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Molecular Genetics of Extracellular Polysaccharide Biosynthesis in Vascular Phytopathogenic Bacteria

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Production of polysaccharides outside of the cell wall is common in many genera of bacteria. These extracellular polysaccharides (EPSs) can form an organized capsule or glycocalyx around the cell or can be shed into the environment as slime. Although synthesis of EPS may be optional depending on growth conditions, it frequently is a major determinant in the ability of a bacterium to colonize a given niche (Costerton *et al.* 1987). Two properties of exopolysaccharides help to determine their functions (Ferris and Beveridge 1985). First, capsular polysaccharides are highly hydrated. This protects the bacterium against desiccation and prevents hydrophobic molecules from penetrating the capsule and arriving at the cell membrane. For example, encapsulated bacteria are more resistant to the action of detergents and hydrophobic polypeptide antibiotics. Second, the acidic EPSs produced by most plant pathogenic bacteria are highly anionic. As such, they can act as ion exchange resins, concentrating minerals and nutrients in the vicinity of the cell or binding toxic elements (Norberg and Persson 1984). Another important function of capsular polysaccharides, in soil and aquatic environments, is to help bacteria adhere to inert or biological surfaces (Norkrans 1980; Ramphal and Pier 1985; Marcus and Baker 1985), where higher concentrations of nutrients may be found.

Many plant pathogenic bacteria need to grow or survive in soil, in irrigation water, or on plant leaves or roots to complete their disease cycle. Protection against a hostile environment would be reason enough for plant pathogens to produce EPS; however, it can also be just as important a factor in pathogenicity and virulence. EPSs have been shown to play a vital role in attachment and infection by *Agrobacterium tumefaciens* (Kamoun *et al.* 1989) and *Rhizobium* species (Chen *et al.* 1988; Djordjevic *et al.* 1987; Leigh *et al.* 1985). Capsules and slime may act as barriers to host defenses, such as phytoalexins, and block recognition events (Bradshaw-Rouse *et al.* 1981; Romeiro *et al.* 1981). In leaf spots, bacterial slime formed in the intercellular spaces may hold the water and nutrients released from damaged cells and thereby contribute to both water-soaking symptoms and the creation of a favorable environment for bacterial multiplication (Coplin and Majerczak 1990).

EPS has been clearly implicated as a mechanism for vascular occlusion (Braun 1982; Chatterjee and Vidaver 1986; Van Alfen 1982) and symptom expression by wilt-inducing bacteria. However, whether wilting ability is purely a function of EPS size and viscosity is a subject of debate, since very little is known about how the structure of EPS influences pathogenicity. EPS minus (EPS^-) mutants of *Erwinia stewartii* (Bradshaw-Rouse *et al.* 1981; Coplin and Majerczak 1990; Dolph *et al.* 1988), *E. amylovora* (Ayers *et al.* 1979; Bennett and Billing 1978; Steinberger and Beer 1988), *Xanthomonas campestris* (Barrère *et al.* 1986; Ramírez *et al.* 1988; Sutton and Williams 1970; Whitfield *et al.* 1981), and *Pseudomonas solanacearum* (Buddenhagen and Kelman 1964; Denny *et al.* 1988; Husain and Kelman 1958; Staskawicz *et al.* 1983) have been shown to have reduced virulence. However, in a few instances, certain nonmucoid strains retained partial to full virulence (Barrère *et al.* 1986; Boucher *et al.* 1985; Coplin and Majerczak 1990; Xu *et al.* 1990). In these cases, wilting may be due to plugging of vascular elements by tyloses or bacterial cells or by production of alternate virulence factors (including EPSs) *in planta.*

This review will be limited to the molecular genetics of acidic heteropolysaccharide biosynthesis in the vascular pathogens *X. campestris* pv. *campestris*, *P. solanacearum*, *E. stewartii*, and *E. amylovora*. When possible, the structure and biosynthesis of these polysaccharides will also be summarized to provide a framework for interpreting genetic studies.

Biosynthesis of EPS. Plant pathogenic bacteria produce a number of exopolysaccharides. The simplest is the polyfructan, levan, which is found in various *Pseudomonas* species (Fett *et al.* 1989) and *E. amylovora* (Bennett and Billing 1980). It is synthesized extracellularly from sucrose by one enzyme, levansucrase (Gross and Rudolph 1987; Gross *et al.*, in press). Other simple polymers are glucans, produced by some gram-positive bacteria (Stoddard 1984), and alginate (Fett *et al.* 1989; Ohman 1986), produced by pseudomonads. The pathways used to produce these polysaccharides involve relatively few enzymes beyond those needed for the synthesis of nucleotide sugar precursors.

On the other hand, the structure and synthesis of the acidic heteropolysaccharide capsules produced by enteric bacteria and xanthomonads are much more complex. Most of what is known about the synthesis of bacterial capsular heteropolysaccharides comes from studies of "cellfree"

enzyme systems from *Klebsiella aerogenes* (Troy 1979) and *X. campestris* (Betlach *et al.* 1987; Ielpi *et al.* 1981a, 1981b; Vanderslice *et al.* 1989) and by analogy to similar studies on lipopolysaccharide synthesis in *Salmonella typhimurium* and sialic acid production in *Escherichia coli* (Troy 1979; Vimr *et al.* 1989). The biosynthesis of these polysaccharides has been reviewed (Sutherland 1977; Troy 1979) and involves five stages: 1) synthesis of nucleotide sugar diphosphate intermediates, 2) stepwise assembly of the repeating oligosaccharide subunit of the polymer by transfer of monosaccharides from the corresponding nucleotide to the carrier lipid (C-55 undecaprenyl phosphate) located in the cell membrane, 3) extrusion of the subunits, 4) transfer of the growing polysaccharide chain from its carrier lipid to the new subunit, and 5) addition of "decorations", such as pyruvate, acetate, succinate, hydroxybutyrate, and sulfate. Thus, EPS synthesis requires enzymes for production of each nucleotide sugar precursor, separate transferases for each monosaccharide in the subunit, one or more polymerases, and proteins involved in export of the polysaccharide. Pathways for synthesis of nucleotide sugars and the carrier lipid also provide key intermediates for peptidoglycan and lipopolysaccharide biosynthesis.

Genetics of EPS synthesis. Large clusters of EPS biosynthetic genes have been reported in *E. coli* (Trisler and Gottesman 1984), *X. campestris* NRRL-B1459 (Barrère *et al.* 1986; Harding *et al.* 1987; Hotte *et al.* 1990; Thorne *et al.* 1987; Vanderslice *et al.* 1989), *Rhizobium* sp. strain NGR234 (Chen *et al.* 1988), *R. meliloti* (Glazebrook and Walker 1989; Leigh *et al.* 1985), *Zoogloea ramigera* (Easson *et al.* 1987), *P. solanacearum* (D. Cook and L. Sequeira, unpublished data), and *E. stewartii* (Coplin and Majerczak 1990; Dolph *et al.* 1988). In each case, DNA that complemented EPS mutants was cloned and mapped by subcloning and transposon mutagenesis; regulatory mutants were identified by their phenotype or effect on reporter gene fusions. Only experiments with plant pathogens are reviewed below.

***X. campestris* EPS.** The studies on the xanthan gum of *X. c.* pv. *campestris* provide the most complete model for EPS synthesis, because it is the only system in which both the biosynthetic genes and their enzymatic products are known. To date, most of this excellent work has appeared as abstracts and symposia papers in the chemical engineering literature (Betlach *et al.* 1988; Vanderslice *et al.* 1989). Progress in this system has been greatly aided by a thorough understanding of polymer structure and biosynthesis. The repeating unit of xanthan gum is a pentasaccharide composed of two glucose (Glu), one glucuronic acid (GlcA), and two mannose (Man) moieties. The Glu forms a β-1→4-linked backbone, and a Man-GlcA-Man side chain extends from alternate Glu moieties. The Man residues in the side chain are acetylated and pyruvylated in a specific, alternating pattern.

Genetics and biochemistry of xanthan synthesis. The genes for xanthan gum synthesis are physically separate from those for synthesis of nucleotide sugars. They have been cloned and are organized in a contiguous 16-kilobase (kb) cluster (Barrère *et al.* 1986; Thorne *et al.* 1987; Harding *et al.* 1987; Vanderslice *et al.* 1989). When transferred to *P. fluorescens* and to *P. stutzeri*, the cluster directed synthesis of a small amount of xanthan gum (M. R. Betlach, D. S. Campbell, M. A. Capage, D. H. Doherty, M. Gold, R. A. Hassler, N. M. Henderson, J. K. Ryan-Graniero, R. W. Vanderslice, and C. A. Weaver. Recombinant DNA mediated biosynthesis of xanthan gum in denitrifying pseudomonads under anaerobic conditions, Am. Inst. Chem. Eng. Annual Meeting, San Francisco, CA, Nov. 5-10, 1989), thus indicating that it contains all of the genes necessary for xanthan synthesis. The entire region coding for synthesis of EPS from strain NRRL-B1459 was sequenced by M. Capage and collaborators at Synergen, Inc., Boulder, CO (M. Capage and D. Doherty, personal communication), and the protein products were determined (Vanderslice *et al.* 1989). Twelve open reading frames were found and these have been designated *gumB* through *gumM*. Sequence analysis and transcription mapping have revealed only one promoter region and no internal termination signals (M. Capage, personal communication). This, together with polarity data, suggests that the region is one very large operon. In previous genetic studies of the same strain, however, researchers concluded that the cluster contained five complementation groups (Harding *et al.* 1987). Recently, Hotte *et al.* (1990) identified, by means of Tn*5-lac* mutagenesis of a different strain of *X. c.* pv. *campestris*, a new cluster of 12 complementation groups, each of which are required for EPS production. By analysis of *lac* fusions, the authors determined that not all of the genes were transcribed in the same direction, although the functions of these genes remain unknown.

A pathway for xanthan gum biosynthesis has been proposed by Ielpi *et al.* (1981a, 1981b) based on studies of the order of nucleotide sugar incorporation into EPS using a cellfree system consisting of EDTA-treated cells. The oligosaccharide subunit is assembled upon an isoprenoid lipid carrier in the following order: Glu, Glu, Man, GlcA, and Man (see Fig. 1). The additions are accomplished by glycosyltransferases I through V, respectively. Pyruvate and acetate are added to the oligosaccharide by the corresponding ketolase and acetylase, and the polysaccharide chain is then polymerized. To take place, each step requires the appropriate nucleotide sugar substrate and glycosyltransferase. If either the substrate or the enzyme is absent, the step is blocked and oligosaccharide intermediates accumulate on the carrier lipid; this finding has proven extremely useful for the analysis of mutants that lack specific glycosyltransferases (see below). The activated xanthan molecule is thought to be added to the lipid-linked repeating unit by a "tail-to-head" polymerization. Finally, the carrier lipid is recycled by a dephosphorylation step, and the polymer is released into the medium.

The Synergen group constructed mutations in each open reading frame of the *gum* cluster. Characterization of the altered polysaccharides and/or lipid-linked oligosaccharide intermediates produced by each mutant in a cellfree system or *in vivo* enabled them to classify their mutants and assign functions to most of the genes of the *gum* cluster in the following manner: transferase I (*gumD*) mutants did not incorporate any label; transferase II (*gumM*) mutants charged the carrier lipid with Glu; transferase III (*gumH*) mutants produced cellobiose in the lipid fraction;

transferase IV (*gumK*) mutants incorporated label into both soluble and lipid fractions and produced a polytrimeric gum both *in vivo* and *in vitro*; and transferase V (*gumI*) mutants produced a less viscous polytetrameric gum *in vivo* and *in vitro*, which lacked the terminal Man. Transferase I, II, and III mutants did not incorporate UDP-GlcA or GDP-Man, and transferase IV mutants did not incorporate UDP-GlcA. Polymerase mutants (*gumB, gumC*, and *gumE*) incorporated all three nucleotide sugars but only into the lipid fraction. *gumF* and *gumG* mutants were defective in acetylation, and *gumL* mutants did not add pyruvate. Similar mutants producing altered xanthans have also been reported by Tait and Sutherland (1989) and Whitfield *et al.* (1981).

It is unfortunate that the effect of these mutations on the virulence of *X. campestris* has not been examined, since the detailed biochemical and genetic data for these mutants make them ideal tools for such an analysis. However, Ramírez *et al.* (1988) examined a range of chemically induced *gum* mutants of NRRL-B1459 for a relationship between gum quality and virulence on *Brassica oleracea*. They found a positive correlation between lesion area and the final viscosity of the culture, the viscosifying capacity of the polymer, and the amount of acetyl substituents in the gum. Although this study found that EPS was necessary for pathogenesis when bacteria were inoculated via hydrathodes, Daniels *et al.* (1989) found that it was not necessary on turnip seedlings when bacteria were inoculated by wounding the stem.

Regulation of xanthan production. Suprisingly, none of the studies on NRRL-B1459 have yielded regulatory mutants (D. Doherty, personal communication). Perhaps, this commercial strain was selected for a high level of xanthan production and may not have normal regulation. Work in M. J. Daniels' laboratory, however, suggests that at least two positive regulators and a negative regulator control xanthan gum synthesis in a wild-type strain of *X. c.* pv. *campestris*. Daniels *et al.* (1989) isolated nonpathogenic mutants and found one of them to be defective in the production of extracellular protease and polygalacturonic acid lyase and severely depressed in synthesis of EPS, amylase, and endoglucanase. Cloning, Tn*5-lac* mutagenesis, promoter cloning, and DNA sequencing of the corresponding locus revealed a cluster of five positive regulatory genes (A–E) that have coordinate effects on all four degradative enzymes, EPS, and pathogenicity (Daniels *et al.* 1989). The sequence of gene C has strong homology with both the "sensor" and "effector" members of several procaryotic two-component regulators. From amino acid residues 138 to 376 there is strong homology with the conserved carboxy-terminal domains of the EnvZ and PhoR proteins (sensors), and from residues 408 to 563 there is homology to the effectors NtrC, OmpR, SpoOF, CheY, and PhoB. The putative C protein has significant hydrophobic regions in its amino-terminal portion, so it may be a transmembrane protein. Thus, it appears that EPS and other pathogenicity factors in *X. c.* pv. *campestris* are controlled by a two-component regulatory system in which the sensor and effector are part of the same protein. This system appears to be balanced, in turn, by a parallel negative regulatory gene. When cloned in a multicopy plasmid, this gene coordinately represses extracellular

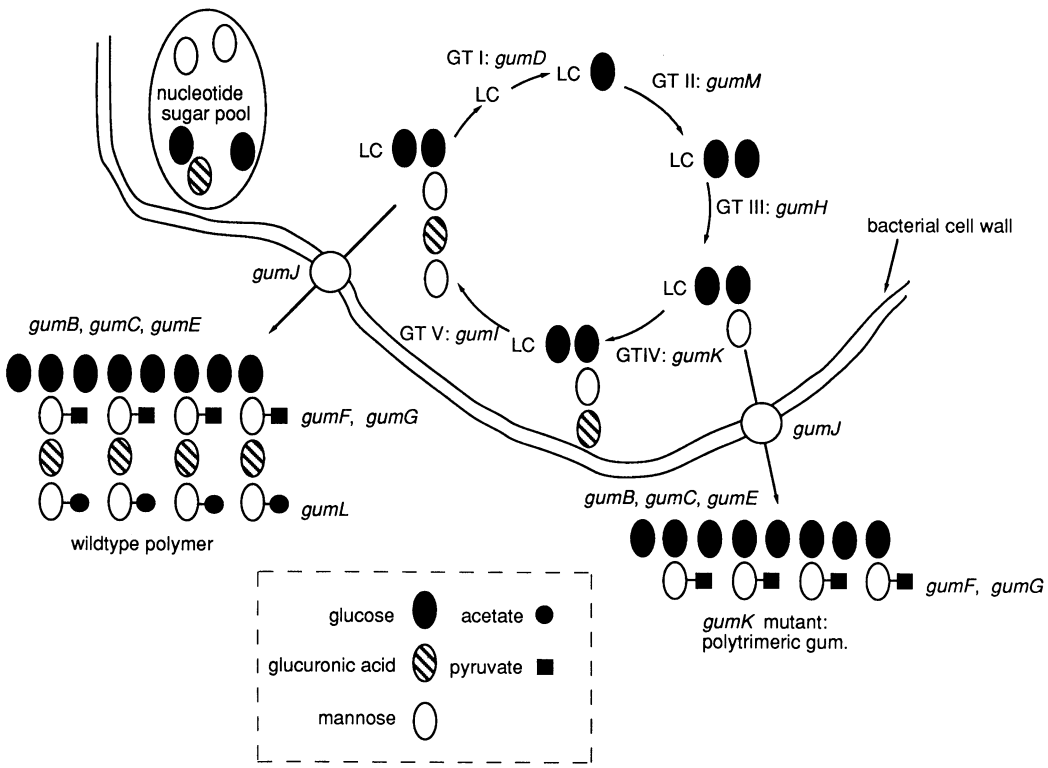

Fig. 1. Simplified model of xanthan gum biosynthesis. Lipid-linked oligosaccharides are assembled intercellularly from nucleotide sugars extruded from the cell and polymerized. GT, glycosyltransferase; LC, lipid carrier.

enzyme and polysaccharide synthesis (Tang *et al.* 1990). Disruption of the gene by Tn*5* mutagenesis and marker exchange of the mutations into the wild-type chromosome produced a pathogenic strain with elevated levels of degradative enzymes and EPS. An additional two-component regulatory system, which more specifically regulates EPS synthesis, was identified in an *X. c.* pv. *campestris* library by means of oligonucleotide probes to conserved regions of known regulators (Osbourn *et al.* 1990). This gene pair also has extensive homology with both the sensor and effector proteins of known two-component systems. Mutants of the effector gene, constructed by marker exchange, were affected in EPS production and resistance to salt and chloramphenicol.

***P. solanacearum* EPS.** EPS produced by *P. solanacearum* forms a loosely associated slime that is produced by all wild-type virulent strains. Since the pathogen is limited primarily to xylem elements, where it produces copious amounts of slime, the EPS is thought to cause wilt symptoms by occluding xylem pit membranes (Husain and Kelman 1958; Van Alfen 1982; Van Alfen *et al.* 1983). However, EPS may also contribute to virulence by other means. For example, Young and Sequeira (1986) observed that EPS^- mutants of *P. solanacearum* are rapidly agglutinated by plant cell wall fragments and that EPS can prevent this process. Consequently, EPS may function to prevent binding of the bacteria to the plant cell wall, thereby aiding in systemic movement of the bacterium.

Biochemical characterization of *P. solanacearum* EPS. The structure of *P. solanacearum* EPS is not known, but several compositional analyses have been conducted. Akiyama *et al.* (1986) suggested that the EPS is a homopolymer of *N*-acetylgalactosamine (GalNAc). Although Glu and rhamnose have been reported as minor components (Akiyama *et al.* 1986; Drigues *et al.* 1985; Denny *et al.* 1988), the presence of these sugars is likely to be the result of incomplete purification. Instead, two amino sugars, bacillosamine (2,4-diamino-2,4,6-trideoxy-glucose) and galactosaminuronic acid, are probably integral components of the EPS; the later sugar being responsible for the acidic nature of the molecule (A. Trigalet, personal communication).

Very little information on the biosynthesis of *P. solanacearum* EPS is available. However, recent studies at the University of Wisconsin, Madison, indicate that UDP-GalNAc is likely to be a key intermediate in EPS synthesis. It is one of the predominant sugar nucleotides in wild-type cells and is rapidly incorporated into a chloroform-methanol soluble fraction, which is indicative of the formation of lipid-linked intermediates for EPS synthesis (D. Cook and L. Sequeira, unpublished results). However, the possibility that labeled UDP-GalNAc may be incorporated into lipid-linked precursors of lipopolysaccharide or peptidoglycan via interconversion to UDP-*N*-acetylglucosamine cannot be ruled out.

Studies with EPS mutants of *P. solanacearum*. Most of the work on the genetics of *P. solanacearum* EPS has involved EPS-deficient transposon mutants. Denny *et al.* (1988) obtained two classes of mutants both of which lack EPS when grown on rich culture medium. Subcloning and complementation analysis revealed that the two classes of mutants resulted from Tn*5* insertions in separate complementation units, which are about 12 kb apart (T. Denny, personal communication). Class I mutants are severely reduced in virulence, but class II mutants retain moderate to high levels of virulence. The differential ability of these strains to induce wilting is explained by the fact that class II mutants produce EPS *in planta*, and on minimal medium, whereas class I mutants do not (T. Denny, unpublished results). Thus it appears that EPS production in class II mutants is dependent on the nutrient status of the environment and that the affected gene(s) may not be essential for EPS production under all conditions.

In a related study, D. Cook and L. Sequeira (unpublished results) used Tn*3* mutagenesis of wild-type cosmid clones and marker exchange to identify a 6.5-kb gene cluster that is required for normal EPS production and virulence. It has not yet been determined if these genes are linked to T. Denny's class I and class II mutations (above). Five separate complementation units were identified by the inability of plasmid-borne Tn*3* insertions to complement certain chromosomal mutations. Four of the five complementation units are required for the wild-type fluidal colony morphology. Mutations in *epsA—epsC* yield strains that produce no visible EPS, whereas *epsD*::Tn*3* strains have an intermediate colony morphology (EPS-impaired). Tn*3* insertions in the fifth complementation unit, designated *rvrA* for reduced virulence, do not affect colony morphology. Analysis of EPS by gas-liquid chromatography of alditol acetate derivatives revealed that all of these mutants are affected in EPS production, even those with apparently wild-type colony morphologies. The quantity of EPS produced in broth culture is positively correlated with virulence and the ability to grow in the plant. Data obtained from β-glucuronidase gene fusions suggest that these genes are expressed in a constitutive fashion; they are expressed continuously in rich media, and expression can be detected in both minimal media and in the plant. It is likely that this region contains genes directly involved in EPS biosynthesis, since all five complementation units are required for EPS production and clustering is typical of genes for EPS synthesis. The intermediate colony morphology of *epsD* mutants is similar to that of *X. campestris* mutants affected in glycosyltransferases required for side-chain assembly or of mutants affected in acetylation and pyruvylation of EPS (see above).

Regulation of EPS production in *P. solanacearum*. Most bacteria that produce EPS spontaneously give rise to variant colony types (Bartlett *et al.* 1988; Easson *et al.* 1987; Flynn and Ohman 1988; Hoiseth *et al.* 1985). EPS-deficient strains of *P. solanacearum* are obtained following prolonged maintenance in still broth cultures (Kelman and Hruschka 1973). In addition to their obvious EPS deficiency, these strains are affected in other characteristics including production of polygalacturonase, endoglucanase, indole-3-acetic acid, and melanin-like pigment, and in virulence and motility (Morales *et al.* 1985; Buddenhagen and Kelman 1964). It is not likely that these strains arise via simple mutation, since reversion to the wild type has never been observed despite repeated attempts. T. Denny (personal communication) has obtained a possible Tn*5*-induced regulatory mutation in strain AW1 that confers

the same pleiotropic phenotype as spontaneous EPS mutations. The corresponding cosmid clone, which complements the Tn*5* mutant, restores wild-type characteristics to all spontaneous mutants of strain AW1, and to some but not all spontaneous variants of strains K60 and GMI1000. The change to this pleiotropic EPS$^-$ phenotype has been termed "phenotype conversion", and the complementing gene was labeled "*phcA*" (for *ph*enotype *c*onversion). The fact that *phcA* complements only a subset of spontaneous mutants indicates that it is probably not the sole factor in the pleiotropic shift. In strains K60 and GMI1000, spontaneous EPS mutants are a phenotypically heterogenous group (D. Cook and L. Sequeira, unpublished data) and are therefore unlikely to have all arisen from mutation at a single locus. Nevertheless, for those spontaneous mutants complemented by *phcA*, regulation of *eps* genes appears to be affected. T. Denny (personal communication) constructed EPS$^-$, Lac$^+$ fusion mutants corrresponding to the class I and class II mutants discussed previously. These strains became Lac$^-$ by a subsequent phenotype conversion mutation and were restored to Lac$^+$ by the *phcA*$^+$ plasmid, indicating that *phcA* is required for the expression of these genes (T. Denny, personal communication).

EPS synthesis in *P. solanacearum* may be under negative as well as positive control. Huang and Sequeira (in press) obtained a cosmid clone that when present in the low copy number plasmid pLAFR3 represses EPS production. The functional unit was subcloned to a 1.3-kb fragment that appears to encode a single gene, designated *epsR*. Strains carrying multiple copies of *epsR* are not only affected in EPS production but are also reduced in virulence and overproduce melanin. Since these are some of the phenotypes associated with spontaneous EPS$^-$ mutants, it is tempting to speculate that an imbalance between PhcA and EpsR might mediate some aspect of the spontaneous phenotype shift.

Although the studies cited above indicate that EPS is important for the virulence of *P. solanacearum*, a recent report of EPS$^-$ mutants that retain nearly wild-type levels of virulence (Xu *et al.* 1990) poses a serious challenge to this long-standing hypothesis. Unlike the conditional class II mutants, which are EPS$^-$ on rich media but produce EPS *in planta*, the virulent EPS$^-$ mutants produced no detectable EPS *in planta*. Although it is possible that EPS is not strictly required for virulence of *P. solanacearum*, this conclusion needs to be examined further because these EPS$^-$, virulent mutants remain largely uncharacterized and may themselves have a pleiotropic phenotype. For example, the overproduction of polygalacturonase is associated with some EPS$^-$ mutants, and the corresponding increase in maceration potential may be sufficient to produce disease symptoms, particularly in stem inoculation assays. Critical evidence for the role of EPS in virulence of *P. solanacearum* requires a more detailed analysis of the various mutant types and of the factors affecting EPS biosynthesis.

***E. stewartii* EPS.** The capsular EPS of *E. stewartii* is very large (45×10^6 Da), viscous, and highly charged (Darus 1980; Gorin and Spencer 1961). Preliminary compositional analysis and ^{1}H and ^{13}C NMR have indicated that the polysaccharide contains Glu, galactose (Gal), and GlcA in a ratio of 4:2:1 and has a repeating unit of seven monosaccharides (J. Costa, D. Horton, and D. Coplin, unpublished data). Methylation analysis of alditol acetate derivatives suggests that the polysaccharide has a β-1→6-linked backbone of Glu and Gal with 1→4 and 1→3 branch points on one of the Gal moieties. One side chain consists of Glu and the other contains GlcA and a terminal Glu. This is the only major EPS that has been detected in culture.

Genetics of capsule synthesis in *E. stewartii*. A 10-kb gene cluster required for capsular polysaccharide biosynthesis (*cps*) has been genetically characterized (Coplin and Majerczak 1990; Dolph *et al.* 1988). This cluster also contains the gene for UDP-Gal-4-epimerase (*galE*). The *cps* genes are arranged in at least five complementation groups (termed *cpsA* though *cpsE*) and are all transcribed in the same direction. All of these genes are necessary for EPS synthesis and wilt induction, and in addition, they can affect water-soaked lesion formation (Coplin and Majerczak 1990). These phenotypes have been confirmed by marker exchange of transposon-induced mutations in each of the complementation groups. The entire region is linked to the *his* operon on the *E. stewartii* chromosome and may be analogous to a similar locus near *his* in *E. coli* that controls synthesis of colanic acid (Trisler and Gottesman 1984). Tn*phoA* mutagenesis of the cluster resulted in alkaline phosphatase-active gene fusions within *cpsB* and *cpsC* (D. Coplin, unpublished). This indicates that *cpsB* and *cpsC* encode membrane, periplasmic, or exported proteins, which could be glycosyltransferases or proteins involved in export of oligosaccharides or polymerases. By means of Tn*5* mutagenesis and complementation analysis, two additional complementation groups, *cpsF* and *cpsG*, have been identified, and *cpsF* appears to be linked to the *cpsA-E* cluster. The finding that the *galE* gene is not part of a *gal* operon in *E. stewartii* is unusual, and its location in the *cps* region may imply that this enzyme's role in supplying precursors for polysaccharide biosynthesis is more important than its role in galactose utilization. A similar organization of the *gal* genes in *Vibrio cholerae* also has been reported (Houng and Cook 1986).

Regulation of polysaccharide production in *E. coli* and *E. stewartii*. The regulation of colanic acid synthesis in *E. coli* (Fig. 2) has proven to be a very useful model for the regulation of EPS synthesis in the nonpectolytic

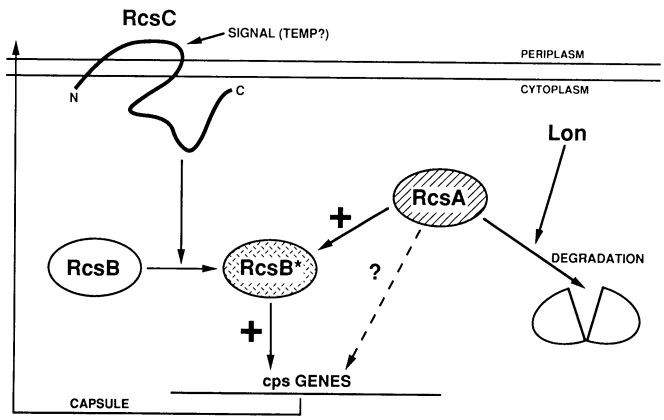

Fig. 2. Model of capsule regulation in *Escherichia coli*. Adapted from Stout and Gottesman (1990).

erwinias. In *E. coli*, *cps* gene expression is regulated positively by the product of the *rcsA* gene, which in turn is rapidly degraded by the Lon protease (Gottesman 1984, 1989; Torres-Cabassa and Gottesman 1987). Additional control is provided by a two-component regulatory system consisting of an effector, RcsB, and a sensor, RcsC (Brill *et al.* 1988; Gottesman *et al.* 1985; Stout and Gottesman 1990). The *rcsBC* locus has been sequenced and is homologous to other two-component regulators. By analogy RcsC may be a phosphorylase/kinase that activates RcsB, which in turn activates *cps* transcription. The promoter of *rcsB* is dependent on the alternate sigma factor, RpoN (Stout and Gottesman 1990). The RcsB protein may also interact with RcsA, stimulating *cps* expression by an as yet unknown mechanism (S. Gottesman, personal communication). Mutation of a different locus, termed *ops*, results in overproduction of colanic acid and thus may negatively regulate polysaccharide synthesis (Zinkewich-Péotti and Fraser 1988).

EPS synthesis in *E. stewartii* has many similarities to that in *E. coli*. By means of *cps*::*lacZ* operon fusions, we have shown that the *cpsA–cpsD* genes in *E. stewartii* are under positive regulation by the product of an *rcsA* gene (Torres-Cabassa *et al.* 1987). We have cloned and sequenced the *rcsA* gene from *E. stewartii* (K. Poetter and D. Coplin, unpublished) and found that it functions in *E. coli* and shares substantial homology with the predicted amino acid sequences of the *rcsA* gene from *E. coli* (S. Gottesman, unpublished) and *K. aerogenes* (Allen *et al.* 1987). Recently, K. Poetter and D. Coplin (unpublished) have cloned an *E. stewartii rcsBC*-like locus, which complements *E. coli rcsB* and *rcsC* mutations and a group of nonmucoid *E. stewartii* Tn*5* mutants. Conversely, the *E. stewartii* mutations are complemented by a clone of the *E. coli rcsB* and *rcsC* genes. Another *E. stewartii* clone has been shown to complement *E. coli lon* mutations (J.-H. Kim and D. Coplin, unpublished). These preliminary findings suggest that EPS synthesis in *E. stewartii* could be under the control of a two-component regulatory system, like RcsB and RcsC, in addition to RcsA and a protease.

Amylovorin. Among the erwinias, the capsule of *E. amylovora* has been the most extensively studied to date. *E. amylovora* produces a large (50 to 150 Mda) capsular polysaccharide, called amylovorin, composed of Gal, GlcA, and pyruvate. Smith *et al.* (in press) have proposed the following structure:

$$\text{Pyr - Gal}_{(\beta 1\rightarrow 4)}$$

$$|$$

$$[\text{GlcA}_{(\alpha 1\rightarrow 3)}\ \text{Gal}_{(\beta 1\rightarrow 3)}\ \text{Gal}_{(\alpha 1\rightarrow 6)}\ \text{Gal}_{(\beta 1\rightarrow 4)}]_n$$

Mutants that cannot produce amylovorin are nonvirulent (Ayers *et al.* 1979; Bennett and Billing 1978; Steinberger and Beer 1988). The wilting ability of *E. amylovora* is correlated with the abnormally high viscosity of its EPS, which is a consequence of both high molecular mass and a high frictional coefficient (Sijam *et al.* 1985). Another function of amylovorin has been suggested by Romeiro *et al.* (1981), who isolated a highly positive-charged protein from apple seeds that agglutinates acapsular strains. A. Karr (personal communication) has determined that the agglutinin is a thioninlike protein, which has bactericidal activity. EPS titrates this protein, and thus may prevent agglutination and killing of capsulated strains.

Genetic studies in *E. amylovora*. *E. amylovora* and *E. stewartii* are closely related and produce similar diseases. This relationship has been exploited to isolate EPS genes from *E. amylovora* by complementation of mutations in *E. stewartii*. Clones from an *E. amylovora* genomic library complemented both *rcsA* (Chatterjee *et al.* 1990; F. Bernhard, K. Poetter, K. Geider, and D. Coplin, unpublished) and *cpsA* to *cpsD* (F. Bernhard, unpublished) mutations in *E. stewartii*. The *rcsA* gene from *E. amylovora* has been sequenced, and it has 82% amino acid homology with *rcsA* from *E. stewartii* and 59% homology with *rcsA* from *E. coli* (F. Bernhard, K. Poetter, K. Geider, and D. Coplin, unpublished). Site-directed mutagenesis was used to create an *rcsA* mutant of *E. amylovora*. This mutant showed more than 90% reduction of amylovorin and levan synthesis, indicating common regulation of the two polysaccharides. Furthermore, rotting of immature pear slices was considerably diminished. A. Chatterjee (personal communication) has also identified *E. amylovora* clones with *rcsB*-like and *lon*-like activity.

Considerations for future research. As obvious a phenotype as capsule and slime production may appear, it has proven difficult to determine the role of EPS in pathogenesis. As indicated above, the literature on plant pathogenic bacteria is full of correlations between virulence and colony type, but only recently have researchers created EPS mutants with a greater range of pathogenic phenotypes. A possible reason for this is that EPS may have multiple functions in pathogenesis. EPS is the product of multienzyme pathways rather than being a direct gene product. Thus, a bacterium can produce more than one EPS or may use alternate pathways and regulatory circuits for the synthesis of the same EPS. In addition, it is difficult to determine, without chemical analysis, if an apparently EPS^- mutant is still producing a small amount of EPS or if it produces EPS *in planta* but not in culture. Furthermore, mucoid variants may synthesize an altered EPS that lacks side chains or modifications, thereby changing charge and viscosity. Important questions that remain to be answered are how much EPS is required for growth and/or symptom production in plants and what features of EPS structure are important for pathogenicity.

Molecular characterization of EPS genes has been aided greatly by the finding that, without exception, the genes for the biosynthetic enzymes are clustered. This type of physical arrangement is common among catabolic genes, but also extends to such idiosyncratic functions as symbiosis and pathogenicity. This may imply that basic sets of EPS genes have evolved together in gram-negative bacteria, moved by horizontal gene exchange to new species, and then diverged to produce different polysaccharides.

Similarities among regulatory systems are also apparent, especially within the Enterobacteriaceae, where common regulatory molecules have been identified in *E. stewartii*, *E. amylovora*, *K. aerogenes*, and *E. coli*. In all cases studied, EPS synthesis is controlled by multiple regulatory systems.

But what is the reason for such complexity? The "demand theory" of gene regulation (Savageau 1983) predicts that frequently needed pathways are in high demand and will be positively regulated, whereas pathways that are used only occasionally are in low demand and will be negatively regulated. In either case, the environment will select against regulatory mutants, because high-demand pathways will be lost or low-demand pathways will be expressed when they are not needed. The theory further predicts that in bacteria, which alternate between two very different niches, some pathways will show both positive and negative regulation if the demand for them changes from one niche to the other. Thus, as the microbe changes niches, the predominant mode of regulation for a pathway will shift.

E. coli is a good example of a bacterium with two niches. Colanic acid is not produced in the mammalian intestine (the primary niche), but instead, it is produced only under conditions of nutrient stress and low temperature, such as *E. coli* would encounter in water, sediments, and soil (the secondary niche). Both positive and negative regulators of colanic acid have been reported, but the external signals that they respond to have not been determined. Plant pathogens typically occupy two or more niches: as pathogens or endophytes within a plant; as epiphytes, rhizobacteria, or soil inhabitants; or as part of the intestinal microflora of an insect vector. In each niche, the demand for EPS and the signals needed for its induction or repression may be different, thereby leading to multiple regulatory systems.

P. aeruginosa varies the epimerization and acetylation of alginate in response to growth conditions (Ohman and Chitnis 1989). Will we find that the EPSs of plant pathogens vary similarly and that this affects pathogenicity? Will the individual operons in an EPS cluster be regulated differently to vary EPS structure or decorations in response to the environment? It is interesting to speculate about what the patterns of EPS gene regulation might tell us about the demand for EPS in a particular situation. Is the net regulation positive or negative *in planta*? In *Rhizobium* sp., only negative systems (Borthakur and Johnston 1987; Doherty *et al.* 1988; Gray *et al.* 1990) have been described so far. Does this mean that the acidic EPS is involved only in the infection process and is not needed for survival in soil or by the bacteroid? EPS synthesis in *X. c.* pv. *campestris* and *P. solanacearum* is even more intriguing because both positive and negative regulation have been reported. In addition to specific controls on its synthesis, EPS in these bacteria appears to be regulated as part of a "pathogenicity regulon" along with many extracellular enzymes. It will be interesting to determine which regulatory circuits are most important in the plant and if the pattern of expression reflects the role of EPS as a virulence factor. Determining the regulatory circuits controlling EPS synthesis and the various signals to which they respond may eventually help to answer many questions about the importance of EPS in pathogenesis.

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