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Planktonic/sessile dimorphism of polysaccharide-encapsulated sphingomonads

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Sphingomonads have acquired diverse metabolic activities to inhabit a wide range of environments. Several strains of *Sphingomonas* display phenotypic dimorphism and can adopt either a planktonic or sessile behavior in liquid media. The sessile state is marked by the presence of a viscous exopolysaccharide capsule. Specific types of these capsular polysaccharides are harvested from large-scale fermentations for use as rheology modifiers in many industrial and food applications. Sensing of environmental stimuli and genetic control over synthesis of the capsule are key events in alternating between these two phenotypes.

Keywords: exopolysaccharide; capsule; motility; dimorphism

Introduction

Many bacteria can adopt either a planktonic or sessile existence depending on environmental conditions. A biofilm is a sessile community of cells embedded in a matrix of exopolysaccharide (EPS) which adheres to a substrate. In a well-studied case the formation of a biofilm by *Pseudomonas aeruginosa* is reversible, allowing it to descend progressively to the lower respiratory tracts in lungs of infected patients [4]. This bacterium secretes alginate which contributes to anchoring the cells to the lung, and at other times secretes an alginate-degrading enzyme which allows the cells to detach in order to move and seek further advantage. The actual environmental signals that are sensed by *P. aeruginosa* and lead to the production of alginate are not known, but may include high osmolarity, nutritional deprivation, physical contact, or communication between cells. In particular, quorum-sensing is a key determinant in the development of this sessile community [5].

Motility and chemotaxis allow planktonic bacteria to survive when local environments become deficient in essential nutrients or oxygen. Diverse bacteria have chemically specific mechanisms to sense and respond to each type of deprivation [1]. *Ralstonia solanacearum* and *Xanthomonas campestris* typify these adaptive responses among phytopathogens. Each secretes an EPS which plays a significant role in pathogenesis, but which is usually not needed for survival in laboratory cultures. In the laboratory, EPS synthesis is generally favored when carbon is in excess supply relative to nitrogen. For *R. solanacearum* the destructive cause of wilt, Kelman and Hruschka [12] observed a conditional phenotypic shift from Eps^-/virulent/non-motile (Mot^-) cells to the opposite of each characteristic. These pleiotropic changes arise in still or non-agitated cultures, and the resulting bacteria become aerotaxic. More recent work has shown the existence in this bacterium of a complex regulatory network including at least two sensor-regulator systems which respond to unknown signals and which activate the production of EPS. Similarly, for *X. campestris*, pleiotropic conversion of Mot^- Che^- (nonchemotactic) wild-type bacteria to swarming Mot^+ Che^+ progeny is also associated with the loss of synthesis of an EPS, xanthan gum [11]. The phenotypic switch is reversible *in planta*, but appears stable in laboratory culture.

Like their pathogenic distant relatives, the sphingomonads also display a phenotypic dimorphism between motile and sessile behavior which is closely associated with the synthesis and secretion of EPS. The cultural dimorphism, the role for EPS, and the mechanism and control of EPS synthesis in strains of *Sphingomonas* are discussed and reviewed in this work.

Exopolysaccharides secreted by strains of *Sphingomonas*

Numerous bacteria have been screened for the production of viscous polysaccharides. Some of the most useful polymers were not only structurally related, but also produced by similar appearing yellow-pigmented, nonfermentative, Gram-negative, rod-shaped bacteria [15,16]. These include EPS S-60 (gellan), S-130 (welan), S-198, S-88, S-657, S-194, NW-11, and S-7. A re-examination of the biochemical and physiological characteristics of the corresponding strains, which were initially assigned to diverse genera, indicated that they each belonged to the genus *Sphingomonas* [17]. The main criteria of the re-examination included metabolic fingerprinting with the Microlog 1 system (Biolog, Inc, Hayward, CA, USA), fatty acid profiles, and pigment spectroscopy. The similar but not identical capsular polysaccharides were named 'sphingans' after the genus. The repeat structure for each sphingan is shown in Figure 1. The backbone contains a common trisaccharide: (D)glucose-(D)glucuronic acid-(D)glucose. The fourth position of the backbone is occupied by either L-rhamnose or L-mannose. Variability is also found in the side chains. The L-mannose is noteworthy due its rarity in nature, and because its synthetic origin remains unknown.

Gellan, welan, and rhamsan were discovered by the

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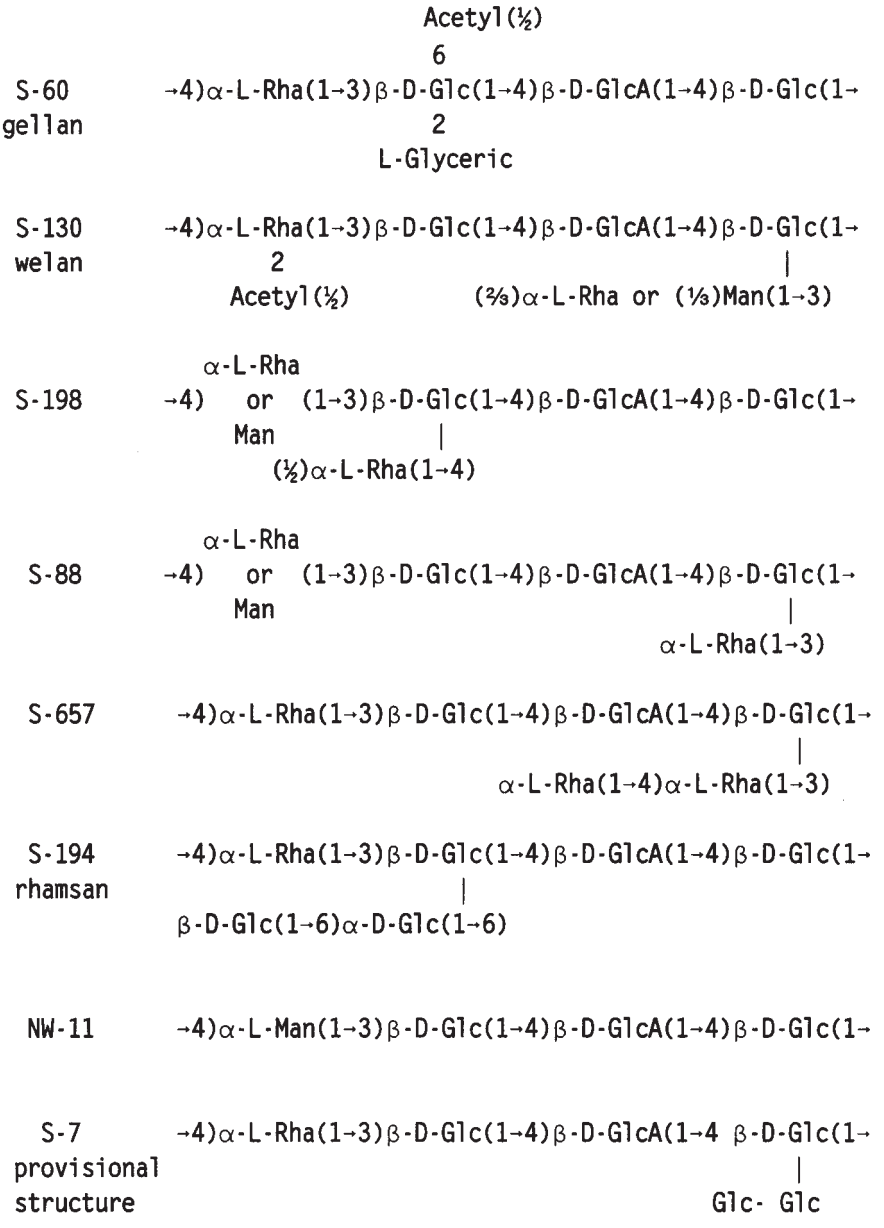

Figure 1 Repeating subunit structures for sphingan polysaccharides. Abbreviations (all pyranosides): Rha, rhamnose; Glc, glucose; GlcA, glucuronic acid; and Man, mannose. For S-130, S-198 and S-88 one position in each subunit has either rhamnose or mannose, and the relative occupancy fraction is indicated. Before polymerization the subunits are attached through a phosphodiester linkage to the lipid carrier C_{55}-isoprenylphosphate at the reducing end (right). The S-7 EPS structure is provisional and not based on a chemical sequence analysis. It was deduced by combining our understanding of the sugar composition of the capsule with the physiological, structural, and genetic relatedness of strain S7 to the other sphingan producers, and by recognizing that its properties are distinct from its closest compositional relative, rhamsan [14,17].

Kelco division of Merck (San Diego, CA, USA) and are now produced by large-scale fermentation by the Nutrasweet/Kelco unit of Monsanto. They are valuable for controlling the viscosity of aqueous solutions in many food and industrial applications. Their specific structural variations confer unique rheological properties. For example, gellan forms a gel in the presence of divalent cations, welan is a heat-stable suspending agent, rhamsan is a salt-tolerant suspending agent, and S-198 is particularly stable to shear [15]. The prospects for isolating other members of this valuable group of aquatic sphingomonads are promising since they are readily isolated by selective growth in M9 minimal salts medium containing arabinose as a carbon source and streptomycin (TJ Pollock, Shin-Etsu Bio, unpublished).

A few other EPS producers are recognized as sphingomonads. *S. paucimobilis* strain I-886 produces an EPS similar to rhamsan [7], but with glucuronic acid replaced by the uncommon 2-deoxy-*β*-D-*arabino*-HexpA (2-deoxyglucuronic acid). A second strain, *S. paucimobilis* GS1, secretes a viscous anionic heteropolysaccharide comprised of the sugars glucose, galacturonic acid and glucuronic acid, and which when deacetylated has gelling properties similar to gellan gum [2]. A third encapsulated strain, *S.*

capsulatus, was originally attributed to this genus in 1962, but was later reported to have lost its capsule in the intervening years [28]. We examined *S. capsulatus* ATCC 14666 and found that it did form mucoid colonies and produced a capsule. We then recovered the polysaccharide from a liquid culture by precipitation with isopropyl alcohol, hydrolyzed the precipitate with trifluoroacetic acid, and separated the monosaccharides by chromatography. The major sugars included glucose, mannose and galactose, but not rhamnose. The presence of glucuronic acid in this EPS is uncertain because of our poor recovery of that monosaccharide after hydrolysis. Although the *S. capsulatus* EPS was shear thinning, the viscosity was low compared to the sphingans. These results indicate that the *S. capsulatus* capsule probably has a different basic repeat structure. In fact, *S. capsulatus* may properly belong to a different genus based on sequence analysis of rRNA [25].

Most of the polysaccharides isolated during the extensive industrial screening programs were not subjected to a complete structural determination, presumably because their properties were not superior to previous isolates. In addition the taxonomic descriptions of the producing strains are either incomplete or are not public.

Mechanism of assembly and genes required for sphingan synthesis

Sphingan assembly follows the mechanisms established for other acidic heteropolysaccharides of Gram-negative bacteria, such as *X. campestris* [10], and *Sinorhizobium meliloti* [20]. The synthetic pathway can be considered in three parts: first, uptake of simple sugars and conversion to nucleotidyl derivatives; then assembly of oligosaccharide repeat subunits attached to a C_{55}-isoprenyl pyrophosphate carrier (PPI); and finally polymerization of the repeat subunits and secretion of the polysaccharide [23].

Certain aspects of the biosynthesis of sphingans, especially gellan, have been studied experimentally because of their commercial value. During fermentation of *S. elodea*, the yield of gellan gum from glucose is about 50% since nearly half of the carbon source is diverted to CO_2. The desirability of improving the yield of gellan stimulated an effort to understand the catabolism of glucose in *S. elodea* and to find a way to reduce CO_2 evolution [26]. Measurement of the relevant enzyme activities suggested that glucokinase and glucose dehydrogenase were the likely routes for initial metabolism of glucose. In addition, cell-free extracts had considerable glucose-6P-dehydrogenase activity (Zwf), which could feed glucose-6P to the pentose phosphate shunt and the tricarboxylic acid cycle. Since enzymes for both of these pathways were detected and would be sources for CO_2, a gene-specific null mutation (Zwf^-) was constructed to limit the diversion of carbon from EPS synthesis. Despite the ingenuity of this approach and execution, the mutant bacterium matched the parent in evolution of CO_2 and failed to channel more carbon to gellan production. It was suggested that an alternative route using glucose dehydrogenase and gluconate kinase might have bypassed the Zwf^- defect [26].

All of the enzymes expected to be involved in the conversion of fructose-6P or glucose-6P to the precursors UDP-glucose, UDP-glucuronic acid, and dTDP-rhamnose were also detected in cell-free extracts: phosphoglucoisomerase, phosphoglucomutase, UDP-glucose pyrophosphorylase, UDP-glucose dehydrogenase, dTDP-glucose pyrophosphorylase, and dTDP-rhamnose synthetase [13]. The activities of the two pyrophosphorylases were elevated when the cells were cultured in medium that favors gellan synthesis (high in carbohydrates, low in amino acids) and reduced for mutants unable to accumulate gellan [13].

The assembly of a sphingan repeat unit begins with the transfer of glucose-1-P from UDP-glucose to isoprenyl phosphate (IP). Availability of the IP substrate is reduced by the antibiotic bacitracin, which selectively binds to and sequesters isoprenyl pyrophosphate, a precursor to IP [21]. We previously showed that among bacitracin-resistant mutants of both *Sphingomonas* strain S88 and *X. campestris*, the majority lost their ability to produce nonessential EPS, as if the mutants survived by committing all of the remaining IP carrier to the synthesis of the essential peptidoglycans of the cell wall [18]. We used the bacitracin-resistant Eps^- mutants as conjugal recipients for cloned segments of bacterial chromosomes and isolated the genes which coded for the corresponding glucosyl transferases: *spsB* from *Sphingomonas* S88 and *gumD* from *X. campestris*. As is the case for *gumD* of *X. campestris* [3,8,24], the *spsB* gene is embedded within a cluster of genes coding for EPS synthesis [29]. A diagram of the multi-gene *sps* (*s*phingan *p*olysaccharide) cluster for strain S88 is shown in Figure 2. It includes four genes coding for the synthesis of one of the precursor nucleotide sugars (dTDP-rhamnose), and several glycosyl transferases and secretion functions. However, not all of the essential sphingan biosynthetic genes are located in this cluster. According to the complete DNA sequence of this cluster, the genes coding for the synthesis of UDP-glucose and UDP-glucu-

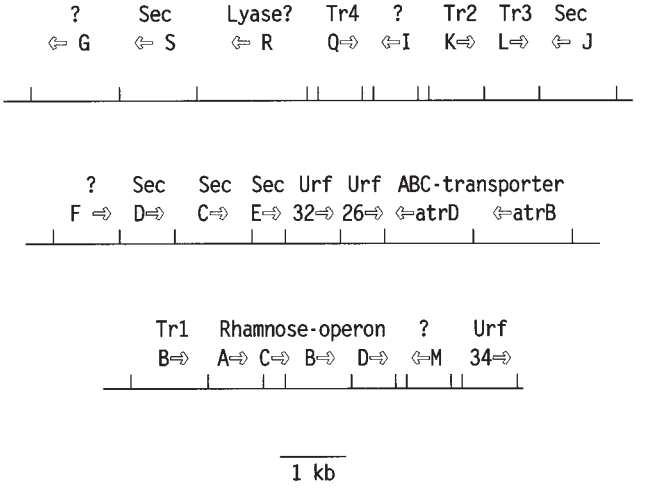

Figure 2 Gene cluster for sphingan S-88 synthesis. The map spanning about 30 kbp is divided into three contiguous segments with the intergenic spaces indicated to scale and the direction of transcription shown by the open arrows. From left to right the *sps* genes are labeled G, S, R, Q, I, K, L, J, F, D, C, E, B and M. The other interspersed genes include the rhamnose operon *rhsACBD*, two components of an ABC-transporter *atrDB*, and three unidentified reading frames urf32, 26 and 34. Presumed functions (abbreviated) are indicated above the genes: Sec, secretion: Tr1, 2, 3 or 4, sugar transferases in the order of assembly.

ronic acid must reside elsewhere in the chromosome, as previously observed for *X. campestris* [9]. Analogous segments from the chromosomes of the other sphingan-producing bacteria have similar but not identical organizations (L Thorne *et al*, Shin-Etsu Bio, in preparation).

To further characterize the sugar transferases of *Sphingomonas*, we determined their substrate recognition properties *in vivo* by transferring cloned genes between the related strains of *Sphingomonas* and also to *S. leguminosarum* and *X. campestris* for which the order of assembly of the monosaccharides was already known. The repeat subunit for the backbone portion of the EPS of *S. leguminosarum* is αDglc(1→4) βDglcA(1→4) βDglc(1→4) βDglc-PPI, and is assembled on the carrier IP beginning at the reducing end and progressing toward the left. The sinorhizobial EPS also has a tetrasaccharide side-chain attached to the glucose residue at the non-reducing end. For *X. campestris* the backbone is βDglc(1→4) βDglc-PPI, with a trisaccharide side-chain attached similarly. Therefore, a small portion of the repeat subunit structures from these two genera are identical to that of the sphingans. We expected that enzymes with identical substrate specificities would substitute for one another, and they did. The results of the intra- and inter-generic reciprocal complementation experiments indicated that SpsK transferred glucuronic acid to glc-PPI and SpsL transferred glucose to glcA-glc-PPI [19]. From these sugar specificities it was then possible to deduce the order of assembly resulting in the intermediate sphingan structure glc-glcA-glc-PPI.

The *in vivo* genetic complementation experiments also revealed an interesting negative phenomenon. When transferred into *Sphingomonas*, certain foreign glycosyl transferase genes which were expected to attach an incorrect sugar to the glc-PPI intermediate in *Sphingomonas* caused inhibition of EPS synthesis or interfered with cell growth [19]. For example the GumM gene product of *X. campestris* normally attaches glucose to glc-PPI, while either SpsK of *Sphingomonas* S88 or PssDE of *S. leguminosarum* attach glucuronic acid to glc-PPI. The foreign GumM glucosyl transferase was deleterious when placed in either *Sphingomonas* S88 or *S. leguminosarum*. Similarly, SpsK and PssDE were deleterious in *X. campestris*. It appears that the unnatural carrier-linked oligosaccharide intermediates are toxic to *Sphingomonas*, perhaps because they are not degraded effectively and accumulate.

Motile/sessile dimorphism

Sphingomonads are generally capable of motility and have a single polar flagellum [28]. However, sphingan-producers (Sps^+), of which strain S88 is typical, grow in aerated liquid medium containing a carbohydrate carbon source as large multicellular sheets containing thousands of cells. The formation of aggregates requires the synthesis of an exopolysaccharide capsule and the cells within the sheets are separated from one another by at least a half-cell width, presumably due to the capsules. These aggregates are reminiscent of a biofilm after detachment from a substrate. Swimming free of the sheets are some planktonic motile cells, usually comprising less than 5% of the total. Thus, under these growth conditions, the wild-type strains are Sps^+, form aggregates, and exhibit both motile (5%) and nonmotile (95%) behavior. However, at this time we do not know if the minority motile cells remain encapsulated with polymer, or if removal of the capsule must precede movement. As discussed below, motility and aggregation appear to be alternative behaviors controlled by environmental signals rather than by genetic mutation.

The relation between motile or sessile behavior and capsule formation is complex and not yet well understood. Nonmotile cells are not simply the consequence of a capsule being present, since it is possible to prevent capsule synthesis and retain nonmotility. We have isolated several mutant derivatives of strain S88 which are unable to synthesize sphingan due to the loss of a specific biosynthetic enzyme activity. For example, SpsB260 and SpsB265 are both defective in the glucosyl transferase required for attaching glucose-1P to the carrier IP, the initial step in assembly of the repeat subunit. Both mutants are largely nonmotile and indistinguishable from wild-type in this regard, but they do not aggregate. The few motile cells in the SpsB260 and SpsB265 cultures are not simply due to the presence of spontaneous Sps^+ revertants, since the frequency of such mutations in *Sphingomonas* is less than 10^{-4}. Likewise, the presence of some (5%) motile cells in a wild-type culture is not simply due to Sps^- mutants since a comparable mutation frequency applies.

The absence of a sphingan capsule is probably not sufficient to cause motility, since Sps^- mutants such as SpsB265 are largely nonmotile. Additional factors are necessary for motility, especially synthesis, maintenance and operation of the flagellum. Nevertheless, we suspect that *Sphingomonas* must shed a large fraction of its capsule in order to move, but we do not know this for certain.

We can cause the disaggregation of *Sphingomonas* cells under specific conditions. Aggregation is affected by the concentration of salt in the medium. In liquid cultures sheets are larger in size with fewer freely motile cells when grown in a high-salt medium compared to a low-salt medium. Similarly, when adherent Sps^+ cells are scraped from agar plates and suspended in liquid containing less than 5–10 mM NaCl, the cells disaggregate completely within 1–2 h. Conversely, transfer of cells from a low-salt to a high-salt medium appears to instantly inhibit motility for strain S88.

The level of oxygen in shake-flask cultures also affects aggregation of *Sphingomonas*. Strain S88 was cultured in four separate 125-ml flasks, containing 60 ml of YM medium, and then the exponentially dividing cultures were pooled and redistributed into identical shake-flasks, such that a shift up or down in oxygen availability was obtained based on the volumes of the original culture added to each flask. Standard YM medium contains 3 g yeast extract, 3 g malt extract, 5 g peptone, and 10 g glucose per liter of water. A shift down in oxygen, from an increase in the culture volume from 60 ml to 80–130 ml, caused the rafts to disintegrate within 90 min, a period less than the cell doubling time. After this short interval in the low-oxygen medium, so many cells were motile that they could not be the progeny of pre-existing motile cells. A phenotypic shift had occurred. These motile *Sphingomonas* cells are probably also capable of aerotaxis since on occasion they have

been observed to actively concentrate around air bubbles trapped under the cover slip on a microscope slide.

Aggregation and motility appear to be two alternate phenotypes for wild-type *Sphingomonas*, with control over expression coordinated in some way. Evidence for coordinate control over motility and capsule synthesis comes from a unique class of *Sphingomonas* mutants that arose when we incubated wild-type cultures of strain S88 without agitation at 30°C for several days. As in the 'still' cultures of *R. solanacearum* [12], our *Sphingomonas* mutants also do not synthesize polysaccharides (Sps^-). In addition these mutants are fully motile (Mot^+). This contrasts with other Sps^- mutants like SpsB265 which is defective in a glucosyl transferase and which is nonmotile. We reasoned that the combination of low oxygen availability and sphingan biosynthesis from glucose might be incompatible or deleterious in *Sphingomonas*, and might bring about the selection pressure favoring the growth of the Sps^- Mot^+ mutants. If this is true, then elimination of sphingan biosynthesis by removal of glucose from the medium should prevent appearance of the Sps^- Mot^+ mutants. As a test, an aerated culture of S88 was grown overnight in standard YM medium (with 1% glucose) and then a sample was inoculated into full test tubes containing YM medium lacking added glucose, and incubated for several days without shaking at 30°C. (The final sugar concentration from the malt extract in YM is 0.2%.) A film of aggregated bacteria formed at the liquid-air interface and the upper one-third of the liquid was also turbid. The cultures were then mixed and plated for single colonies onto agar plates containing YM medium (with 1% glucose). Colonial phenotypes were distinguished by eye: Sps^+ colonies are elevated, opaque and rubbery, while Sps^- colonies are flat, translucent and watery. The frequency of Sps^- Mot^+ colonies decreased by over 100-fold in the absence of glucose. Sphingan synthesis, driven by high levels of glucose, combined with the low oxygen levels appears to select for Sps^- Mot^+ mutants.

Genes involved in control of capsule synthesis and motility

A large segment of the *Sphingomonas* chromosome was selected from a cosmid library of clones based on its ability to complement the Sps^- Mot^+ mutants described above (Yamazaki *et al*, in preparation). A preliminary description of the cloned segment which restores the wild-type phenotype is given here. The complementing region was localized by restriction mapping, inactivation with transposons, and subcloning. When the complementing DNA was sequenced, we identified two tightly linked open reading frames. We named these genes *spsAZ*, and amino acid sequences were deduced from the DNA sequences. The SpsA and Z gene products are similar to the proteins comprising two-component regulatory systems of other bacteria. In a typical sensor-regulator system the first protein is a histidine kinase and senses an environmental or intracellular stimulus, while the second protein becomes phosphorylated by the kinase and modulates transcription of a target gene or set of genes. The SpsA protein is most similar to VirA of *Agrobacterium* [27], FixL of *Sinorhizobium* [6], and RcsC of *Escherichia* [22]. EPS synthesis is also among the metabolic properties controlled by each of these related regulatory genes.

Conclusion

The encapsulated sphingomonads described in this report display a conditional dimorphism between planktonic and sessile behavior when cultured in the laboratory. A closely associated component of this dimorphism is the synthesis of a capsular polysaccharide. We have identified genes that appear to be involved in regulating the synthesis of the sphingan polysaccharides and which probably sense an environmental or cellular signal before activating EPS synthesis. This general scheme is supported by a simple comparison of the SpsAZ proteins to the amino acid sequences of sensor-regulators identified in other bacteria. However, at this time we do not know the nature of the signal, nor which *sps* genes are the primary targets for regulation. We are now investigating these questions, and paying particular attention to the possibility that oxygen availability or cell number might be the critical extracellular signal for controlling the planktonic/sessile dimorphism.

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