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ORIGINAL PAPER

Identification and organization of genes for diutan polysaccharide synthesis from *Sphingomonas* sp. ATCC 53159

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Abstract A cluster of genes for diutan polysaccharide synthesis was isolated from a library of *Sphingomonas* sp. ATCC 53159 genomic DNA by complementation of glucosyl-isoprenylphosphate transferase-deficient mutants of *Sphingomonas elodea* ATCC 31461 (producing gellan) and *Xanthomonas campestris* (producing xanthan). The synthesis of polysaccharide in these strains shares a common first step, transfer of glucose-1-phosphate from UDP-glucose to the isoprenylphosphate lipid. The cluster of 24 genes was compared to genes for biosynthesis of gellan, and S-88 sphingan from *Sphingomonas* sp. ATCC 31554. Diutan, gellan and S-88 sphingan have a common four-sugar backbone but different side chains, one rhamnose for S-88 sphingan, a two-rhamnose side chain for diutan and no side chain for gellan. The genes for biosynthesis of diutan, gellan and S-88 sphingan were similar in general organization but differed in location of some genes, in particular, *dpsG* (putative polymerase), *dpsR* (putative lyase) and *dpsS* (putative repeat unit transporter). An unidentified reading frame *urf31*, present in the gene clusters for diutan and S-88 sphingan but not gellan, had similarity to glycosyl transferase group 2 proteins, and was detrimental when cloned in *Sphingomonas elodea* producing gellan that lacks a side chain, but not in *Sphingomonas* ATCC 31554 producing S-88 sphingan with a rhamnose side chain. Gene *urf31* could possibly encode a side-chain rhamnosyl transferase. Another gene *urf31.4* was unique to the diutan gene cluster. A plasmid containing 20 of the 24 genes resulted in a slight increase in the amount of diutan produced, but a significant increase in the rheological properties of diutan.

Keywords *Sphingomonas* sp. ATCC 53159 · S-657 · Diutan polysaccharide · Diutan biosynthesis genes

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Introduction

A number of bacteria of the genus *Sphingomonas* produce polysaccharides called sphingans that have related structures with a generally conserved tetrasaccharide backbone structure and different side chains [2, 17, 27, 31]. The sphingans gellan, welan, rhamsan and diutan are produced commercially for use in food, industrial, oilfield or personal care applications. The value of sphingan polysaccharides lies in their ability to modify the rheology of aqueous solutions, i.e., to thicken liquids, suspend solids, stabilize emulsions or form gels and films. Although the backbone structure of sphingan polysaccharides, comprised of [→4)-α-L-rhamnose-(1→3)-β-D-glucose-(1→4)-β-D-glucuronic acid-(1→4)-β-D-glucose-(1→] is conserved in most sphingans, variation in the composition and linkage of the side chains produces unique rheological characteristics [2, 27]. Sphingan rheology is also affected by the length of the polysaccharide chain (may be up to several million in molecular weight), intramolecular interactions and interactions with molecules of their environment. Gellan contains acetyl and L-glyceryl substituents and is effective in forming gels [2, 18, 22]. The strength of gellan gels depends upon the type and concentration of cation present as well as the degree of polymer acylation [1]. Welan has acetyl and

an L-rhamnosyl or L-mannosyl side group branching from the same basic repeating unit as gellan [17, 23, 27]. Welan does not gel, but produces viscous solutions stable to high temperatures making it useful as an additive in oil well drilling fluids. High salt and pH tolerance makes welan an ideal viscosifier for cement-related applications where it is desirable to prevent phase separation. Rhamsan has the same backbone structure as gellan and welan but with two D-glucosyl units comprising the side chain and acetyl in an unknown location [17, 23, 27]. The rhamsan polymer can be used for a broad range of industrial applications particularly in paints and coatings, as a thickener and suspending agent.

Sphingomonas sp. ATCC 53159 was isolated from an algal sample taken from a marsh near Eureka, CA, USA [30]. The polysaccharide secreted from this strain, specified as S-657 or diutan, exhibits unique rheological properties in aqueous solutions including high thermal stability, superior suspension properties and the ability to generate high viscosity at low concentrations [30]. Diutan consists of a repeat unit with L-rhamnose, D-glucose, D-glucuronic acid, D-glucose backbone and a two-sugar L-rhamnose side-chain attached to the (1→4) linked glucose residue (Fig. 1) [3]. Two O-acetyl groups are attached per repeat unit to the 2′ and 6′ positions of the (1→3) linked glucose [5]. Since variations in the repeating structure of sphingans produce distinct differences in rheology, it would be beneficial to understand how the *Sphingomonas* polysaccharide biosynthesis machinery functions to synthesize a specific polysaccharide structure. Progress has been made in elucidating the genetics and biochemistry of sphingan biosynthesis. Genes for biosynthesis of sphingans S-88 [33, 41], S-7 [37] and gellan [13, 34] have been identified. Genes for several glycosyl transferases of the backbone structure have been analyzed biochemically [33, 38], as have genes *gelC* and *gelE* potentially involved in secretion and chain length determination [28]. Several of the genes for synthesis of sugar nucleotide precursors have also been elucidated [34]. The genetics and biochemistry of polymerization, secretion and control of polysaccharide molecular length are less well-defined. In the present study, we report the cloning and sequence analysis of a large cluster of genes for diutan polysaccharide biosynthesis, and the effect of multiple copies of this gene cluster on diutan yield and rheology.

Materials and methods

Media and culture conditions

Escherichia coli strains were grown in LB medium [26]. *Sphingomonas* strains were grown in YM (Difco, Detroit, MI, USA) or YEME medium (0.25% yeast extract, 0.025% malt extract) [13]. *Xanthomonas campestris* strains were grown in YM or YT medium [26]. Antibiotics were added in the following concentrations (μg/ml): kanamycin 12.5–50, tetracycline 7.5–15, streptomycin 25, rifampicin 100 and ampicillin 100. All strains were grown at 30–37°C.

Gene library construction

Sphingomonas ATCC 53159 was grown in YEME medium for about 24 h. Chromosomal DNA was purified, partially digested with *Sau*3AI (New England Biolabs, Inc., Beverly, MA, USA) and DNA fragments of 15–50 kb were isolated from agarose, ligated into *Bam*HI digested pLAFR3 [35], packaged into bacteriophage λ using the Gigapack III Gold packaging extract (Stratagene, La Jolla, CA, USA) and transfected into *E. coli* DH5α MCR (Life Technologies, Rockville, MD, USA).

DNA techniques

Restriction endonuclease digestions and ligations were performed using standard methods [25]. DNA isolations were conducted using commercially available kits (Qiagen, Valencia, CA, USA). Plasmids were introduced into *E. coli* by electroporation (BTX ECM® 600) and transferred from

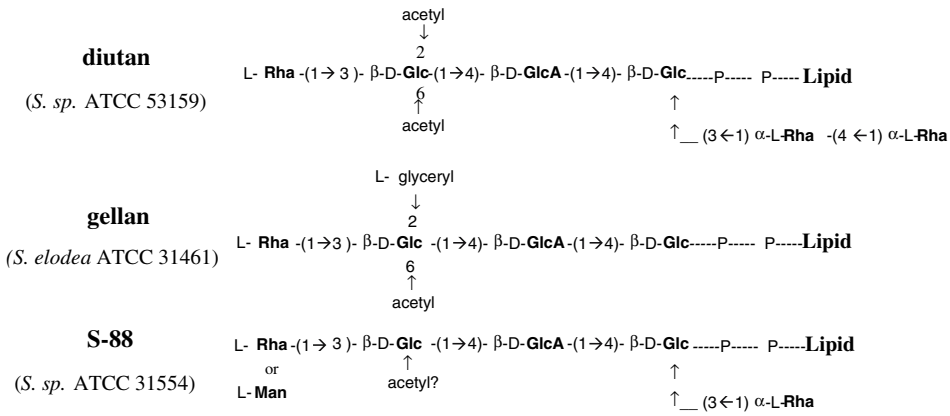

Fig. 1 Repeat unit structures of diutan, gellan and S-88 sphingan. Each repeat unit is depicted attached to the lipid carrier by a phosphodiester bond at the reducing end. Constituents include glucose (Glc), glucuronic acid (GlcA), mannose (Man), rhamnose (Rha), acetyl and glyceryl. The acetyl linkage in S-88 is unknown

E. coli to *Sphingomonas* (streptomycin resistant) and rifampicin resistant *X. campestris* [10] by triparental filter or spot matings [6], as described [13]. The pLAFR3, pLO2, pTR213b and pBBR1MCS-2 vectors are not self-transmissible but can be mobilized in *trans* by pRK2013 [6]. DNA sequencing was conducted by San Diego State University Microchemical Core or at Lark Technologies Inc. (Houston, TX, USA). Reading frames were identified using Clone Manager (Sci Ed Central) and analyzed using the programs BLAST (National Center for Biotechnology Information), and Pfam (Protein Families Database; The Sanger Center). The DNA sequence for the *dps* genes cluster has been deposited in the NCBI GenBank database and assigned accession number EU026118.

Construction of chromosomal gene deletions

Primers (Ransom Hill Biosciences, Inc. Ramona, CA, USA; Table 1) with incorporated restriction enzyme sites were used to amplify DNA fragments flanking targeted genes, by PCR, using HotStarTaq Master Mix Kit (Qiagen) and plasmid pX6 as template. DNA fragments were cloned into pLO2 [24] or pTR213b, transferred into *E. coli* DH5α (Life Technologies) then into *Sphingomonas*. Insertion of the plasmid into the *Sphingomonas* chromosome was selected by kanamycin resistance. After growth without antibiotic, recombinants were isolated by selecting for loss of sucrose (10–12.5%) toxicity encoded by the *sacB* gene [24], followed by screening by PCR. Cloning vector pTR213b was derived from pK18mobGII [20] by addition of the *Bacillus subtilis sacB* gene (unpublished results). Plasmid pRC106, containing a deletion of 1,892 bp encompassing genes *urf31.4* and *urf31* was constructed using primers P57-1 and 2 and P57-3 and 4 and cloning into pTR213b. Plasmid pRC111, containing a deletion of 582 bp of the 816 bp *urf31* gene was constructed using primers P57-11 and 12 and P57-13 and 14 and cloning into pTR213b. Plasmid pRC117, containing a deletion of 949 bp, including 742 bp of the 837 bp *urf31.4* gene and its start codon, was constructed using primers P57-15 and 17 and P57-18 and 19 and cloning into pLO2.

Fermentation, composition and rheological analysis

Sphingomonas strains were grown for 72 h in shake flasks, or in Applikon 20L fermentors, in media containing corn syrup as carbohydrate source, nitrogen sources and salts [30]. Broth viscosity was measured with a Brookfield viscometer at 60 rpm with the no. 4 spindle. Diutan was precipitated with two volumes of isopropanol, steam-dried at 40°C for 16 h and weighed to determine yield of total precipitated material. Samples were reconstituted at 1.5% (w/v) in deionized water, re-precipitated with isopropanol, steam-dried, milled and hydrolyzed with 1 M trifluoroacetic acid for 16 h at 100°C. The hydrolysate neutral sugars were quantitated by high-performance anion-exchange chromatography using the Dionex BioLC 300 instrument and the Dionex CarboPac PA1 column [4]. Organic acids

Table 1 Oligodeoxynucleotide primers used to construct chromosomal gene deletions in *Sphingomonas* sp. ATCC 53159

Primer	Sequence (5′–3′)	Restriction site	Binding region	Purpose
P57-1	GGGTCTAGAGGTTGATCAGCCCTTGTTTG	*Xba*I	5′ *urf31*	*Δurf31.4–urf31*
P57-2	ATTGGATCCGCCCGGCCCCAGCGTGACC	*Bam*HI	5′ *urf34*	*Δurf31.4–urf31*
P57-3	CACTGCAGTGATCCGGCTGGCCTGGGTC	*Pst*1	3′ *rmlD*	*Δurf31.4–urf31*
P57-4	CCTCTAGATCGCCGGCTGGAGCGAGC	*Xba*1	3′ *urf31.4*	*Δurf31.4–urf31*
P57-5	TCGGCCAGAGCTACAATGTC	None	3′ *rmlB*	Deletion diagnostic
P57-6	ACAGCGAGAACCAGAACACC	None	3′ *urf34*	Deletion diagnostic
P57-7	CGCGCCGATCATCCACCTGT	None	5′ *rmlD*	Integration diagnostic
P57-8	CACTTTATGCTTCCGGCTCGTATGTTGT	None	pTR213-b	Integration diagnostic
P57-9	GCCCTTGCGCCCTGAGTGCT	None	pTR213-b	Integration diagnostic
P57-10	GTGCCCCGGCTGGACGAC	None	5′ *urf34*	Integration diagnostic
P57-11	CGGGATCCCTTCCAGGCGCCAGTTGAC	*Bam*HI	5′ *urf31.4*	*Δurf31*
P57-12	GGTCTAGAGCGGCCGGCGCGATTCTGT	*Xba*I	3′ *urf31*	*Δurf31*
P57-13	GGTCTAGAGCTCGGCAGGCAGAACTG	*Xba*I	5′ *urf31*	*Δurf31*
P57-14	CCGTCGACGGACGAGGGGGCGACAC	*Sal*I	5′ *urf34*	*Δurf31*
P57-15	GGGTCTAGAAAGAAGAAGAAGTCCGAAGTA	*Xba*I	3′ *urf31*	*Δurf31.4*
P57-17	GGGGAGCTCGGCGGGCAACTCTACACC	*Sac*I	3′ *urf31*	*Δurf31.4*
P57-18	CAGTCGACTGATCCGGCTGGCCTGGGTC	*Sal*I	3′ *rmlD*	*Δurf31.4*
P57-19	CCTCTAGAAAATCGCCGGCTGGAGCGAGC	*Xba*I	3′ *urf31.4*	*Δurf31.4*

The underlined sequences (6 bases) are the restriction enzyme recognition sequences (where present)

were quantitated by high-performance ion-exclusion chromatography using the Dionex BioLC 300 instrument and the Dionex HPICE-ASI column (Dionex, Sunnyvale, CA, USA).

For the seawater viscosity tests, 0.86 g diutan was added to 307 g synthetic seawater (419.53 g of Sea Salt, ASTM D-1141-52, Lake Products Co., Maryland Heights, MO, USA, in 9,800 g water) and mixed at 11,500 rpm in a Fann Multimixer (Model 9B5). For the 3-rpm reading, viscosity was measured with a Fann viscometer (Fann model 35A; Torsion spring MOC 34/35 F0.2b; Bob B1; Rotor R1) and the shear stress value read from the dial and recorded as sea water viscosity (SWV) 3 rpm dial reading (DR). The 0.3-rpm viscosity was measured with a Brookfield LV DV-II viscometer with a LV-2C spindle and expressed in centipoises (cP). For the PEG LSRV (low shear rate viscosity) test, 0.75 g diutan was dispersed in 4.5 g Polyethylene Glycol 200 (CAS 25322-68-3), 299 g of standard tap water (10 g NaCl and 1.47 g $CaCl_2{\cdot}2H_2O$ in 10 l deionized water) was added, mixed for 4 h at 800 rpm and viscosity (cP) measured using a Brookfield LV viscometer, with a 2.5 + torque spring at 3 rpm using the LV 1 spindle.

For analysis of intrinsic viscosity and composition, diutan samples were precipitated with alcohol, re-hydrated (4 g/300 ml deionized water), treated for 2 h each with hypochlorite, glucoamylase (Optidex) at pH 4.5, 40°C, lysozyme at pH 9, and protease (Protex), and then precipitated with 4:1 CBM, dried and milled. CBM is an azeotropic isopropanol/water mixture with 82% isopropanol. A 0.2% diutan solution prepared in 0.01 M NaCl on a moisture corrected basis (determined with a Mettler HB 43 halogen moisture balance) was diluted to 0.004, 0.008, 0.010 and 0.012% with 0.01 M NaCl. Viscosity measurements were made using the Vilastic® VE System, at 2 Hz, a strain of 1 and shear rate of 12 s^{-1}, at 23°C. Data for the average of five measurements for each sample were used to calculate intrinsic viscosity. Neutral sugars and organic acids were quantified after hydrolysis with trifluoroacetic acid.

Results

Isolation of genes for diutan biosynthesis

The *Sphingomonas* ATCC 53159 gene library was transferred into glucosyl-isoprenyl phosphate transferase deficient, polysaccharide-negative strains of *Sphingomonas elodea* (Gps2) [13] and *X. campestris* (Xps109) [10]. These strains share a common first step in polysaccharide biosynthesis: transfer of glucose-1-phosphate from UDP-D-glucose to the isoprenylphosphate lipid carrier by glucosyl-isoprenyl phosphate transferases [13, 16, our unpublished results]. Transconjugants of Xps109 were obtained at a frequency of 3.8×10^{-2} per recipient, of which 0.8% were mucoid; for Gps2, 3.7×10^{-3} transconjugants per recipient were obtained, of which 2×10^{-4} were mucoid. Plasmid DNA was isolated from 12 mucoid Xps109 transconjugants and digested with *Hind*III and *Eco*RI to excise the entire insert DNA. Nine different inserts were detected, which had in common 4.4, 1.2, 2.0 and 0.6 kb fragments. Since isolation of plasmids from *Sphingomonas* is difficult, plasmid DNA from eight mucoid Gps2 transconjugants was transferred into *E. coli* DH5α, purified and digested with *Hind*III and *Eco*RI. Six different inserts were detected; five had 6.8 and 5.5 kb fragments in common, and all but one (pS6) had a 4.4 kb fragment.

Sequencing of diutan biosynthesis genes

Plasmid pX6 was used for double-stranded shotgun sequencing. Subclones of pS8 (6.8, 4.9 and 2.7 *Hind*III fragments in pBluescript II KS+ , Stratagene) were also sequenced. The diutan polysaccharide synthesis (*dps*) genetic map is shown in Fig. 2. Gene functions were designated based on homology to other genes in databases, in particular to the genes for biosynthesis of S-88 sphingan [33, 41], GenBank accession number U51197, and gellan, GenBank AY217008 and AY220099 [13] and AY242074 [34]. The minimum region in common in all plasmids isolated contained the *dpsB* gene. Thus, the presence of this gene allowed restoration of gellan and xanthan synthesis in glucosyl-isoprenyl phosphate transferase deficient mutants of *Sphingomonas elodea* and *X. campestris*, indicating that *dpsB* encodes this enzyme activity.

Of the 24 predicted protein coding regions, genes were identified that encode the transferases for the four sugars of the backbone (*dpsB, L, K* and *Q*), four enzymes for dTDP-L-rhamnose synthesis (*rmlA, C, B* and *D*), and proteins for secretion of polysaccharide (*dpsD, C, E*). Three genes putatively encode a polymerase (*dpsG*), a repeat unit transporter (*dpsS*) and a lyase (*dpsR*) [13, 34]. Gene *dpsG* encodes a hydrophobic membrane protein with strong homology to other membrane proteins involved in polysaccharide synthesis and a portion of the protein has homology to polymerases. The gene *dpsS* is incomplete, but the portion of the gene cloned encodes a protein with homology to proteins for polysaccharide export and O-antigen transporter or "flippase". The predicted protein for *dpsJ* was presumed to be involved with secretion and transport based on similarity to the AAA-superfamily of ATPases (CD0009) and to ABC transporter proteins (Pfam00005). The *atrB* and *atrD* proteins show similarity to a number of ABC-type transporter proteins from diverse genera of bacteria. Genes *dpsM*, *dpsN* and *dpsI* may be involved in attachment of the polysaccharide to the cell [14]. Functions for genes *dpsF* and *urf31.4, urf31* and *urf34* are unknown.

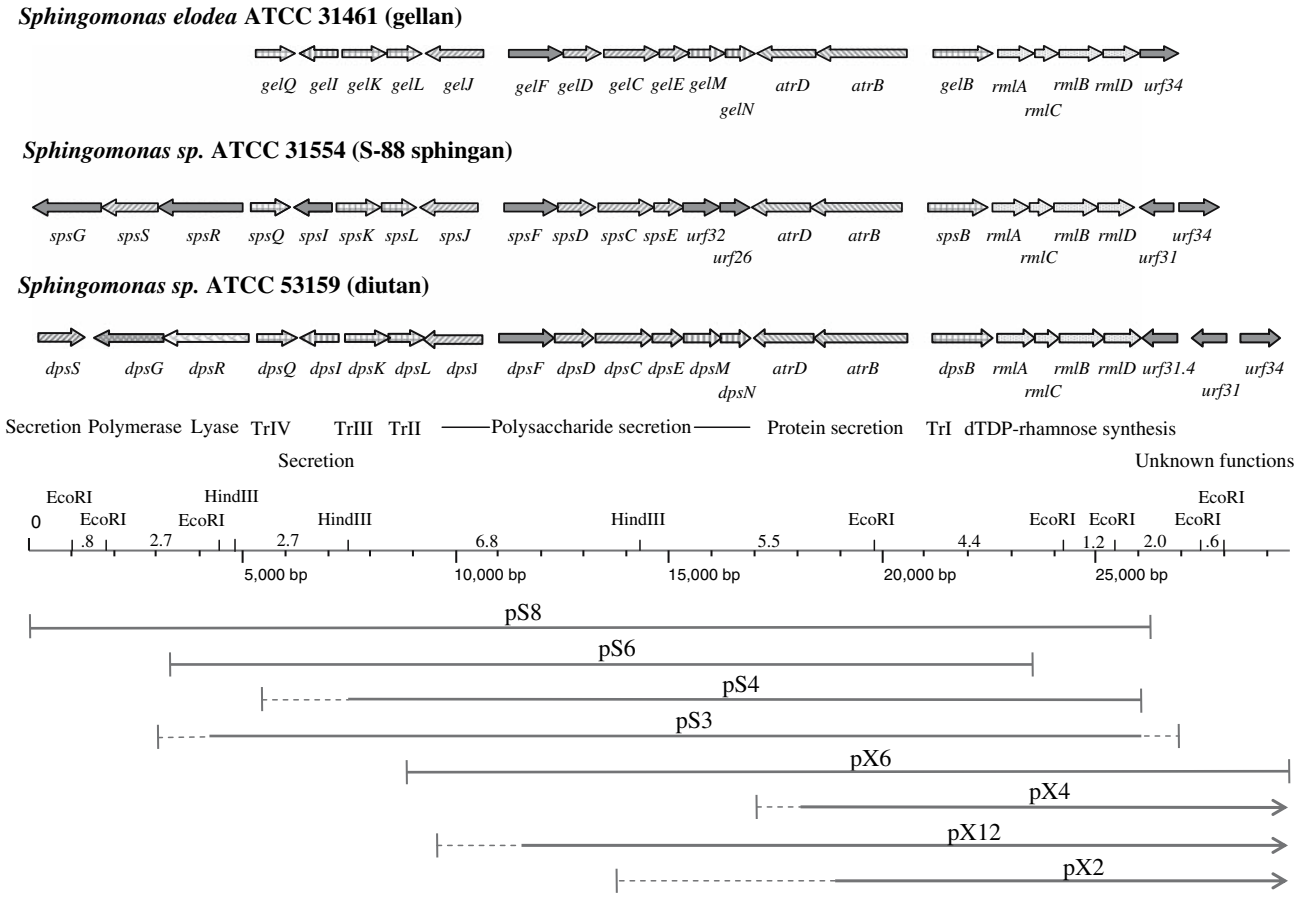

Fig. 2 Genes (*dps*) for diutan polysaccharide synthesis. The genes for diutan biosynthesis are aligned relative to the genes for synthesis of S-88 sphingan (GenBank accession number U51197, adapted from Yamazaki et al. [41] and to the genes for gellan synthesis (AY217008), adapted from Harding et al. [13]. Putative or known gene functions are indicated. Genes of unknown function are designated *urf* (unknown reading frame) and a number indicative of the molecular weight (kDa) of the encoded protein (following the convention of Yamazaki et al. [41]. Restriction endonuclease sites for *Eco*RI and *Hin*dIII are indicated. DNA segments cloned on plasmids are shown; where the ends of the inserts are not known exactly the probable lengths are indicated by *dashed lines*, and *arrows* indicate that the inserts include additional DNA beyond that shown in the figure

Comparison of the diutan polysaccharide synthesis gene cluster to the genes for biosynthesis of gellan [13, 34] and S-88 sphingan [33, 41], showed that although generally conserved in terms of genetic organization, notable differences exist, including: (1) gene *urf31.4* that is not found in the polysaccharide gene clusters of S-88 or gellan, (2) a homolog to S-88 *urf31* not found in the gellan cluster, (3) the *dpsG* and *dpsR* genes are adjacent to other genes in the cluster, as in S-88, while the corresponding genes for gellan are in a separate region of the chromosome [13] and (4) the *dpsS* gene is in a different order and orientation with respect to *dpsR* and *dpsG*. Another *Sphingomonas* strain ATCC 21423 that produces sphingan S-7 was reported to lack genes homologous to *spsS* and *spsR* in the sphingan biosynthetic gene cluster based on homology to S-88 gene probes [37]. Comparison of the predicted proteins with ATCC 31554 (S-88 sphingan) and ATCC 31461 (S-60 gellan) homologues (Table 2), suggests that the diutan biosynthesis genes are more closely related to those for S-88 sphingan than for gellan, as might be expected since S-88 sphingan and diutan contain single and double rhamnose side chains, respectively, and gellan has no sugar side chain. Since the three strains produce different repeat units, some divergence in enzymes for polymerization and secretion could be expected. The genes with the least similarity are *dpsS* (putative repeat-unit transporter), *dpsG* (putative polymerase), *dpsR* (putative lyase), *dpsQ* (transferase IV) and *dpsF* (unknown function essential for polysaccharide synthesis).

Comparison of the diutan and S-88 *urf31* homologs to the Pfam Database and *Psi* Blast produced weak hits to glycosyl transferase group 2, a diverse family of glycosyl transferases. Urf31 proteins of both strains had a DXD consensus sequence (amino acids 111–113) that has been identified in a large family of glycosyl transferases including the glycosyl transferase group 2 family [40], and consists of aspartic acid residues essential for activity in other β-glycosyl transferases including *Sinorhizobium meliloti exoM* [7]. The *urf31.4* gene, unique to the diutan

Table 2 *Sphingomonas* S-88 and S-60 homologs of predicted S657 proteins

S657 protein	Amino acids	Predicted function	% Identity (% similarity)	
			S-88	S-60
Sugar transferase enzymes				
*dps*B	470	Glucosyl-isoprenylphosphate transferase I	88 (95)	87 (94)
*dps*K	348	Beta-1,4-glucuronosyl transferase II	93 (95)	88 (92)
*dps*L	288	Glucosyl transferase III	86 (91)	76 (85)
*dps*Q	312	Rhamnosyl transferase IV	63 (74)	60 (72)
Polymerization/secretion				
*dps*R	665	Putative polysaccharide lyase	53 (69)	49 (63)
*dps*G	541	Putative polysaccharide polymerase	53 (66)	42 (62)
*dps*S partial	[350]	Putative flippase or repeat unit transporter	19	25 (38)
*dps*I	290	Polysaccharide secretion/attachment	79 (86)	78 (87)
*dps*J	462	Polysaccharide secretion (ATPase)	89 (96)	87 (93)
*dps*D	305	Polysaccharide export protein	78 (84)	74 (82)
*dps*C	448	Export protein (chain length determinant)	87 (93)	82 (92)
*dps*E	235	Export protein (tyrosine kinase)	88 (95)	88 (94)
dpsM	293	Polysaccharide secretion/attachment	85 (91)	84 (90)
dpsN	232	Polysaccharide secretion/attachment	90 (93)	87 (92)
*atr*D	464	Secretion protein	89 (93)	86 (93)
*atr*B	728	ABC transporter	94 (96)	93 (96)
dTDP-rhamnose synthesis				
*rml*A	292	Glucose-1-phosphate thymidylyltransferase	97 (99)	94 (97)
*rml*C	188	dTDP-6-deoxy-D-glucose-3-5-epimerase	96 (98)	94 (96)
*rml*B	353	dTDP-D-glucose-4,6-dehydratase	98 (99)	98 (98)
*rml*D	288	dTDP-6-deoxy-L-mannose-dehydrogenase	88 (94)	89 (94)
Unknown functions				
*dps*F	432	Unknown, essential for polysaccharide synthesis	74 (86)	57 (74)
urf31.4	278	Unknown	None found	None found
urf31	271	Putative glycosyl transferase	76 (87)	None found
urf34	310	Membrane transport protein	88 (92)	85 (87)

gene cluster, encodes a protein with a domain (223–278 amino acids) with homology to a domain of unknown function DUF1289 and has the four conserved cysteines of this domain. Another region of the protein (55–173 amino acids) has homology to EGL-9, a predicted proline hydroxylase, involved in posttranslational modification, protein turnover and chaperones. The protein encoded by diutan *urf34* was homologous to a number of hypothetical proteins from Sphingomonads and other bacteria. A portion of the protein (amino acids 160–294) had homology to COG2510, a predicted membrane protein. Urf34 is likely a membrane protein involved in transport, however, it is not known if this protein is involved in sphingan biosynthesis.

Transfer of ATCC 53159 genes into *Sphingomonas elodea* and *Sphingomonas* sp. ATCC 31554

Selected pX (complemented Xps109) and pS (complemented Gps2) plasmids were transferred into *Sphingomonas elodea* strain S60wtc [13]. Plasmids pS3, 4, 6 and 8 resulted in no change in phenotype; however, plasmids pX2, 6, and 12 resulted in dramatic changes in phenotype, predominantly small, slightly mucoid colonies and some highly mucoid, soft, runny colonies, suggesting that genes present in the pX plasmids and not in the pS plasmids, i.e., *urf31.4, urf31* or *urf34*, are disruptive to cell growth and production of polysaccharide in *Sphingomonas elodea*. Gene *urf34* is probably not detrimental since *Sphingomonas elodea* contains a homolog. To determine whether the *urf31.4–urf31* region was responsible for the detrimental phenotype, a 2.9 kb *Sal*I fragment spanning from within *rmlD* to within *urf34* was cloned into vector pBBR1MCS-2 [21]. This plasmid, designated pBBsal2.9, when transferred into S60wtc produced the detrimental phenotype, while the vector control did not. Some mucoid colonies were mixed in with a majority of small, slow growing colonies. The large colony phenotype was stable, while restreaking small colonies produced a mixture of small and large mucoid colonies.

Plasmid pBBsal2.9 was transferred into *Sphingomonas* sp. ATCC 31554 (S-88) to determine if expression of these genes is detrimental in this strain that has an *urf31* homolog and produces a polysaccharide with the same backbone structure as diutan but with a single rhamnose side-chain instead of the double rhamnose side-chain of diutan (Fig. 1). No altered colony phenotype was observed. pBBsal2.9 plasmids were isolated from the *E. coli* DH5α, the *Sphingomonas* sp. ATCC 31554 (S-88), and both the S60wtc mucoid and small colony transconjugants and analyzed by *Eco*R1 and *Pst*1 double digestion (Fig. 3). Plasmid pBBsal2.9 isolated from the S-88/pBBsal2.9 transconjugants produced a similar digestion pattern to pBBsal2.9 isolated from *E. coli* DH5α. However, the *Eco*R1/*Pst*1 fragments produced from pBBsal2.9 isolated from both the S60wtc/pBBsal2.9 mucoid and small colony transconjugants indicated DNA alterations or deletions had occurred within the *urf31.4–urf31* coding regions, represented by the 175, 401, 772 and 1,062 bp fragments. The pBBsal2.9 DNA isolated from the S60wtc/pBBsal2.9 mucoid transconjugant most likely deleted the entire 2.9 kb *Sal*1 fragment, while pBBsal2.9 isolated from the S60wtc/pBBsal2.9 small colony transconjugant appeared to have a smaller DNA alteration within the 3′ end of *urf31* that resulted in the loss of an *Eco*R1 restriction site. The *urf31* sequence may encode a glycosyl transferase responsible for addition of a side-chain rhamnose. The small, slow growing S60wtc/pBBsal2.9 transconjugants could be producing non-native, lipid linked-intermediate subunit structures, thus interfering with polysaccharide polymerization and cell growth. The *urf31.4–urf31* region was not detrimental in *Sphingomonas* sp. ATCC31554. The presence of the first rhamnosyl transferase would be redundant. To determine if a second rhamnose was added, polysaccharide was obtained from two S-88/pBBsal2.9 transconjugants, a pBBR1MCS-2 (vector only) transconjugant, and the parental strain by duplicate shake flask fermentations to examine neutral sugars and acetate composition. The polysaccharide produced by these isolates did not have increased rhamnose content indicating that a second rhamnose had not been added (data not shown).

Construction of chromosomal deletions

Individual and double *urf31.4* and *urf31* deletions were constructed on plasmids and introduced into both ATCC 53159 wild-type and a nonmucoid derivative, Dps1. Dps1 was isolated by selecting for bacitracin resistance and screening for colonies exhibiting a nonmucoid phenotype [32, 36] and able to be complemented by plasmids expressing glucosyl-isoprenyl phosphate transferase. Attempts to isolate an *urf31.4–urf31* double deletion with plasmid pRC106, or an individual deletion of *urf31* with plasmid pRC111, in either the wild-type or Dps1 strains were unsuccessful. Although the insertion of the plasmids into the chromosome was confirmed by kanamycin resistance and by PCR amplification using primers hybridizing to chromosomal regions outside of the cloned sequences coupled to primers hybridizing to vector DNA (P57-7 to 8 and P57-9 to 10), all second recombinants were restored to wild-type rather than deletion. These results suggest that the *urf31* gene may be required for growth, or that *urf31* may encode a side-chain rhamnosyl transferase and deleting this gene results in the production of non-native

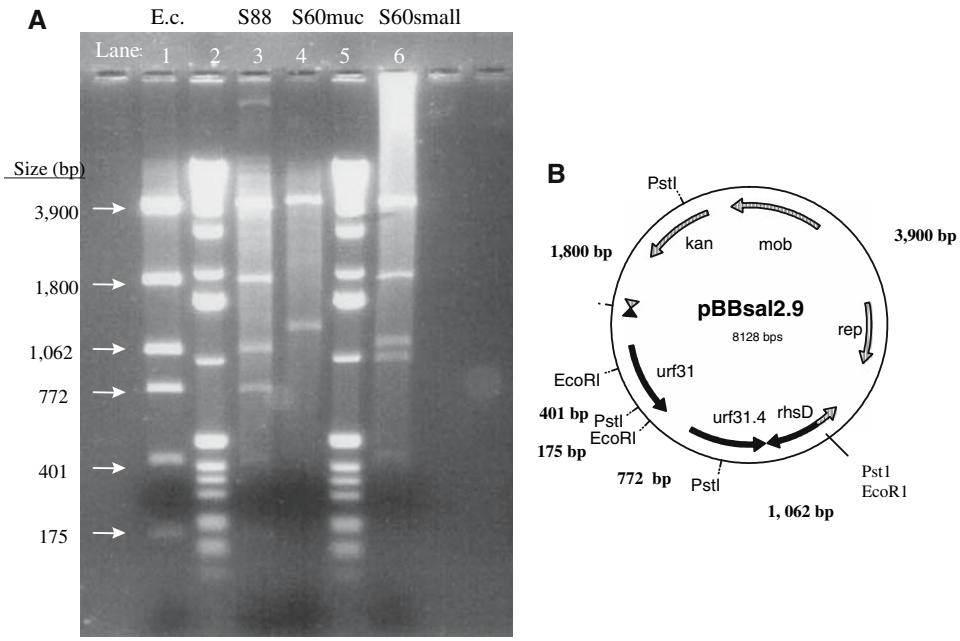

Fig. 3 **a** DNA fragments produced by *Eco*R1 and *Pst*1 double-digestion of pBBsal2.9 DNA isolated from *E. coli* (*Lane 1*), *Sphingomonas* sp. ATCC 31554 (*Lane 3*), *Sphingomonas elodea* mucoid colony (*Lane 4*) and small colony (*Lane 6*) transconjugants. *Lanes 2 and 5* contain 1 kb DNA ladder, on a 1.5% agarose gel, **b** Plasmid pBBsal2.9 *Eco*R1 and *Pst*1 restriction enzyme map and expected fragment sizes

subunits missing one or both rhamnose side groups, thus producing a lethal phenotype. However, this deletion would not have been expected to be lethal in Dps1. Introduction of the *urf31.4* deletion from plasmid pRC117 into both ATCC 53159 wild type and Dps1 was successful. Phenotypically, the ATCC 53159 *urf31.4* deletion mutants appear similar in growth and polysaccharide production to the parent. Composition analysis of diutan produced by shake flask fermentations of parental and two *urf31.4* mutant strains showed that deletion of the *urf31.4* gene did not affect the diutan composition (data not shown).

Effects of gene amplification on diutan production and rheology

Several plasmids (pX4, pX6, pS6 and pS8) were tested for ability to enhance diutan production of the wild-type strain, *Sphingomonas* ATCC 53159 (S657). Plasmid pX6 contains 17 genes including *dpsB* encoding glycosyl transferase I, genes for secretion and for dTDP-rhamnose synthesis, but lacks the genes for transferases II, III and IV and the putative genes for polymerase and lyase. Plasmid pS8 contains 20 genes including genes for all four backbone sugar transferases, the four genes for dTDP-rhamnose synthesis, genes for secretion of the polysaccharide, and the putative genes for polymerase and lyase, but lacks the genes *urf31.4*, *urf31* and *urf34* and has a partial *dpsS*. Plasmid pS6 contains genes for secretion and the four sugar transferases but does not have all genes for dTDP-rhamnose synthesis or the gene for polymerase. Plasmid pX4 contains only a small part of the *dps* region but includes the gene encoding transferase I and the four genes for dTDP-rhamnose synthesis that were reported by Pollock et al. to be sufficient to result in an increase in yield of polysaccharide in *Sphingomonas* strains [37]. Strains S657/pS8, S657/pS6, S657/pX6 and S657/pX4 were compared to wild-type S657 without a plasmid in three sets of fermentations in Applikon fermentors. Somewhat higher diutan yield was obtained with all four plasmids (Table 3). However, with the pS8 plasmid, there was an increase in broth viscosity relative to the increase in yield indicating that some factor in addition to increased amount of diutan was affecting the viscosity.

These diutan samples were analyzed in terms of rheological properties useful in two applications: oilfield additives for oil recovery and cement additives for water retention and quick set-up. The oilfield industry uses a SWVtest, as an indicator of the effectiveness of a polymer to increase viscosity in brine (for example, to replicate recovery from seabeds). For cement applications, the PEG LSRV test (a low shear rate viscosity using polyethylene glycol as dispersant) provides an indication of effectiveness of a viscosity modifier. Diutan from S657/pS8 had significantly increased rheological parameters compared to diutan from

Table 3 Fermentation and rheology of diutan from plasmid-containing strains

Strain	No. of runs	Fermentation parameters						Rheology of Recovered Diutan								
		TPM av.	c.v.%	Increase %	Av. broth viscosity cP	c.v.%	Increase %	SWV 3 rpm DR	c.v %	Increase %	SWV 0.3 rpm[a] cP	c.v.%	Increase %	PEG LSRV cP	c.v. %	Increase %
S657	3	33.5	3.3	–	5,217	5.9	–	24	8.6	–	26,500	11.2	–	2,750	16.0	–
S657/pS8	3	36.1	2.4	8.0	6,783	1.7	30.0	44	6.0	80.8	40,150	4.8	51.5	4,873	2.8	77.2
S657/pX6	3	36.5	7.2	9.1	5,592	10.5	7.2	27	7.8	9.6	27,050	10.7	2.1	3,177	8.9	15.5
S657/pS6	1	37.6	–	12.4	6,675	–	28.0	22	–	−9.6	n.d.			2,270	–	−17.5
S657/pX4	1	36.4	–	8.8	5,525	–	5.9	25	–	0.7	n.d.			2,950	–	7.3

TPM total precipitable material, *c.v.* coefficient of variation (standard deviation/mean), *SWV* sea water viscosity, *LSRV* low shear rate viscosity

[a] For SWV-0.3 rpm $n = 2$

wild-type, by all three methods (81% in 3 rpm SWV, 52% in 0.3 rpm SWV and 77% in PEG LSRV, Table 3). Plasmids pX6, pS6 and pX4 resulted in only slight or insignificant increases. The substantial increase in the sea water and PEG low shear rate viscosity measurements cannot be attributed to the increase in yield alone since other plasmids resulted in similar yield results. This increase in viscosity may be due to an increase in the molecular weight (i.e., chain length) of the diutan molecule. As an indicator of molecular weight, the intrinsic viscosity was determined, by plotting the reduced viscosity (viscosity normalized for concentration) versus the solution concentration, and extrapolating to zero concentration. Five diutan samples, two from wild-type S657 and three from S657/pS8 were evaluated. S657/pS8 produced diutan with significantly higher intrinsic viscosity, 165.2 ± 4.7, versus that of the wild-type, 140.7 ± 3.3 (Fig. 4), suggesting that diutan from S657/pS8 is higher in molecular weight. The purified samples used for intrinsic viscosity measurements were used for neutral sugars and organic acid analyses. The composition of diutan from S657/pS8 (32% rhamnose, 18.3% glucose, 8.6% acetate) was nearly identical to that of wild-type (31.5% rhamnose, 19% glucose, 8.65% acetate). Although both results were different from theoretical values (indicating samples were not 100% pure), these results indicated that the composition of diutan from S657/pS8 was the same as that for wild-type and that the increase in viscosity imparted by the pS8 plasmid may be due to higher molecular weight, i.e., longer chain length.

A comparison of the genes cloned in the tested plasmids suggested a gene possibly responsible for the increase in molecular weight might be *dpsG*, since this gene is present in pS8 and not in the other plasmids. The function of *dpsG* has not been determined biochemically; however amino acids 280-472 have homology to O-antigen polymerases (pfam04932 or *wzy*). A 4.7 kb *Bam*HI–*Xba*I fragment from pS8 spanning from within *dpsS* to within *dpsQ*, including *dpsG, dpsR* and the upstream region likely to include a promoter region was cloned into plasmid pLAFR3-MCS193, constructed by replacing the multiple cloning site of pLAFR3 with the multiple cloning site from pNEB193 (New England Biolabs) to provide an *Xba*I site needed for cloning. Plasmid pS8 was prepared from a *dam* and *dcm* deficient strain (SCS110, Stratagene) to avoid modification near the *Xba*I site. The resulting plasmid pLAFR3193-*dpsG* was transferred into S657 and evaluated in fermentors (data not shown). However, yield of diutan and rheological parameters were comparable to the wild-type and not to that of S657/pS8. In another attempt to identify genes possibly contributing to the improved rheology, a region containing genes *dpsFDCE* (linked genes for secretion) was amplified by PCR, cloned into pLAFR3, transferred into S657 and evaluated in fermentors. No improvement in rheological parameters was observed. Thus, a combination of genes may be required. A previous report with *Sphingomonas* sp. ATCC 21423 showed an increase in S-7 sphingan conversion from glucose with a plasmid containing the gene for glucosyl transferase I and genes for dTDP-rhamnose synthesis, and a further increase in yield (to 17.9 g/l versus 16.5 g/l) and broth viscosity with a plasmid that contained most of the sphingan biosynthetic genes [37]. Effect on molecular weight was not determined.

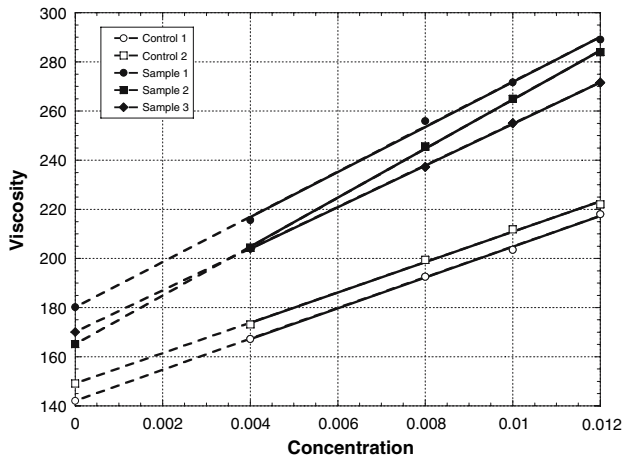

Fig. 4 Intrinsic viscosity of diutan purified from S657 wild-type strain (control) and S657/pS8 (samples). Intrinsic viscosity was determined as: S657 control no 1 = 138.3, S657 control no 2 = 143, for an average of 140.7 ± 3.3 for diutan from wild-type S657; S657/pS8 sample no 1 = 170.7, sample no 2 = 162.2 and sample no 3 = 162.8, for an average of 165.2 ± 4.7 for diutan from S657/pS8

Discussion

Restoration of polysaccharide synthesis in polysaccharide-deficient mutants of *X. campestris* and *Sphingomonas elodea* with a library of *Sphingomonas* sp. ATCC 53159 chromosomal DNA resulted in the isolation of a cluster of 24 putative *Sphingomonas* sp. ATCC 53159 genes. Heterologous complementation of the glucosyl-isoprenyl phosphate transferase of *X. campestris* supports the identification of *dpsB* (gene in common in all complementing plasmids) as the gene encoding the glucosyl-isoprenyl phosphate transferase. Similarly, a plasmid pYP3.6XVBam containing the *Sphingomonas elodea gelB* also complemented a glucosyl-isoprenyl phosphate transferase deficient mutant of *X. campestris* [13]. Twenty-two of the 24 cloned genes had homologs in the gene clusters for biosynthesis of both gellan and S-88 sphingan, including four genes involved in the synthesis of dTDP-L-rhamnose sugar nucleotide precursor, four genes that encode the glycosyl transferase enzymes responsible for addition of sugars to the common sphingan tetrasaccharide repeating backbone

structure, and genes for polymerization and secretion of the polysaccharide and protein transport, and a putative gene for lyase. Homologs to *dpsF* and *urf34* are present in all three clusters, but their functions are unknown. Gene *urf31* had homology to a gene in the cluster of genes for S-88 sphingan synthesis, but not that for gellan. Another gene *urf31.4*, did not have a homolog within either the S-88 or gellan polysaccharide synthesis gene clusters.

Comparison of the predicted protein sequences to gellan and S-88 homologs suggests a well-conserved mechanism for biosynthesis and secretion of capsular polysaccharide among these strains. Most of the homologous genes in these three strains are similar (80–98% amino acid identity). However, several genes, e.g., *dpsS, G, R, Q and F* have much lower similarities (19–63% identities). In general, the genes for diutan biosynthesis are more closely related to those for S-88 sphingan than those for gellan, as may be expected since diutan structure is more similar to S-88 sphingan than to gellan. Diutan has a two-sugar rhamnose side chain, S-88 has one rhamnose side chain, while gellan has no sugar side chain. Taxonomic studies (unpublished data) indicate that the *Sphingomonas* strains producing polysaccharides S-88, S-198 and diutan, which all have rhamnose side chains, are closely related to each other and to the strain *Sphingomonas trueperi* based on 16S ribosomal RNA homology. *Sphingomonas elodea* producing gellan is most closely related to *Sphingomonas pituitosa*.

Since the diutan and S-88 sphingans structures have a two and one sugar rhamnose side chain, respectively, it would be expected that the protein responsible for addition of the first side-chain rhamnose would be similar and would be present in S-88 but not in the gellan gene cluster. A likely candidate would be *urf31*. The predicted Urf31 protein shows similarity to conserved glycosyl transferase motifs. Introduction of cloned *urf31.4–urf31* genes had no apparent effect in *Sphingomonas* ATCC 31554 (S-88), but in *Sphingomonas elodea* was detrimental to growth and gellan polysaccharide production, and resulted in DNA alterations in or near the *urf31* gene. One reason for the detrimental effect could be the addition of a rhamnose side-chain sugar to the gellan subunit to produce a non-native structure. Attempts to delete *urf31* in *Sphingomonas* ATCC53159 wild-type or a nonmucoid mutant were unsuccessful. It was reported by Pollock *et al.* that inactivation of the S-88 *urf31* homolog did not affect polysaccharide synthesis or cell growth [41]. Possibly, for the S-88 polysaccharide, the absence of the rhamnose side chain may not prevent polymerization. In ATCC 53159, however, if inactivation of *urf31* prevents addition of both rhamnose side-chain sugars, non-native oligosaccharide structures may accumulate that cannot be polymerized, thus being lethal. Other studies have demonstrated an association between inhibition of cell growth and polysaccharide production with incomplete repeat unit structures or with the introduction of non-native glycosyl transferase genes whose products compete with endogenous glycosyl transferases to attach different sugars to the same substrate, e.g., S-88 *spsK* in *X. campestris* or *spsL* in *Rhizobium leguminosarum* [9, 33]. Formation of non-native subunit structures linked to lipid carrier may be detrimental to cells because the carrier cannot be released to support other cellular functions if the polymerization machinery does not recognize non-native substrates. In this study, all plasmids isolated in *Sphingomonas elodea* did not contain DNA beyond the *urf31* gene, while plasmids isolated in *Xanthomonas* lacked the genes for transferases II, III and IV. Deletion of *urf31* may also be disruptive to polymerization/export protein complexes on the plasma membrane. Studies of *E. coli* polysaccharide capsule synthesis indicate that proteins involved in polysaccharide subunit synthesis, polymerization, and export form a tight complex within the plasma membrane. A number of proteins play an important role in the formation and stabilization of this complex [39]. Disturbances of this capsule assembly complex at sites of adhesion between the inner and outer membrane may be detrimental to growth and polysaccharide synthesis.

Alternatively, the diutan side-chain rhamnosyl transferases may not be within the main polysaccharide gene cluster. Generally, genes required for synthesis and secretion of polysaccharide in other acidic heteropolysaccharide-producing Gram-negative bacteria such as *X. campestris* [10, 15, 19] and *Sinorhizobium meliloti* [7–9] are clustered. The *X. campestris gum* cluster contains genes for all transferase enzymes required to assemble the sugars, add acetate and pyruvate to the pentasaccharide subunit, and genes required for polymerization and secretion of xanthan [10, 19]. The genes for synthesis of the precursor sugar nucleotides are not linked to this cluster [11]. The *R. meliloti exo* gene cluster contains glycosyl transferase genes, genes for the addition of the acetyl, succinyl and pyruvyl modifications, and genes for polymerization of the octasaccharide subunits, and export of succinoglycan [7–9]. In *Sphingomonas* sp. ATCC 31554, ATCC 53159 and *Sphingomonas elodea* all of the glycosyl transferases responsible for addition of the four sugars comprising the backbone structure are found within the main polysaccharide gene cluster [13, 34, 41]. However genes *gelR, gelS* and *gelG* in *Sphingomonas elodea* are in a different region of the chromosome [13] and the gene for acetylation of gellan is also not adjacent to either of the *gel* gene clusters [12]. The identification of diutan side-chain rhamnosyl transferases would require more than sequence comparison since glycosyl transferases tend to share limited homology [7, 8, 33, 40]. For example, in vitro assays employing permeabilized *Sphingomonas* cells carrying plasmids with *Sphingomonas* DNA incubated

with radiolabeled sugar nucleotides could be used to characterize lipid-linked intermediates [15, 16, 33].

Four plasmids containing different portions of the *dps* gene cluster were shown to slightly increase the production of diutan in fermentation. However, plasmid pS8 harboring 20 of the 24 genes identified, resulted in a significant increase in viscosity of diutan measured at low shear rates in saline solution and in a PEG dispersion. The increase in viscosity was attributed to increased molecular weight based on intrinsic viscosity measurements. Since the composition of the diutan produced in the strain with plasmid pS8 was the same as that of the wild-type, it is likely that this increase in molecular weight is due to an increase in chain length. Attempts to determine specific genes on pS8 that could achieve this improved rheology were unsuccessful. Potential genes that may affect molecular weight could be *dpsG* (which is present on pS8 but not on the other plasmid tested) and has homology to polymerases, or genes *dpsD, dpsC* and *dpsE*, which are homologous to genes *gumB* and *gumC* that have been shown to increase the molecular weight of xanthan when amplified on a multicopy plasmid [29] and homologous to other genes that control polysaccharide chain length [28]. However individual amplification of *dpsG* or of the *dpsFDCE* cluster on multicopy plasmids did not increase diutan viscosity. Other genes may be required in combination with *dpsG* or *dpsD, dpsC, dpsE* to achieve the observed increase in viscosity, e.g., genes *dpsB, dpsL, dpsK* and *dpsQ* encoding the sugar transferases I, II, III and IV, in particular the gene *dpsB* which encodes transferase I that adds the first sugar of the repeat unit to the lipid carrier.

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