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Increasing the yield and viscosity of exopolysaccharides secreted by *Sphingomonas* by augmentation of chromosomal genes with multiple copies of cloned biosynthetic genes

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Certain bacteria of the *Sphingomonas* genus secrete structurally related capsular polysaccharides. Due to their unique properties, three (gellan, welan and rhamsan) are produced commercially by submerged fermentation and are used as modifiers of aqueous rheology and as gelling agents. However, conversion of glucose into these polysaccharides is relatively inefficient. To identify general methods for increasing the productivity of *Sphingomonas*, we augmented the normal chromosomal copy of the phosphoglucomutase gene (*pgm*) and the cluster of genes (*sps*) required for assembly of the carbohydrate repeat unit for strain S7 with multiple copies of plasmids carrying these genes. Although a sixfold increase in Pgm activity only lead to a small percentage increase in conversion of glucose to the S-7 polysaccharide, multiple *sps* genes caused a nearly 20% increase in the yield from glucose and an even larger increase in culture viscosity. The increased viscosity was accompanied by a change in the sugar composition of the secreted polymer. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 49–57.

Keywords: *Sphingomonas*; exopolysaccharide; productivity; biosynthetic-gene augmentation

Introduction

Several members of the bacterial genus *Sphingomonas* secrete capsular polysaccharides with similar but not identical structures [26,28]. Synthesis of the capsule is controlled by a genetic system that links environmental sensing to a regulatory response and is an important factor in whether *Sphingomonas* adopts a sessile or a motile behavior [29]. As diagrammed in Figure 1, the main chains of the polymers are largely conserved, while the side chains vary in composition and location. We refer to this group of related polysaccharides as "sphingans" after the common genus. L-Mannose, a rare sugar in nature, partially replaces L-rhamnose in some of the sphingans and in one case, *Sphingomonas* NW 11, only L-mannose is found [27]. The sugars that make up the repeat unit for polysaccharide S-7 (Pollock, unpublished) are the same as previously determined for a polysaccharide of *Sphingomonas paucimobilis* I-886 [11], including a rare 2-deoxyglucuronic acid in the main chain. However, polysaccharide S-7 is different from I-886 due to a β-linked glucose in the side chain.

Gellan (S-60), welan (S-130), and rhamsan (S-194) are produced commercially by large-scale submerged fermentation and are sold as gelling and rheology-control agents for aqueous solutions. Each possesses a unique array of useful rheological properties, responding in different ways to temperature, pH, ionic conditions, and shearing. Compared to *Xanthomonas campestris*, which converts nearly 60–80% of the sugar in its culture medium into xanthan gum, sphingan-producing strains are relatively inefficient in making polysaccharides. A typical yield of gellan from glucose for *Sphingomonas* S60 is about 40–50%, with a large portion of carbon being diverted to CO_2 [33]. Our interest in the mechanism and control of biosynthesis of the sphingans derives from their commercial importance and the possibilities for yield enhancement.

For sphingan-producing cells, sugars such as glucose are either converted into exopolysaccharides or into additional cell mass by alternative routes of intermediary metabolism (Figure 2). The involvement of specific enzymes was inferred from the enzymatic activities detected in cell-free extracts of *Sphingomonas* S60 [23,25,33]. We attempted to genetically manipulate the flow of carbon through these alternative pathways in order to enhance polysaccharide production. As a model we used *Sphingomonas* S7 because it also showed inefficiencies in carbon conversion similar to strain S60 and the other sphingan producers, and because of its future commercial potential as a producer of an industrial viscosifying agent. Our approach was to augment the chromosomal genes by introducing additional copies of the biosynthetic genes inserted in plasmids. This included the phosphoglucomutase (E 5.4.2.2) gene (*pgm*), which occupies a key position in directing carbon flow toward sphingan assembly, and a cluster of biosynthetic genes (*sps*) which code for the assembly of a lipid-linked carbohydrate repeat unit and secretion of the polymer. We obtained an increase in conversion efficiency and also an increase in the viscosity of the S-7 polymer.

Materials and methods

Bacterial strains and culture conditions

Bacterial strains and plasmids are described in Table 1. YM medium contained 3 g of Bacto yeast extract, 3 g of Bacto malt extract, 5 g Bacto peptone and 10 g D-glucose (all from Difco) per liter of tap water. 1/4 YM was YM diluted with three volumes of tap water. "YM with low amounts of glucose" refers to YM medium containing only the small amount of glucose present in

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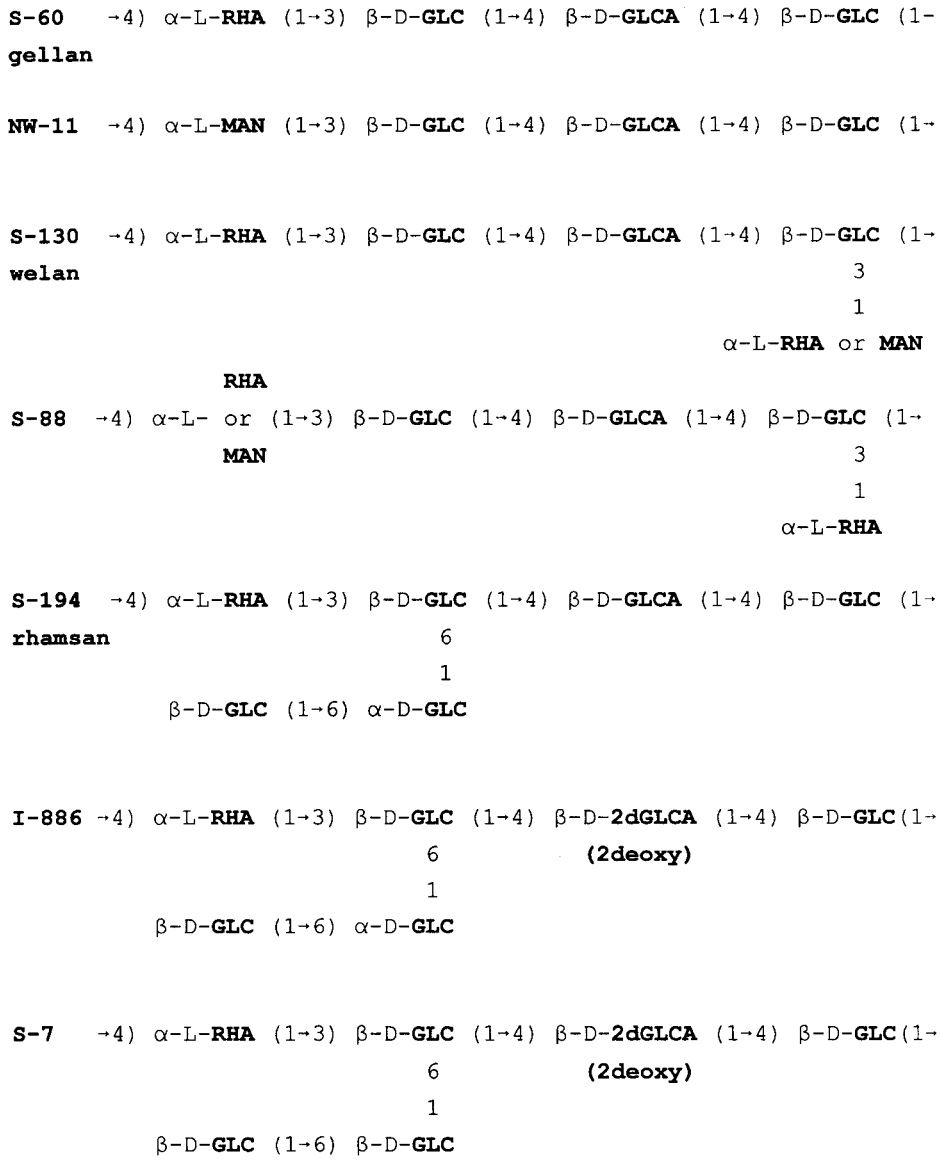


Figure 1 Structures of repeat units for polysaccharides S-60, NW-11, S-130, S-88, S194, I-886 and S-7 [11,15–18, 27]. Rhamnose predominates as the side chain of S-130 [19]. Acetyl groups are present in each polymer but the position was determined only for S-130 [32]. The reducing end of each repeat is to the right, and during assembly the IP carrier is attached through a phosphodiester linkage to a reducing end. Abbreviations: Rha, rhamnose; Glc, glucose; GlcA, glucuronic acid; Man, mannose; and 2dGlcA, 2-deoxy-glucuronic acid.

malt extract and without the large amount of additional glucose. Solid medium contained agar (Difco) at 15 g/liter. Antibiotics (Sigma) were added at the following concentrations: bacitracin at 73 units/mg or in the range 0.01 to 8 mg/ml; kanamycin at 25 μg/ml; rifampin at 50 μg/ml; streptomycin at 25 to 100 μg/ml; tetracycline at 6 to 12 μg/ml; and chloramphenicol at 25 μg/ml. Colonies of bacterial strain NW11 which grew on YM plates containing 20 μg/ml bacitracin were screened for non-mucoid appearance on YM plates lacking the drug and then tested for complementation by plasmids carrying the cloned *spsB* gene of strain S88 [30]. The resulting isolate, NW11B, was found to be defective for transferase I in sphingan assembly since the normal mucoid appearance was restored by the cloned *spsB* gene. The fermentation medium contained 1 g of NH_4NO_3, 3.2 g of K_2HPO_4, 1.6 g of KH_2PO_4, 0.2 g of $MgSO_4{\cdot}7H_2O$, 1 ml of 1000× trace minerals, 0.5 g of soluble soy peptone (Marcor), and 30 g of D-glucose per liter of tap water. 1000× trace minerals contained 270 mg of $FeCl_3{\cdot}6H_2O$, 136 mg of $ZnCl_2$, 198 mg of $MnCl_2{\cdot}4H_2O$, 24 mg of $CoCl_2{\cdot}6H_2O$, 24 mg of $Na_2MoO_4{\cdot}2H_2O$, and 25 mg of $CuSO_4{\cdot}5H_2O$ per 100 ml of deionized water, and was autoclaved. Cells that were frozen as starter cultures were grown in the presence of tetracycline (6 μg/ml). No antibiotics were added to seed shake flasks or during the 4-l fermentations since the plasmids were stable. Less expensive insoluble soy proteins also supported the growth and productivities reported here. The fermentors (New Brunswick BioFloIII and 3000) were stirred by a downward-flowing three-bladed marine impeller located about 1 in. under the liquid surface and by two six-bladed Rushton impellers, one

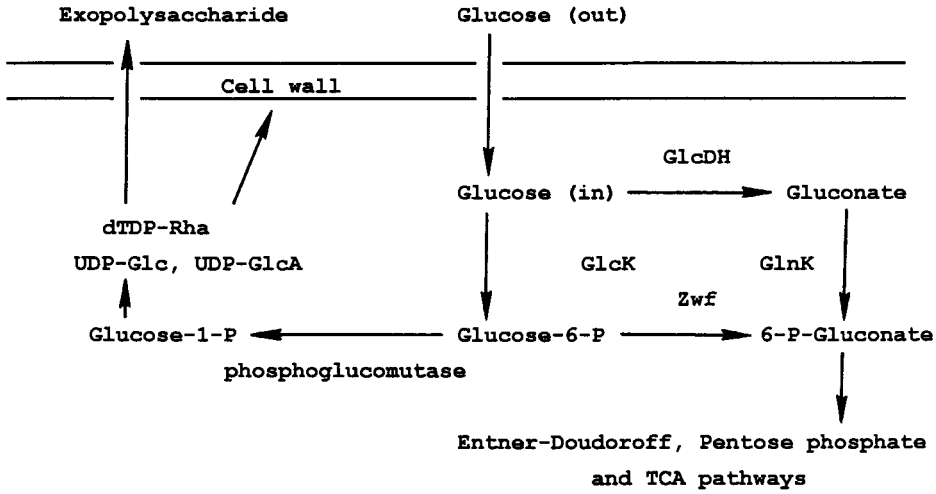


Figure 2 Conversion of glucose into intermediary metabolites, cell walls and exopolysaccharides. Abbreviations: GlcDH, glucose dehydrogenase; GlnK, gluconate kinase; GlcK, glucose kinase; Rha, rhamnose; Glc, glucose; and GlcA, glucuronic acid.

located at the midpoint in the culture and one near the bottom. One volume per minute of air was supplied through a sparger below the lower impeller, and the speed of agitation (50–1000 rpm) was controlled automatically to maintain dissolved oxygen (DO) at about 30%. Baffled Erlenmeyer flasks were inoculated with an aliquot of frozen cells and shaken overnight for about 16 h at 30°C and then transferred to the fermentors to give inocula of about 5%. The medium was the same for each stage of growth except that the initial glucose for the seed cultures was 20 g l^{-1}. Residual glucose was determined by the toluidine-binding method and kit from Sigma (catalogue no. 635). Ammonium was assayed with the Spectroquant® kit from EM Science (catalogue no. 14752-2) and nitrate was measured by the cadmium reduction method and kit from LaMotte (catalogue no. 3649-SC).

Construction of Sphingomonas gene library and genetic complementation

Strain S7 was cultured in YM medium by shaking at 30°C to yield encapsulated cells. A sample of this culture (5 ml) was lysed by the SDS-proteinase K-CTAB procedure [35] and the resulting high molecular weight DNA was partially digested with *Sal*I endonuclease and then treated with Klenow DNA polymerase to add dCMP and dTMP to the cohesive ends as described [22]. Plasmid

Table 1 Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype[a]	Source (reference)
Sphingomonas		
S7	Str^r Sps^+	ATCC21423
NW11	Str^r Sps^+	ATCC53272
NW11 B	Str^r Bac^r $SpsB^-$	This work
S88	Str^r Sps^+	ATCC31554
S88m265, m260	Str^r Bac^r $SpsB^-$	[30]
E. coli K-12		
DH5α	F^- ϕ80d*lacZ*$\triangle$M15 *recA1 endA1 gyrA96 thi-1 relA1 supE44 hsdR17*(rK^- mK^+) $^{\triangle}$(lacZYA-argF)U169	Bethesda Research Laboratories
Plasmids		
pRK2013	*ori*(colE1) Kan^r *oriT* Tra^+	[12]
pRK311	*oriV*(RK2) Tet^r *oriT* Tra^- λ*cos lacZ*(α)	[8]
pS7c6	*spsGQIKLFDCEBrhsACB* (S7 DNA in pRK311)	This work
pS7c1	*spsQIKLFDCEBrhsACBD* (S7 DNA in pRK311)	This work
pS7-Brhs	*spsBrhsACBD* (subclone of pS7c1)	This work
pS7-PGM	*pgm*	This work
pS7-BrhsPGM	*spsBrhsACBD pgm* (subclone of pS7c1 and pS7-PGM)	This work
pS7c6PGM	*spsGQIKLFDCEBrhsACB pgm* (composite)	This work
pSEB26	*ori*(colE1) Cam^r Amp^r Kan^r *oriT* Tra^- Mcs	[36]
pS88c3	*spsQIKLJFDCEB rhsACBD* (S88 DNA in pRK311)	[36]

[a]Abbreviations: Str, streptomycin; Kan, kanamycin; Tet, tetracycline; Cam, chloramphenicol; Amp, ampicillin; Bac, bacitracin; Mcs, multiple cloning site; Sps, sphingan polysaccharide synthesis; and Tra, transfer by conjugation.

pRK311 was isolated by the alkaline procedure [5] and digested with *Bam*HI endonuclease, purified by phenol extraction and ethanol precipitation, and then treated with Klenow DNA polymerase to add dGMP and dAMP [22]. Equimolar amounts of plasmid and insert were ligated (T4 DNA ligase), packaged into bacteriophage λ (Gigapack IIXL; Stratagene), and transferred into *Escherichia coli* DH5α. A pooled library representing more than 1000 tetracycline-resistant (Tet^r) colonies was frozen. The Tet^r colonies contained plasmids with inserts of 25 to 30 kbp. DNA was transferred from *E. coli* to *Sphingomonas* by triparental conjugal mating [9]. Mixtures of donor cells containing Mob^+ Tra^- recombinant plasmids, helper cells containing Mob^+ Tra^+ pRK2013 plasmid, and sphingan-polysaccharide-negative (Sps^-) recipient cells in the ratio of about 5:2:10 were spotted onto YM plates with low glucose and incubated for 6–16 h at 30°C. Exconjugants were isolated by spreading the cells onto plates containing streptomycin to select against the donor and helper cells and tetracyline to select for entry of the recombinant pRK311 plasmid. Genetic complementation was observed as a restoration of a mucoid Sps^+ colonial phenotype on YM plates with low glucose or normal YM plates. Individual clones were purified by isolating the DNA from each recipient and transferring the DNA back into *E. coli* by transformation [13].

Qualitative and quantitative analysis of polysaccharides

Exopolysaccharides were separated from the culture medium by precipitation with two to three volumes of isopropyl alcohol (IPA), dried at 80°C, and weighed. IPA precipitates of culture samples taken at 48 h contain less than 20% by dry weight as cells. Cells were separated from the capsular polysaccharides by heating a diluted culture broth in an autoclave at 121°C for several minutes with the pH at about 5.5–6.5 and then centrifuging the cells. For acid hydrolysis 7.5–10 mg of polysaccharide were suspended in 200 μl of 2 M (v/v) trifluoroacetic acid and heated for 4.5 h at 100°C, dried under vacuum, suspended in 100 μl HPLC water, dried again, and then suspended in 200 μl HPLC water. Samples and sugar standards were separated on a CarboPac PA-1 anion-exchange column and the sugar compositions were determined with a Dionex DX500 HPLC system as described [6]. Assays for acetyl [14] and pyruvyl [10] groups have been described. Viscosities were measured at room temperature with a Brookfield LVTDV-II viscometer.

DNA hybridization

DNA was purified as described above but without the CTAB precipitation, digested with restriction endonucleases, electrophoresed through 0.8–1.2% agarose gels in Tris–acetate–EDTA buffer, and then transferred from the gel to Zeta Probe membranes. Hybridization probes were prepared from two overlapping segments of DNA cloned from strain S88 (plasmids pRK311-S88c2 and pRK311-S88c3) and labeled with a Genius® kit (Boehringer Mannheim). The nucleotide coordinates for the probes are from the DNA sequence of S88 (GenBank accession number U51197): *G* 656–1752; *S* 2225–3291; *R* 4480–5246; *Q* 5799–6433; *I* 6693–6900; *K* 7987–8332; *L* 8723–9362; *J* 10212–10927; *F* 11783–12380; *E* 15391–15585; *32* 15949–16429; *26* 17050–17283; *atrD* 18114–18807; *atrB* 20249–20708; *rhsA* 23653–23788;*rhsC* 24446–24648; *rhsB* 24906–25368; *rhsD* 26035–26291; *31* 26945–27496; and *34* 27864–28476 [36].

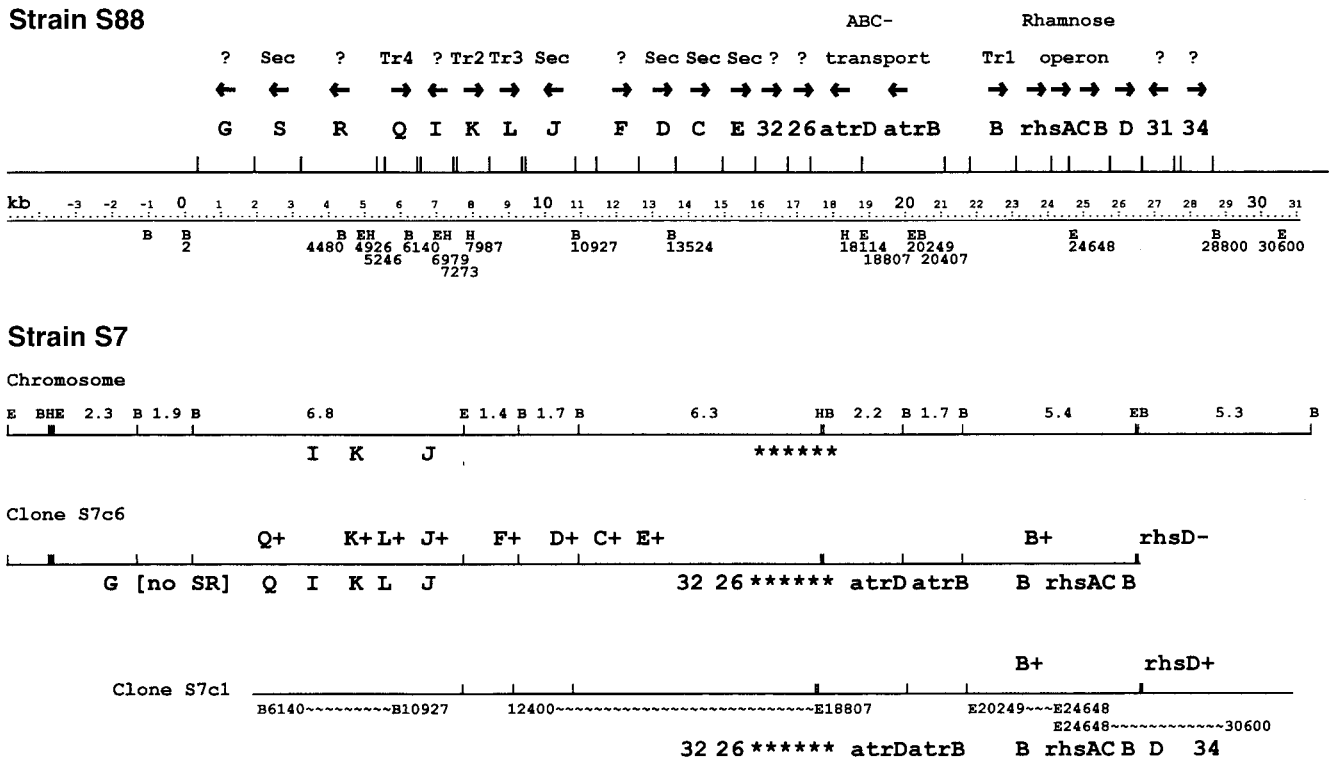


Figure 3 Hybridization and genetic complementation between DNA from strains S7 and S88. Abbreviations: Sec, secretion; Tr, transferase; kb, kilobase; B, *Bam*HI restriction endonuclease; E, *Eco*RI; H, *Hin*dIII; ★★, segment of S7 DNA not found in S88 cluster shown; and B∼∼∼B, restriction site and nucleotide coordinates for multigenic hybridizing fragments.

Assay of phosphoglucomutase (EC 5.4.2.5) activity

Cells were cultured in 1–2 ml of fermentation medium (to an A600 of 1–2), centrifuged, suspended in 100 mM NaCl, centrifuged again, and then equivalent numbers of washed cells were suspended in a solution containing 10 mM Tris–HCl pH 7.6, 5 mM EDTA, 10% glucose, and 0.5 mg/ml egg white lysozyme. After 1 h at 37°C and one cycle of freezing and thawing about 90% of the cells were lysed as observed microscopically. Debris was removed by centrifugation at 4°C in a microfuge and the supernatants were immediately assayed for enzyme activity by mixing reagents directly in a spectrophotometer cuvette in a final volume of 0.2 ml. The phosphoglucomutase assay of Martins and Sá-Correia [24] detects the conversion of glucose-1-P to glucose-6-P by measuring the reduction of NADP by glucose-6-P dehydrogenase in a coupled reaction. Initial reaction rates were calculated from the linear increase in absorbance at 340 nm and then normalized for the total protein concentration in the crude extract, which was measured with a BioRad kit.

Nucleotide sequence accession numbers

The *pgm* gene of strain S7 and the *sps* genes of strain S88 have GenBank accession numbers AF214113 and U51197, respectively.

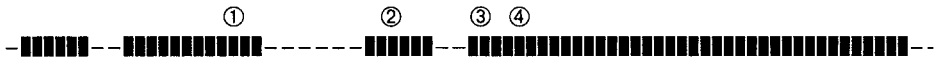
Results

Cloning and analysis of the sphingan-synthesis (sps) genes from Sphingomonas strain S7

A library of cloned segments of chromosomal DNA from strain S7 was prepared in a matable broad-host-range plasmid vector (pRK311) and maintained in *E. coli*. The library was transferred in mass to a sphingan-negative bacitracin-resistant mutant of *Sphingomonas* NW11 (NW11B) by conjugal mating. Strain NW11B was chosen as the recipient in place of strain S7 because the distinction between Sps^+ and Sps^- colonies is easier to detect by eye with strain NW11B and because it yields more exconjugants. Bacitracin-resistant mutants in *Sphingomonas* are usually defective in the *spsB* gene, which codes for a sugar transferase required for synthesizing sphingan polysaccharides [30]. The cloned DNA segments from strain S7 that restored the Sps^+ phenotype to strain NW11B contained the gene for the SpsB transferase in addition to about 20–25 kbp of adjacent DNA.

Similarly, the DNA of a chromosomal cluster of sphingan biosynthetic genes from strain S88 was previously cloned and sequenced, and several genes were identified as coding for specific glycosyl transferases, the dTDP-rhamnose biosynthetic operon, and secretory proteins [31,36].

We used DNA hybridization between labeled probes corresponding to internal segments of the sequenced genes from strain S88 and specific restriction fragments of the newly cloned DNA from strain S7 to construct a physical map of the S7 cluster of genes (Figure 3). The cluster includes genes for four glycosyl transferases (*spsQ*, *K*, *L*, and *B*) and at least four secretion functions analogous to those of strain S88. When the target S7 restriction fragment was large, such as the 6.8 kbp *Bam*HI–*Eco*RI fragment, we ordered the genes within the fragment according to the S88 genetic map. Subcloning and hybridization localized the *QKLJ* region of strain S7 and indicated that the *Q* and *K* genes of S7 and S88 were in the same positions (not shown). The S7 DNA also contained an insertion of 1200–1400 nucleotides in the region of genes *spsE*, *urf32*, *urf26*, and *atrD* which was not found in S88. The insertion in S7 was confirmed by sequencing about 1100 nucleotides (asterisks in Figure 3) and finding two open reading frames, neither of which coded for a recognizable amino acid sequence or function. With the exception of genes S, R, and urf31, all of the clustered genes from strain S88 were located in the S7 DNA and in the same order with no other gaps or insertions.

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                    ①                              ②
S7   Pgm   ...GGIQITGSHNPGNYN.........SYKIGWDAGNGAAGPVIEKL...
S60  Pgm   ...GGIQITGSHNPGNYN.........AYKIGWDAGNGNNGPVIEKL...
Zm   Pmm   ...GGIQITGSHNPAEYN.........EYRIGWDAGNGVGGPVLEKL...
Pa   AlgC  ...SGVMLTGSHNPPDYN.........PMKVVVDCGNGVAGVIAPQL...
Xc   XanA  ...GGIEVTASHNPMDYN.........PLKLVVNAGNGGAGLIVDLL...
Ec   CpsG  ...GGVMVTASHNPMDYN.........PLKLVVNSGNGAAGPVIDAI...
Ec   Pgm   ...DGIVITPSHNPPEDG.....................................
              G...T.SHNP                      GNG..G
             (Active site)

                    ③                              ④
S7   Pgm   ...VDGNFPNHHPDPTE.............LDFGLAFDGDGDRLGA...
S60  Pgm   ...VDGNFPNHHPDPTE.............LDFGLAFDGDGDRLGA...
Zm   Pmm   ...VDGRFPNHHPDPTV.............LDFGFAFDGDADRIGA...
Pa   AlgC  ...VDGNFPNHHPDPGK.............ADLGLAFDGDGDRVGV...
Xc   XanA  ...PDGNFPNGIPNPLL.............ADFGIAWDGDFDRCFF...
Ec   CpsG  ...PDGNFPNGIPNPLL.............ADMGIAFDGDFDRCFL...
Ec   Pgm   ...............................FDLAFANDPDYDRHGI...
                                              D.D.D
                                           (Active site)
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Figure 4 Fingerprint of conserved amino acids in the phosphoglucomutase family. Abbreviations: Zm, *Zymomonas mobilis*; Pa, *Pseudomonas aeruginosa*; Xc, *X. campestris*; Ec, *E. coli*. Key: ▮▮, about 60% conserved; – –, about 25% conserved.

The physical hybridization map was confirmed for a subset of the genes by in vivo genetic complementation of specific mutations in strain S88 with cloned segments of S7 DNA. The mutated S88 genes that were complemented by the S7 segments are labeled with a "+" adjacent to each gene in Figure 3. The unmarked genes were untested due to a lack of S88 mutants. Sugar compositions of the capsular polysaccharides made by the complemented mutants were similar to the wild-type S88 host, containing rhamnose, glucose, mannose and glucuronic acid in amounts typical of strain S88 (not shown). This contrasts with sphingan from strain S7 which not only lacks mannose but also has a rare 2-deoxy-glucuronic acid which is completely destroyed during hot acid hydrolysis. Complementation of the SpsK-defective S88 mutant by the S7 cloned DNA might indicate that the S7 SpsK activity is able to recognize either of two similar substrates, GlcA or 2-deoxy-GlcA. However other formal possibilities cannot be ruled out at this time.

Cloning and analysis of the phosphoglucomutase (pgm) gene from Sphingomonas strain S7

The library of S7 genes was also transferred into a mutant of *E. coli* [1] deficient in phosphoglucomutase. The mutation disrupts the normal metabolism of maltose and galactose and results in intracellular accumulation of amylose which can be detected by staining colonies black with iodine. Some white recombinant colonies with a Pgm^+ phenotype were obtained after iodine staining, and the *pgm* gene from strain S7 was subcloned to obtain the DNA sequence (GenBank accession number AF214113). A likely open reading frame coding for 462 amino acids was identified based on a skewed pattern of G and C nucleotides in the three codon positions typical of *Sphingomonas* genes. The overall G+C composition for the presumptive *pgm* gene was 66%, with 69% in the first position of the codon, 42% in the second, and 88% in the third. The sequence preceding the open reading frame contained a likely ribosome-binding site and ATG start codon for translation. The codon preference for the *pgm* gene of strain S7 matched the pattern identified earlier for the approximately 23 *sps* genes of strain S88 [36].

A comparison of the inferred amino acid sequence of this open reading frame to known Pgm proteins indicated that the gene cloned from strain S7 also coded for Pgm [2]. The regions of sequence similarity covered nearly the entire length of the protein. Among these known proteins the greatest match for identical amino acids at each position (98.5%) was with the phosphoglucomutase of *Sphingomonas* strain ATCC31461 [34]. The latter bacterium is closely related to strain S7 and secretes gellan gum. The amino acid sequences and locations of four peptide segments which are useful to identify Pgm proteins (eMotif Search, Bioinformatics Group, Stanford University) are shown in Figure 4. The first and fourth segments contain the amino acids (G...T.SHNP and D.D.D, respectively) believed to comprise a highly conserved site residing in a deep crevice of the enzyme which is involved in phosphate transfer [7].

Pgm activity was measured in crude extracts derived from a *Sphingomonas* strain carrying multiple copies of the cloned *pgm* gene, and compared to the parent strain with its single chromosomal copy. The copy number for plasmid RK2 and its derivatives, including the cloning vector pRK311, is about five. Two assays using separately grown and lysed cells showed that the recombinant with multiple copies of the *pgm* gene had six- and sevenfold more Pgm activity per microgram of total protein than the parent strain S7.

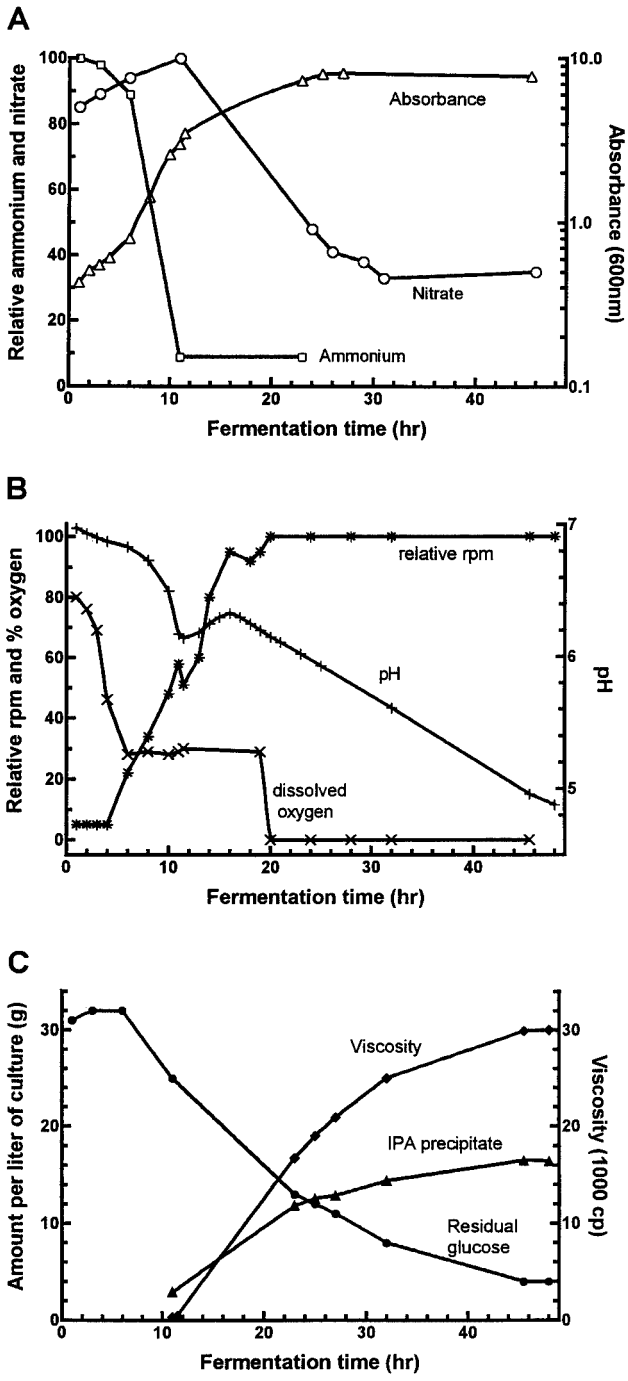

Figure 5 Time course of fermentation for *Sphingomonas* S7 with plasmid pS7c6. Panel A: a relative ammonium of 100 equals 225 mg NH_4/l and a relative nitrate of 100 equals 775 mg NO_3/l. Panel B: relative rpm of 100 is 1000 rpm and a relative % oxygen of 100 is the amount of oxygen dissolved in water at 30°C after saturation with air. Panel C: viscosity was measured at a shear rate of 2.508 s^{-1}.

Fermentation of recombinant microorganisms

Optimal conditions for culturing *Sphingomonas* S7 were determined in shake flasks and preliminary submerged stirred-vessel fermenta-

Table 2 Eps production by fermentation of wild type and recombinant S7 strains

Strain:plasmid	Broth at 48 h			After IPA precipitation of broth			
	A600	Viscosity[a]	Glc[b]	Eps[b]	% Yield[c]	Glc:Rha	Viscosity[d]
S7 wild type	10.9	22 250	0	16.6	52	5.55	–
	10.2	22 700	0	16.4	51	5.36	2770
S7:PGM	11.5	19 500	3	15.5	53	5.20	–
	11.2	22 200	3	16.4	57	5.27	2860
S7:Brhs	9.2	18 400	5	15.2	56	5.61	2270
	9.4	22 400	4	16.7	60	5.44	–
S7:BrhsPGM	9.1	21 500	4	16.2	58	5.01	2500
	9.2	25 900	0	18.0	56	4.83	–
S7:S7c6	7.8	30 000	4	16.4	59	4.22	3810
	7.8	31 200	1.5	17.9	59	4.18	–
	9.2	34 000	0	19.4	61		
S7:S7c6PGM	8.7	33 100	3	17.2	59	4.54	3410
	9.4	32 000	4.7	16.9	62	5.09	3020
S7:S7c6 (42 l)	8.7	37 700	0	18.8	61	–	–

[a]Centipoise for spindle 4 at 12 rpm.
[b]Grams per liter.
[c](g Eps produced/l) / (g Glc consumed/l) ×100%; initial Glc was 32 g/l.
[d]Centipoise for spindle 4 at 60 rpm for 1.0% (w/v) Eps in 0.1 M KCl.

tions at the 4-l scale by varying agitation, aeration, temperature, pH, and the types and concentrations of medium components. Then the parent and each of the genetically modified strains were fermented using the same procedure, and measurements were made of the absorbance at 600 nm, pH, amount of base added, air flow, speed of agitation, % DO, temperature, residual glucose concentration, residual ammonium and nitrate concentrations, viscosity, and the amount of alcohol-precipitable polysaccharide. The time course of changes in these parameters shows a reproducible pattern for the recombinant strain S7:S7c6 (Figure 5). A notable feature was the near coincidence at around 9 h after inoculation between the interim pH minimum of about 6–6.1, the depletion of ammonium, the end of exponential culture growth, and the onset of viscous polymer production. Nitrate consumption began after the ammonium was depleted and coincided with a temporary rise in pH which was then followed by a steady decline. The DO-controlled agitation usually reached a maximum of 1000 rpm as the indicated DO dropped to 0%. This was followed by a period of slower polysaccharide accumulation until the glucose was exhausted.

A summary of the results of duplicate fermentations is given in Table 2. A yield from glucose of 52% was observed for the wild-type strain S7 with complete exhaustion of the substrate within 48 h. A small but reproducible increase in yield to 55% was observed when additional copies of the *pgm* gene on a multicopy plasmid were added, and about one-half of this increase was due to the increase in cell density in the cultures. Multiple copies of the entire S7c6 cluster of genes or the *spsB-rhsACBD* subset increased the yield from glucose to higher levels, to 60% for S7c6 and 58% for *spsB-rhsACBD*. The *spsB* gene codes for the initial transferase activity, and transfers glucose-1-P from UDP-glucose to the isoprenoid carrier. The *rhs* genes code for the synthesis of dTDP-rhamnose. Contrary to the results with the additional *pgm* genes, these cultures accumulated proportionately fewer cells. We also added multiple *pgm* genes to the multiple *sps* genes, but the yield was not increased over the S7c6 and Brhs genes alone. As observed before for the multiple *pgm* genes alone, the combinations resulted in increased cell densities.

A more interesting and unexpected result of introducing multiple copies of the entire *sps* cluster (S7c6) was the approximately 40% (on average) increase in viscosity of the culture broths and of the alcohol-precipitated polysaccharides. The viscosity of the culture broths reached more than 30,000 cP (spindle 4 at 12 rpm). By contrast, the amount of polysaccharide (on average) increased by only about 10%. This increased viscosity was associated with a decrease in the ratio of glucose to rhamnose compared to the parental S7 polymer.

We suspected that the large increase in viscosity for the recombinants limited the availability of oxygen, which at least partly depends on the degree of agitation. Therefore the fermentation of S7:S7c6 was also carried out at a larger scale (42 l) under conditions that were similar but not identical to those for the smaller cultures. The larger equipment allowed better mixing of the viscous broth, especially at viscosities exceeding 20,000 cP. The inoculum course included seeding a shake flask with frozen cells, inoculating a 4-l fermentor at the 5% level, growth for 17 h, and then transfer of 2 l of culture to the larger fermentor. With the recombinant strain S7:S7c6 the culture broth had a viscosity of 37,700 cP, and the glucose was exhausted within 36 h. For comparison, a 4-l fermentation was seeded with a portion of the same inoculum that went into the 42-l fermentor and at the smaller scale a viscosity of 31,100 cP was attained after 48 h.

Discussion

Part of the reason for the commercial success of xanthan gum produced from the bacterium *X. campestris* is a relatively high

conversion efficiency, between 60% and 70% in a large scale fermentation. By comparison, yields of sphingan polysaccharides from glucose for strains of *Sphingomonas*, including strain S7 reported here, are only about 50% or less. We know of two attempts to increase the conversion efficiency for *Sphingomonas* by genetic means. In the first case, random mutagenesis was used to stop the synthesis of poly-β-hydroxybutyrate (PHB) by *Sphingomonas* S60, which is fermented commercially to produce the polysaccharide gellan [3]. Although the competing PHB synthesis was eliminated, the available carbon source was still not used efficiently for gellan synthesis [33]. In the second attempt, site-specific mutagenesis was used to inactivate the *zwf* gene, which codes for glucose-6-P dehydrogenase, in an attempt to divert the flow of carbon toward the gellan synthetic pathway. However, after rigorously cloning the *zwf* gene and using it to create a site-specific chromosomal Zwf^- mutation, the parent and mutant strains showed the same (41% and 42%) conversion of glucose to gellan, and CO_2 evolution was not changed [33]. It appeared that either the alternative GlnK route was dominant over the Zwf route or it was able to compensate for the loss of Zwf activity.

Using *Sphingomonas* S7 as a model sphingan producer, we also tried to manipulate the flow of carbon toward the synthesis of polysaccharides by augmenting the normal cellular phosphoglucomutase activity with additional *pgm* genes. However, we did not observe a large increase in yield, if any, and there was a simultaneous increase in cell numbers in the cultures. The increased cell growth probably limited the amount of glucose that could be converted into the S7 polymer, since the synthesis of cell wall polysaccharides would also depend on the availability of sugar nucleotide precursors such as UDP-glucosamine. In addition, we augmented the chromosomal genes specifically involved in sphingan biosynthesis with genes from the cloned S7c6 cluster including genes for the four glycosyl transferases required for assembling the tetrameric repeat unit of the main chain, the dTDP-rhamnose operon, several secretion functions, and some other essential but uncharacterized genes. We compared augmentation of the entire cluster with just the *spsB-rhsACBD* genes and found that the entire S7c6 cluster increased the yields to 59–61% while the smaller subset increased yields to about 56–60%. Using a larger fermentor and more effective aeration the conversion for the S7:S7c6 recombinant remained 61% but was complete in only 36 h. The conversion efficiency appears to be an inherent property of the strain and not simply due to limited availability of oxygen when the cultures become highly viscous.

What is a reasonable conversion efficiency for making the S7 polysaccharide? If we compare the yields for S7 and xanthan gum, we must account for the different oxidation states of the carbohydrates in the two polysaccharides. The partial oxidation of UDP-glucose to UDP-glucuronic acid for inclusion in each pentasaccharide repeat unit of xanthan was calculated to provide about 25% of the energy consumed in synthesizing the polymer [20]. Strain S7 polymer also contains uronic acids [21], however, in the partially reduced form of 2-deoxy-glucuronic acid (Pollock, unpublished). Assuming that the reduction coincides with the oxidization of a single NADPH, the energetic benefit from the uronic acid should be lost and synthesis of S7 polymer should require consumption of an additional glucose per hexasaccharide repeat unit. Therefore, compared to a yield of 70% for xanthan, a yield much greater than about 60% for S7 might not be possible.

Does the capsular form of the polysaccharides in *Sphingomonas* limit productivity? It is unlikely that the viscosity of the capsule limits its own productivity, since the viscosity per gram of the S7 polymer exceeds that for xanthan by about twofold and yet the yield for S7 can be as high as 60%, compared to 60–70% for xanthan gum. This is consistent with our measurements (not shown) of nearly identical rates and extents of growth in shaking flasks in medium rich in glucose for Sps+ and Sps− S7 bacteria, as well as for Eps+ and Eps− *X. campestris*. Although capsule viscosity does not appear to form an appreciable obstacle to uptake of carbon and nitrogen substrates nor to limit synthesis and secretion of polymer, it is possible that there might be a limit to how much polymer can be attached to surface components on the cell.

A viscosity increase was previously observed for the "polytrimer" form of xanthan gum produced by a genetically modified strain of *X. campestris* [4]. This polytrimer is missing two of the three side-chain sugars attached to the cellobiose main chain of the pentasaccharide repeat unit of xanthan. The absence of a complete side chain is associated with a greatly reduced yield of xanthan from *X. campestris*. The clustered S7 genes cloned here appear to code only for the synthesis of the main chain of the repeat unit. The glycosyl-transferases responsible for attaching the two side-chain glucose residues are probably located at a different chromosomal locus. Assuming that this is correct, then the additional copies of the main-chain genes on the S7c6 segment in the absence of additional copies of the side-chain genes might lead to a polymer lacking some of the side chains. Although a direct structural analysis is needed, the decreased ratio of glucose to rhamnose observed here for the polymers made by the strains carrying S7c6 plasmids is consistent with the possibility of missing side chains.

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