystis</i> sp.	GDP-mannose dehydratase‡	55.2/83.4 (308)	NC000911
			Gmd	<i>Escherichia coli</i> O157	GDP-mannose dehydratase	55.7/84.9 (354)	AAC32345
WbqR	215		YvfD	<i>Bacillus subtilis</i>	Serine O-acetyltransferase‡	47.2/83.1 (204)	CAB15429
			WlaI	<i>Campylobacter jejuni</i>	Acetyltransferase‡	37.9/83.4 (145)	CAA72358
			NeuD	<i>Escherichia coli</i>	Acetyltransferase‡	32.4/77.2 (206)	AAC43301
			WbdR	<i>Escherichia coli</i> O157	N-Acetyltransferase‡	30.3/72.2 (210)	AAC32350
Per	346		SpsC	<i>Synechocystis</i> sp.	Spore coat polysaccharide synthesis‡	50.0/86.1 (332)	BAA18051
			Mth334	<i>Methanothermobacter thermoautotrophicus</i>	Perosamine synthetase‡	46.4/82.4 (341)	H69142
			Per	<i>Escherichia coli</i> O157	Perosamine synthetase‡	45.4/82.4 (345)	AAC32343
			Per	<i>Vibrio cholerae</i>	Perosamine synthetase‡	42.3/80.1 (346)	CAA42137
WbqZ	346	F4, F12 F15, F19	WbaZ-1	<i>Archaeoglobus fulgidus</i>	Mannosyltransferase‡	24.3/69.8 (227)	AAB91187
			Mth332	<i>Methanothermobacter thermoautotrophicus</i>	LPS biosynthesis‡	24.5/68.6 (162)	AAB84838
			WbaZ	<i>Salmonella enterica</i>	Mannosyltransferase I	19.6/62.0 (299)	CAA43914
			ExpE4	<i>Sinorhizobium meliloti</i>	Glycosyltransferase‡	25.0/40.7 (220)	CAB01935
WbqY	345	F11	WbaZ	<i>Salmonella enterica</i>	Mannosyltransferase I	26.5/89.7 (226)	CAA43914
			WbaZ-2	<i>Archaeoglobus fulgidus</i>	Mannosyltransferase‡	24.5/64.6 (199)	AAB90633
			WbaZ	<i>Escherichia coli</i>	Mannosyltransferase I	24.5/64.5 (296)	AAD21571
			WbaZ-1	<i>Archaeoglobus fulgidus</i>	Mannosyltransferase‡	24.2/66.5 (185)	AAB91187
WbaX	434		MtfA	<i>Archaeoglobus fulgidus</i>	Mannosyltransferase A‡	25.5/50.3 (396)	E69255
			WbdA	<i>Klebsiella pneumoniae</i>	Mannosyltransferase A	24.3/48.6 (263)	AAF04383
			ORF704	<i>Escherichia coli</i>	Mannosyltransferase I‡	23.9/41.3 (263)	BAA28328
WbqA	430	F20	WbdA	<i>Escherichia coli</i>	Mannosyltransferase III	19.4/66.2 (357)	BAA07750
			AF0617	<i>Archaeoglobus fulgidus</i>	LPS biosynthesis protein‡	24.8/69.9 (147)	AAB90623
			Mth370	<i>Methanothermobacter thermoautotrophicus</i>	LPS biosynthesis protein, RfbU-like	29.0/65.7 (167)	AAB84876
ManB	498	F9, F23	AlgC	<i>Pseudomonas aeruginosa</i>	Phosphomannomutase	36.0/57.4 (448)	P26276
			PGM	<i>Neisseria gonorrhoeae</i>	Phosphomannomutase	32.9/50.6 (446)	P40390
			PmmA	<i>Mycobacterium</i>	Phosphomannomutase	38.0/54.2 (449)	CAB08353
			PGM	<i>Neisseria meningitidis</i>	Phosphomannomutase	35.0/53.5 (450)	P40391
ManC	441	F14, F25	XanB	<i>Xanthomonas campestris</i>	Phosphomannose isomerase	38.3/71.7 (393)	P29956
			ManC	<i>Yersinia enterocolitica</i>	Mannose pyrophosphorylase	33.2/64.0 (384)	AAC60775
			ManC	<i>Vibrio cholerae</i>	Mannose pyrophosphorylase	33.2/63.4 (316)	CAA42134
			ManC	<i>Escherichia coli</i> O157	Mannose pyrophosphorylase	32.6/65.9 (399)	AAC32348
LpsI	356	F1	CcpA	<i>Bacillus megaterium</i>	Catabolite control protein‡	34.9/74.2 (312)	P46828
			CcpA	<i>Bacillus subtilis</i>	Catabolite control protein	33.1/74.5 (314)	P25144
			DegA	<i>Bacillus subtilis</i>	Degradation activator	33.1/74.9 (337)	P37947
			Lacl	<i>Bacillus subtilis</i>	Lacl repressor like protein‡	30.0/72.9 (310)	CAB15608
WbqP	187	F24	LpsB1	<i>Rhizobium etli</i>	Galactosyltransferase‡	59.7/71.0 (154)	AAB66671
			CapM	<i>Staphylococcus aureus</i>	Capsule synthesis	45.7/79.6 (154)	P39862
			RfbV	<i>Vibrio cholerae</i>	Galactosyltransferase‡	47.2/79.8 (143)	Y07788
			PssA	<i>Rhizobium leguminosarum</i>	Galactosyltransferase‡	34.6/69.2 (198)	AAA81573
WbqV	601	F3	WlaL	<i>Campylobacter jejuni</i>	Amino sugar epimerase‡	43.8/79.6 (458)	CAA72360
			WblL	<i>Bordetella pertussis</i>	LPS biosynthesis‡	31.0/64.4 (465)	CAA62256
			LpsB2	<i>Rhizobium etli</i>	dTDP-glucose 4,6-dehydratase‡	25.9/39.4 (431)	AAB66669
			CAPD	<i>Bacillus subtilis</i>	Capsule synthesis‡	26.5/69.9 (459)	CAA96491
WbqL	394	F26	CPS23FV	<i>Streptococcus pneumoniae</i>	Rhamnosyltransferase‡	29.8/51.7 (211)	AAC38761
			CPS23FI	<i>Streptococcus pneumoniae</i>	LPS biosynthesis	29.8/51.7 (211)	AAC69532
			ORF51x5	<i>Vibrio anguillarum</i>	Unknown	26.7/45.0 (71)	AAB81629

* Distinct, separate Tn5 insertions.

† Percentage similarity represents identical amino acids and conserved substitutions for the specified number of overlapping amino acids.

‡ Putative function.

terminator after *rsaE* as predicted previously (Awram & Smit, 1998). The Gmd sequence has high similarity over its entire length to GDP-mannose 4,6-dehydratases from *Pseudomonas aeruginosa* and *E. coli*. (Table 1). These homologous enzymes convert GDP-mannose to GDP-4-keto-6-deoxymannose (Stevenson *et al.*, 1996) as part of polysaccharide biosynthetic pathways.

WbqR is similar to N-acetyltransferases

The gene *wbqR* follows *gmd* by 2 bp, suggesting that these genes are transcriptionally coupled. The protein encoded by the gene shows significant similarity to WlaI

from *Campylobacter jejuni* and NeuD from *E. coli* (Table 1). WlaI is involved in the synthesis of the LPS O antigen (Fry *et al.*, 1998) while the function of NeuD is not clear, but is thought to be involved in N-acetylated neuraminic acid transfer (Annunziato *et al.*, 1995). These proteins also show some similarity to the LpxA genes from *E. coli* and *Salmonella enterica*. The LpxA proteins are UDP-N-acetylglucosamine O-acetyltransferases that are involved in the first step of Lipid A biosynthesis and have 24–26 unique hexapeptide motifs, starting with an isoleucine, leucine or valine residue often followed by a glycine (Vaara, 1992; Vuorio *et al.*, 1994). WbqR, WlaI and NeuD also contain several of

these hexapeptide repeats. The protein WbdR from *E. coli* O157 also contains these hexapeptide repeats and has 72.2% sequence similarity to WbqR. WbdR is thought to encode an *N*-acetyltransferase which converts GDP-perosamine to GDP-*N*-acetylperosamine (Wang & Reeves, 1998). Since the data presented here suggest that the genes involved in perosamine synthesis in *E. coli* O157 are also present in *C. crescentus* it is postulated that WbqR may acetylate GDP-perosamine, like WbdR. Preliminary proton NMR studies of the S-LPS have also indicated *N*-acetylation of the 4,6-dideoxy-4-amino-hexose identified (W.-R. Abraham, unpublished).

Per appears to be a perosamine synthetase

The gene encoding Per starts 74 bp 3' of *wbqR*, but no promoter sequence was found between *wbqR* and *per*. Per has considerable identity over its entire length to the *rfbE* and *per* gene products that are thought to synthesize perosamine (Table 1). These proteins likely catalyse the conversion of GDP-4-keto-6-D-deoxymannose to GDP-perosamine (4-amino-4,6-dideoxymannose) in *Vibrio cholerae* and *E. coli* O157 (Strocher *et al.*, 1995; Wang & Reeves, 1998) and show similarity to two classes of pyridoxal-binding proteins involved in the synthesis of amino sugars similar to perosamine. Based on the similarity to these genes, it is likely that Per is a perosamine synthetase.

WbqZ and WbqY resemble glycosyltransferases

The gene for WbqZ followed *per* by 6 bp and the gene for WbqY followed *wbqZ* by 13 bp, suggesting that all three genes are part of a polycistron. Both WbqZ and WbqY have significant similarity to the WbaZ proteins (Table 1). These proteins also have similarity to the RfbU related proteins, but size and amino acid similarity suggest that the WbaZ-like proteins are a separate family. WbaZ is a mannosyltransferase in *S. enterica* (Liu *et al.*, 1993); as such it seems likely that WbqZ and WbqY function to link perosamine monomers (a derivative of mannose) to the O antigen with each providing a different form of linkage.

WbqA is similar to perosamine transferases

The gene for WbqA is separated from the other LPS synthesis genes by *rsaF*, the outer-membrane component gene required for the secretion of RsaA (P. Awram & J. Smit, unpublished), and is transcribed in the opposite orientation. WbqA, like WbqZ and WbqY, may be a mannosyltransferase, but has greater similarity to the RfbU family of mannosyltransferases. The similarity to mannosyltransferases is much less than that seen with WbqZ and WbqY, but it does have significant similarity to the C terminus of the *E. coli* mannosyltransferases WbdB and WbdA (Kido *et al.*, 1998; Sugiyama *et al.*, 1998), and RfbU from *V. cholerae* (Wang & Reeves, 1998). RfbU from *V. cholerae* is known to transfer a perosamine residue onto the growing O antigen chain.

These proteins contain a signature motif that is also found in WbqA (Fig. 3). This motif consists of the sequence EX[XF]GXXXXE[AG] with a serine preceding the motif by 3–5 residues (Geremia *et al.*, 1996; Rocchetta *et al.*, 1998). Again, like WbqZ and WbqY, it seems likely that WbqA acts to add perosamine residues onto the O antigen.

ManB is similar to phosphomannomutases

Two Tn5 insertion mutants interrupted ManB genes. The ManB gene does not appear to be linked to any of the other LPS genes. This protein has very high identity along its entire length to a number of phosphomannomutase enzymes, suggesting that this is the function of ManB (Table 1). Phosphomannomutase converts mannose 6-phosphate to mannose 1-phosphate and is implicated in perosamine synthesis (Strocher *et al.*, 1995; Wang & Reeves, 1998).

ManC may have a dual function as a phosphomannoisomerase and mannose-1-phosphate guanylyltransferase

Two shedder mutants have Tn5 insertions within *manC* which result in loss of proper O antigen production. ManC has significant identity over its entire length to a large family of enzymes that have dual functions as a phosphomannoisomerase and mannose-1-phosphate guanylyltransferase (Table 1). Both functions are required for the synthesis of perosamine (Strocher *et al.*, 1995) and are predicted to also be performed by ManC in *C. crescentus*.

LpsI has similarity to the LacI repressor family

The Tn5 insertion in mutant F1 interrupts *lpsI*. Southern blot analysis indicated that this insertion was linked to the RSA locus. Mutant F1 has a phenotype different from every other shedder Tn5 insertion described here. Analysis of the S-LPS by SDS-PAGE and silver staining revealed that reduced amounts of O antigen were produced by this mutant. Analysis of LpsI indicated that the highest degree of identity was with CcpA, the catabolite control protein in *Bacillus subtilis*. CcpA represses carbohydrate utilization enzymes such as α -amylase and acetyl coenzyme A synthetase and has a positive regulatory effect on proteins involved in excess carbon excretion such as acetate kinase (Henkin *et al.*, 1991). Lower sequence identity was found to a number of LacI repressor-like proteins (Table 1). Analysis of the genes adjacent to *lpsI* revealed the presence of *C. crescentus* analogues of glucokinase, 6-phosphogluconate dehydratase and glucose-6-phosphate 1-dehydrogenase enzymes involved in the Entner–Doudoroff central metabolic pathway. From the position of *lpsI* we hypothesize that LpsI has a regulatory effect on the synthesis of glucokinase. Interruption of LpsI by the F1 insertion may alter the expression of glucokinase, which in turn would affect perosamine synthesis, resulting in the phenotype seen in the F1 mutant (i.e. less O antigen).

Fig. 3. CLUSTAL W alignment of WbqA with a number of known mannosyltransferases. The mannosyltransferase motif is boxed. The conserved serine is marked with an asterisk. RfbU Vc, RfbU from *V. cholerae* (accession no. Y07788); RfbU Ec, RfbU from *E. coli* (BAA31838); WbdA and WbdB from *E. coli* (D43637). Identical and similar residues are boxed. Identical residues have dark shading. Similar residues have light shading.

Fig. 4. Partial CLUSTAL W alignment of WbqV showing the NAD binding motifs. RfbV from *V. cholerae* (accession no. Y07788); WblL from *B. pertussis* (X90711); WlaL from *C. jejuni* (CAA72360). (a) Alignment of amino acids 151–200 showing the GXGXXG motif. (b) Alignment of amino acids 301–350 showing the GAGGSIG motif.

WbqP is similar to galactosyltransferases

The Tn5 insertion F24 interrupts a gene with sequence similarity to several galactosyltransferases. These enzymes appear to transfer the first sugar residue (usually a galactose) to undecaprenol phosphate, the lipid precursor. RfbW is one of these enzymes and its sequence is 47.2% identical and 79.8% similar to WbqP over 144 aa. RfbW is involved in the synthesis of the perosamine homopolymer making up the O antigen of *V. cholerae* O1 (Fallarino *et al.*, 1997), suggesting that RfbW may transfer the first perosamine to the lipid precursor. In *C. crescentus*, WbqP may initiate the formation of the O antigen by attaching the first sugar residue (presumably perosamine) to the undecaprenol phosphate 'carrier lipid'.

WbqV has sequence similarity to amino sugar synthesis enzymes

The mutant F3 had an interruption in *wbqV*. WbqV is similar to a number of large proteins, usually larger than 600 aa. There is considerable similarity, especially in the middle of the protein, to WlaL, RfbV and WblL from *C. jejuni*, *V. cholerae* O1 and *Bordetella pertussis*. These proteins contain five hydrophobic, predicted trans-membrane domains in the N terminus. The central portion contains an NAD-binding site and is homologous to UDP-glucose 4-epimerases. Two motifs have been implicated in binding of NAD in these proteins, GXGXXG and GAGGSIG (Fallarino *et al.*, 1997). As seen in Fig. 4, the second motif is found in all the

proteins, but the first only occurs in RfbV and WblL, suggesting that not all members of this family contain this motif. The C-terminal 300 aa of these proteins have identity with dTDP-glucose 4,6-hydratases (Bechthold *et al.*, 1995; Linton *et al.*, 1995). These proteins are usually associated with synthesizing amino-6-deoxy and dideoxy sugars involved in LPS synthesis or extracellular polysaccharides and probably perform multiple functions accounting for the three domains. WbqV was not found linked to the other O antigen synthesis genes. This may indicate that WbqV is involved in the synthesis of a core sugar, possibly the terminal core sugar. Interruption of this gene may prevent attachment of the O antigen to the core, resulting in the observed shedding phenotype. The presence of three domains may also account for the small amount of full length O antigen that is seen. The Tn5 insertion occurs very close to the N terminus of the ORF and would only interfere with the first domain. If a truncated product were formed it may still have some function that would result in a small amount of O antigen being produced.

WbqL may be a glycosyltransferase

The WbqL amino acid sequence is 29.8% identical and 51.7% similar over a range of 87 aa to a putative rhamnosyltransferase in *Streptococcus pneumoniae* (Table 1). Rhamnose is a 6-deoxy derivative of mannose, as is perosamine, suggesting that WbqL may be another perosamine transferase. The mutant F26 has an insertion in *wbqL*. Full-length O antigen was still produced in small amounts in this mutant (Fig. 1b); this suggests that

Fig. 5. Proposed perosamine biosynthetic pathway (adapted from Stroehler *et al.*, 1995).

the Tn5 insertion does not prevent all the O antigen formation. As the Tn5 insertion is close to the N terminus, a truncated product with some functionality may be produced, allowing a small amount of full length O antigen to be formed.

The functions of some of the Tn5-interrupted genes are still unidentified

The Tn5 insertions F22 and F10 interrupt an ORF with no identity to any known protein. But 5' of this ORF are two ORFs corresponding to an ABC-2 transporter. These transporters are known to transport extracellular polysaccharides and O antigens through the cytoplasmic membranes (Whitfield, 1995). Unlike the ATP binding cassette (ABC) transporters of type I protein secretion systems, the ABC and transmembrane domains reside on separate proteins. Alternatively, ABC-2 transporters are often found adjacent to genes involved in polysaccharide synthesis. This appears to be the case here as *wbqL* was found only two ORFs removed from the F22 and F10 Tn5 insertions and three ORFs from the ABC-2 transporter; therefore it may be that the ORF interrupted by the F10 and F22 mutants is also involved in polysaccharide synthesis. Alternatively, the Tn5 insertion may interfere with the expression of the ABC-2 transporter and cause the loss of O antigen. This may be the more likely explanation since the Tn5 insertions are 135 and 382 bp from the start codon of a 945 bp ORF. This position suggests that any protein made from the gene is less likely to have function with most of its N terminus interrupted. As the Western immunoblot showed that some O antigen was still be made (Fig. 1b), this suggests that the Tn5 insertions are affecting transcription of the ABC-2 transporter. O antigens are elongated at either the reducing terminus or the non-reducing terminus. If synthesis of the O antigen occurs

at the non-reducing terminus, the chain elongates in the cytoplasm and an ABC-2 transporter is required to transport the O antigen chain across the cytoplasmic membrane (Whitfield, 1995). If the ABC-2 transporter upstream of the F10 and F22 insertions is involved in the transport of the O antigen, this suggests that the O antigen is elongated by polymerization at the non-reducing terminus.

The Tn5 insertion F6 interrupts an ORF which has similarity to a chemotaxis receptor (Ward *et al.*, 1995). Analysis of the surrounding genes does not suggest that any are related to LPS synthesis and this Tn5 insertion may simply be unrelated to the observed phenotype.

In both of these cases, because a direct connection to LPS synthesis is not obvious, it is possible that these ORFs have nothing to do with LPS synthesis and the Tn5 insertions may not cause the shedding phenotype. Instead, a second spontaneous mutation may cause the altered phenotype.

Homologues to all the genes required for perosamine synthesis were found in *C. crescentus*

The S-LPS of *V. cholerae* and *E. coli* O157 contain perosamine and a pathway has been proposed for its synthesis (Stroehler *et al.*, 1995; Wang & Reeves, 1998). All the corresponding homologues have been described here. The pathway functions by converting fructose 6-phosphate to perosamine and is shown in Fig. 5. The corresponding *C. crescentus* homologues are described here as ManC, ManB, GMD and Per.

Glucokinase and phosphoglucisomerase were not included as part of the perosamine pathway described by Stroehler *et al.* (1995), but need consideration here when required by *C. crescentus* (Fig. 5). Bacteria using the Embden–Meyerhof–Parnas pathway require phospho-

glucoisomerase as part of the glycolysis process, but *C. crescentus* uses the Entner–Doudoroff glycolytic pathway (Riley & Kolodziej, 1976) and as such would not need phosphoglucoisomerase to convert glucose 6-phosphate to fructose 6-phosphate. But to make perosamine by the pathway described here, *C. crescentus* requires phosphoglucoisomerase (Fig. 5). None of the Tn5 hits were found in such a gene, so the TIGR *Caulobacter* genome database was searched for a phosphoglucoisomerase analogue and one was found with up to 67% similarity over its entire length to a number of *pgi* homologues in the database. A second enzyme, glucokinase, is required for converting glucose to glucose 6-phosphate. A glucokinase analogue was found next to the F1 Tn5 insertion in the potential repressor *lpsI*. These data suggest that *C. crescentus* contains all the genes necessary for the synthesis of perosamine. Furthermore, five separate Tn5 insertions in three of the ORFs cause loss of O antigen synthesis, strengthening this argument. Perosamine is not commonly found in O antigens; as far as we are aware only *V. cholerae*, *Brucella melitensis* and *E. coli* O157 contain perosamine residues in the O antigen (Strocher *et al.*, 1995; Wang & Reeves, 1998).

The S-LPS of *C. crescentus* is a complex linked polymer

Six of the Tn5 insertions occur in putative glycosyltransferases (*wbqZ*, *wbqY*, *lpsF*, *wbqP* and *wbqL*) (Table 1). This suggests there may be a number of different linkages between the sugars in the O antigen (and preliminary proton NMR of purified S-LPS also indicates this). A number of Tn5 insertions were found in genes with similarity to mannosyltransferases. Since perosamine is a derivative of mannose, the transferases are probably highly similar. This has been found to be the case with the perosamine transferase RfbV from *E. coli* O157. The large number of glycosyltransferases found suggest that the O antigen is a complex polymer, even though potentially composed of a single sugar, and the glycosyltransferases are important factors in the proper assembly of the S-LPS molecule.

The Tn5 insertions interrupt synthesis of the S-LPS

While it has not been proved that the ORFs listed here are required for O antigen synthesis, the presence of multiple Tn5 insertions in some of the ORFs indicates that Tn5 must be responsible for causing the defective S-LPS phenotype and the interrupted ORF is very likely a gene involved in S-LPS synthesis. In a number of cases it is also unlikely that the Tn5 insertion is having a polar effect and that Tn5 is interrupting the gene directly involved in S-LPS synthesis. For example, the transcript orientation of *wbqA* is opposite to *rsaF*, suggesting that there are no polar genes to affect downstream of *wbqA*. Another example is that a polar effect by Tn5 insertions in *wbqY* would interfere with production of RsaF and stop RsaA secretion, which does not happen, showing that insertion must only be affecting *wbqY*.

The genes *per*, *gmd*, *lbqZ* and *lbqY* all resulted in a

shedding phenotype when interrupted with Tn5 and were found closely linked to the RsaA transport genes. LPS genes have also been implicated in the proper formation of the transport complex in some type I secretion signals and it is thought that LPS is required for proper insertion of the outer-membrane protein (OMP) into the outer membrane (Wandersman & Létoffé, 1993). The close physical proximity of S-LPS and RsaA transport genes may be a reflection of coordinated expression, perhaps not only for optimal S-layer attachment but also for optimal assembly of the secretion apparatus.

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