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Bacterial exopolysaccharides : Their nature, regulation and role in host-pathogen interactions

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I. INTRODUCTION

In their natural environment bacteria are normally surrounded by a glycocalyx. The term glycocalyx refers to any polysaccharide-containing component outside of the cell wall (Costerton et al., 1987). The primary component of the glycocalyx is usually high molecular weight anionic exopolysaccharide (EPS), but can also be uncharged EPS or S layers, regular arrays of glycoprotein subunits (Sleytr and Messner, 1988). EPS can occur in the form of a tightly-held capsule or a loosely-held slime and EPS fibers can extend 3 μm or more away from the cell surface. In addition, free lipopolysaccharides (outer membrane components of gram-negative bacteria) and glycolipids may also be present as well as proteins and nucleic acids.

It has been proposed that in nature the synthesis and excretion of EPS protects bacterial cells from desiccation and UV irradiation, mediates adhesion to living and nonliving surfaces and mediates the formation of adherent bacterial biofilms (Costerton et al., 1987; Dudman, 1977). Such biofilms may aid in trapping nutrient ions and molecules due to their charged surfaces, protect bacteria from attack by bacteriophage and predators such as amoebae and protozoa as well as from biocides, allow for concentrated enzyme activity, and in clinical situations protect against antibiotics, phagocytosis and antibody attack. The formation of dental plaque is mediated by EPS (Cerning, 1990)

and EPS produced by nitrogen-fixing bacteria may act in signal transduction, may form part of the infection thread matrix and may assist in the evasion or suppression of plant defense responses (Reuber et al., 1991). Synthesis of EPS may also have a variety of important functions for plant pathogenic bacteria during their interaction with plants as discussed herein.

The majority of bacterial EPS's are heteroglycans composed of oligosaccharide repeating units (Kenne and Lindberg, 1983). The repeating units normally contain from 2 to 6 sugar moieties, but up to a heptadecasaccharide unit has been reported (O'Neill et al., 1992). Some bacterial species are capable of producing numerous EPS's of differing chemical structures. For example, over 70 different EPS structures have been found for *Escherichia coli* (Pazzani et al., 1993). Other species are capable of producing only a very limited number of EPS's or a single EPS.

The primary bacterial genera whose members cause plant diseases are *Agrobacterium*, *Clavibacter*, *Erwinia*, *Pseudomonas*, and *Xanthomonas*. This review will focus on the nature of the EPS's produced by these bacteria, EPS synthesis and regulation as well the role these polysaccharides play in host-pathogen interactions. Other bacterial oligosaccharides and polysaccharides (e.g., β -1,2 glucans and lipopolysaccharides) which are released into the environment and may also play a role in host-pathogen interactions will not be discussed.

II. NATURE OF EXOPOLYSACCHARIDES

A. *Agrobacterium*

The genus *Agrobacterium* contains four plant pathogenic species: *A. tumefaciens*, *A. rhizogenes*, *A. rubi* and *A. vitis* (Young et al., 1992). A nonpathogenic form of *A. tumefaciens* (due to the loss of the tumor-inducing or Ti-plasmid) is a common soil-inhabitant and is often designated as *A. radiobacter* even though *A. tumefaciens* takes taxonomic precedence (Young et al., 1992). *A. tumefaciens* causes a disease called crown gall on a very wide range of dicotyledonous plants. The disease is characterized by the formation of galls on the infected plant, primarily at the soil line, but sometimes on roots or on aerial organs. *A. rhizogenes* has an intermediate host range and causes hairy root disease, the primary symptom being the formation of numerous small roots at the site of infection. *A. rubi* and *A. vitis* have much narrower host ranges, inducing the formation of galls on the canes of *Rubus* spp. and on grapevines, respectively.

Agrobacterium are capable of producing both a water soluble acidic EPS, succinoglycan (Hisamatsu et al., 1980; Zevenhuizen, 1973), and two water insoluble neutral EPS's, curdian and cellulose (Hisamatsu et al., 1977; Matthyse et al., 1981). The term succinoglycan refers to a family of closely related polysaccharides, all composed of identical octasaccharide repeating units containing glucose, galactose, and pyruvate (1-carboxyethylidene) (7:1:1) (Fig. 1), but differing in esterification with either acetate or succinate present. Succinoglycan is present as a loosely-held water soluble slime with an estimated molecular weight of 4.2 MDa (Gravinis et al., 1987). *Agrobacterium* also can excrete the octasaccharide repeating unit of succinoglycan (Hisamatsu et al., 1982). Curdian is a linear β -1,3-glucan (Fig. 1) with an estimated molecular weight of 22 kDa (Harada et al., 1968). It is present as a capsule and has been demonstrated to be produced by *A. radiobacter* and *A. rhizogenes* (Hisamatsu et al., 1977). Succinoglycan and curdian can be produced together in culture and their synthesis is not

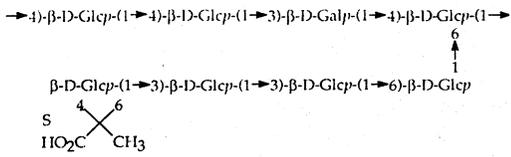
restricted to the *agrobacteria*. Their synthesis was originally reported for *Alcaligenes faecalis* var. *myxogenes* and they are also produced by *Rhizobium* spp. (Harada, 1977; Zevenhuizen, 1973; Sutherland, 1983). Curdian from *Alcaligenes* was estimated to have a molecular weight of 22 kDa (Harada et al., 1968).

Cellulose (a linear β -1,4-glucan) is produced by *A. tumefaciens* as extracellular fibrils (Matthyse et al., 1981). A variety of other bacteria including *Rhizobium* spp. are also capable of cellulose synthesis (Hotchkiss, 1989; Ross et al., 1991).

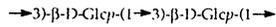
A. tumefaciens has not yet been reported to produce curdian and *A. rubi* and *A. vitis* have not been examined for ability to produce high molecular weight EPS. A strain of *A. tumefaciens* produces a high molecular weight (650 kD) acidic EPS distinct from succinoglycan (El-Sayed et al., 1983). This EPS contained glucose, pyruvate, succinate and acetate (6:1:1:1).

B. *Clavibacter*

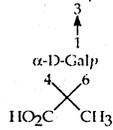
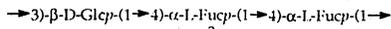
There are five plant pathogenic species of *Clavibacter* (*iranicus*, *michiganensis*, *rathayi*, *tritici* and *xyli*) with *C. michiganensis* (*C. m.*) and *C. xyli* further divided into subspecies (Young et al., 1992). Members of this genus produce multi-component EPS and the structures of two EPS's have been reported. *C. m. insidiosus*, causal agent of bacterial wilt of alfalfa (*Medicago sativa*) (Bradbury, 1986) produces an acidic EPS with a trisaccharide repeating unit composed of glucose, galactose, and fucose, and substituted with pyruvate (1:1:2:1) (Fig. 1) (Gorin et al., 1980). In a later study, five additional strains of *C. m.* subsp. *insidiosus* were reported to produce multi-component EPS whose composition was both strain and culture medium dependent (Van Alfen et al., 1987). Two acidic components (I and II) appeared to be identical to the EPS reported by Gorin et al. (1980). Component I was determined to be an aggregate of component II (5 MDa). The composition of components I and II varied only slightly from strain to strain and for different culture media. Protein was tightly associated with component I, but was not covalently bound.



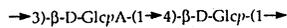
Agrobacterium spp. (succinoglycan)



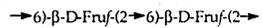
Agrobacterium spp. (curdlan)



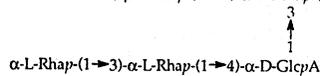
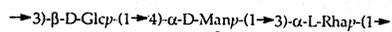
C. m. subsp. *insidiosus*



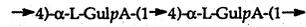
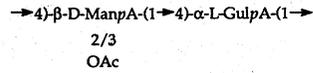
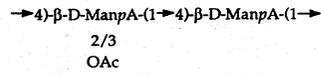
C. m. subsp. *sepedonicus*



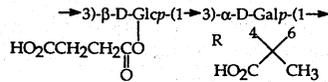
E. amylovora and *Pseudomonas* spp. (levan)



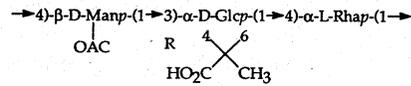
E. c. pv. *zoeae*



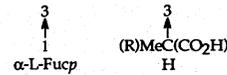
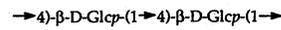
Pseudomonas spp. (alginate)



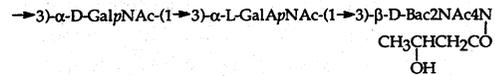
P. marginalis type A (marginalan)



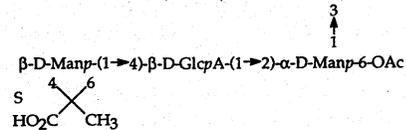
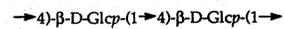
P. marginalis type B



P. marginalis type C



P. solanacearum



Xanthomonas spp. (xanthan gum)

Fig. 1. Exopolysaccharides of plant pathogenic bacteria.

Neutral component III (22 kDa) contained rhamnose, fucose, mannose, galactose and glucose. The relative amounts of these sugars were dependent both on the strain and on the culture medium and one of the five strains did not produce component III. The multicomponent EPS was present as a loosely-held slime for all five strains.

Based on analysis of monosaccharides, proton NMR spectroscopy and methylation analysis *C. m.* subsp. *michiganensis* appears to produce an acidic EPS identical to that which has been structurally characterized from *C. m.* subsp. *insidiosus* except for the additional presence of succinyl and O-acetyl groups (van den Bulk et al., 1991). The molecular weight was estimated at 1 to 10 MDa. Additional studies are required for confirmation of the proposed structure. This bacterium causes a vascular wilt of tomato (*Lycopersicon esculentum*) and potato (*Solanum* spp.) (Bradbury, 1986).

The EPS produced by *C. m.* subsp. *sepedonicus*, causal agent of bacterial ring rot of potato, also contains more than one component. Westra and Slack (1992) identified four components all containing fucose, mannose, galactose and glucose. The relative amounts of these sugars were once again strain and culture medium dependent. Components I (greater than 20 MDa) and II (4.5 MDa) appeared to be aggregates of component III (2.1 kDa). All three components were acidic and had a similar neutral sugar composition for a particular strain. The moieties responsible for the acidic nature of the polymer were not identified although all three components lacked uronic acid. Component IV was neutral and primarily composed of mannose. Henningson and Gudmestad (1993) also reported production of multicomponent EPS by this pathogen. Mucoid and strains intermediate in mucoidy produced both high (0.5 to 1.6 MDa) and low (4 to 11 kDa) molecular weight EPS while nonmucoid strains produced only low (1.5 to 11 kDa) molecular weight EPS. The high molecular weight EPS contained galactose, glucose and fucose, the ratios of which were strain dependent. Low molecular weight EPS contained primarily galactose with variable amounts of glucose, rhamnose, mannose and ribose. A distinct acidic EPS containing D-glucose and D-glucuronic

acid (1:1) was also reported to be produced by *C. m.* subsp. *sepedonicus* and its structure was determined (Kocharova et al., 1991) (Fig. 1).

Based on neutral sugar composition (glucose, mannose, \pm rhamnose) and the presence of uronic acids, the EPS's produced by the additional plant pathogens *C. rathayi* and *C. tritici* appear to differ from each other and from those EPS's described above for other *Clavibacter* spp. (Gorin and Spencer, 1961). There are no reports of EPS production by *C. iranicus*.

C. Erwinia

The genus *Erwinia* is not well defined taxonomically (Young et al., 1992). The plant pathogenic members of this group include bacteria that cause soft rots as well as vascular wilts, galls, leaf spots, leaf scorch and stem cankers. The nature of the EPS of only three *Erwinia* species has been reported to date.

E. amylovora, a pathogen of all species of the Pomoideae and the causal agent of fireblight of apples and pears (Bradbury, 1986), produces both a neutral and an acidic EPS. When grown on high levels of sucrose as the carbon source, levan, a neutral EPS, is produced as a slime (Bennett and Billing, 1980; Gross et al., 1992). Levan is a 2,6-linked β -D-fructan with a variable degree of branching through O-1 (Fig. 1). The length of the branches has not been determined. A very wide range of molecular weights (5 kDa to 100 MDa) for bacterial levans has been reported (Avigad, 1968). Levan production is not specific for *E. amylovora* as many additional bacteria belonging to other genera including plant pathogenic pseudomonads are also capable of producing this EPS (Han, 1989). On carbon sources other than sucrose an acidic EPS initially referred to as amylovorin and, more recently, as amylovoran is produced. Amylovoran is present both as a tightly bound capsule and slime and contains galactose, glucuronic acid, acetate and pyruvate (4:1:1:1). Only a tentative structure has been assigned (Smith et al., 1990). The molecular weight of amylovoran was estimated to be between 50 and 150 MDa (Leigh and Coplin, 1992).

E. chrysanthemi pv. *zearae* causes wilts and

soft rots of several host plants including corn (*Zea mays*) (Bradbury, 1986). Large amounts of an acidic EPS are produced by this bacterium on medium containing lactose. The acidic EPS contains rhamnose, glucose, glucuronic acid and mannose (3:1:1:1) and its complete structure has been determined (Fig. 1) (Gray et al., 1993).

E. stewartii causes a bacterial wilt (Stewart's disease) of corn (Bradbury, 1986). The pathogen produces a high molecular weight (45 MDa) acidic EPS, stewartan, which is present both as a capsule and slime (Bradshaw-Rouse et al., 1981; Coplin and Majerczak, 1990; Darus, 1980). The structure has only been partially determined and is proposed to contain a heptasaccharide repeating unit containing glucose, galactose and glucuronic acid (4:2:1) (Costa, 1991).

D. *Pseudomonas*

The genus *Pseudomonas* encompasses a very large and diverse assemblage of bacteria. Five groups of pseudomonads were distinguished based on ribosomal RNA-DNA homologies (Palleroni, 1984). Pseudomonads with the ability to cause plant diseases have been placed in three of these groups (Young et al., 1992). Group I is comprised primarily of what are referred to as fluorescent pseudomonads. These bacteria produce water-soluble iron-binding fluorescent pigments (siderophores) when grown on media deficient in iron. In the future it is likely that the Group I bacteria alone will comprise the genus *Pseudomonas* and the reclassification of members of Groups II to IV to distinct genera is currently underway (Young et al., 1992). The species *P. syringae*, a member of Group I, is further subdivided into 45 pathovars which are distinguished primarily by their host range and/or types of symptoms they cause (Young et al., 1992). The plant pathogenic pseudomonads induce a variety of plant diseases with symptoms ranging from leaf spots and blights to stem cankers, vascular wilts, galls and soft rots (Bradbury, 1986).

Several acidic EPS's have been described for the Group I pseudomonads. Alginates, a group of structurally related polysaccharides, are produced as a loosely-held slime by several members. Alginates are linear polymers containing varying

ratios of the monomers mannuronic acid and its C-5 epimer guluronic acid (Fig. 1) (Gacesa and Russell, 1990). The monomers can be present as homopolymeric block (e.g., polymannuronate or polyguluronate) or heteropolymeric block structures. The relative amounts of the different block structures greatly affects the physical properties of the polymer. Bacterial alginates differ from algal alginates in the apparent absence of polyguluronic blocks and the presence of mono- or di-acetylated mannuronic acid (Skjak-Braek et al., 1986). Bacterial alginate production was first described for *P. aeruginosa* (Linker and Jones, 1966), an opportunistic human and plant pathogen (Rahme et al., 1993), and has recently been found to be produced by the plant pathogens *P. cichorii*, *P. syringae* pathovars, and *P. viridiflava* (Fett et al., 1986, 1989; Gross and Rudolph, 1987a,b; Osman et al., 1986). Selected strains of the saprophytic bacteria *P. fluorescens*, *P. mendocina* and *P. putida* have also been reported to produce alginate (Fett et al., 1989; Govan et al., 1981). In contrast to alginates produced by *P. aeruginosa*, whose ratios of mannuronate to guluronate are independent of growth conditions (Gacesa and Russell, 1990), these ratios are media dependent for other alginate-producing plant pathogenic pseudomonads (Fett et al., 1986). A very wide range of molecular weights (3 kDa to 1.5 MDa) have been reported for *Pseudomonas* alginates (Fett et al., 1986; Osman et al., 1986; Gacesa and Russell, 1990). This wide range may reflect the inherent difficulties in accurately determining the molecular weights of large macromolecules, the tendency of alginates to form aggregates and the production of alginate-degrading enzymes (lyases) by the source bacterium. Among the pseudomonads, alginate production is restricted to the members of group I. Nonpseudomonads known to be capable of alginate production are *Azotobacter vinelandii* (Pindar and Bucke, 1975), *A. chroococcum* (Cote and Krull, 1988) and *A. beijerinckii* (Likhoshertov et al., 1991).

When grown with high levels of sucrose as the primary source of carbon alginate-producing Group I pseudomonads capable of utilizing sucrose synthesize alginate, levan or both of these polymers as EPS's (Fett et al., 1986, 1989; Gross and Rudolph, 1987b,c; Osman et al., 1986).

P. marginalis, a Group I fluorescent pseudomonad which causes soft rot of vegetables (Bradbury, 1986), does not produce alginate *in vitro*, but rather several novel acidic EPS's. Several strains of *P. marginalis* were shown to produce an acidic EPS containing galactose and glucose (1:1) substituted with pyruvate (Fig. 1) (Fett et al., 1989; Osman and Fett, 1989). The EPS has a molecular weight greater than 2 MDa and was named marginalan. Marginalan is also produced by several plant-associated saprophytic strains of *P. fluorescens* (Fett et al., 1989) and very similar EPS's have been reported for the plant symbiont *Rhizobium meliloti* (Glazebrook and Walker, 1989) and for *Achromobacter* spp. (Zevenhuizen and Ebbinck, 1974). A second acidic EPS produced by two strains of *P. marginalis* (PF-05-2 and PM-LB-1) contains mannose, glucose and rhamnose (1:1:1) substituted with pyruvate and acetate (Fig. 1, type B) (Osman and Fett, 1990). A third acidic EPS is produced by the type strain (ATCC 10844) of *P. marginalis* and is composed of glucose and fucose (2:1) substituted with lactic acid (1-carboxyethyl) (Fig. 1, type C) (Osman and Fett, 1993). Marginalan is present only as a slime while the other two novel EPS's appear to be present both as capsules and slime.

Marginalan producers do not synthesize levan when grown in the presence of sucrose in contrast to *P. marginalis* strains ATCC 10844, PF-05-2 and PM-LB-1 which do synthesize levan (Fett et al., 1989; Osman and Fett, 1990, 1993).

The Group II plant pathogenic pseudomonads are *P. andropogonis*, *P. caryophylli*, *P. cepacia*, *P. gladioli*, *P. glumae*, *P. solanacearum*, *P. syzygii* and *P. rubrisubalbicans* (Young et al., 1992). The most important of these pathogens from an economic standpoint is *P. solanacearum* which induces wilt on more than 400 plant species in 30 different families. The bacterial species has been further divided by various researchers into races, biovars and pathotypes (Young et al., 1992). This pathogen produces large amounts of EPS as a slime on solid media. Over the past several years there have been many studies of the nature of this EPS, with highly variable results. Recently, the complete structure of the main acidic component, which

made up 40% of the EPS by weight, was reported for a tomato strain of the pathogen (Orgambide et al., 1991) (Fig. 1). This component contains equimolar amounts of N-acetylgalactosamine and the unusual sugars 2-N-acetyl-2-deoxy-L-galacturonic acid and 2-N-acetyl-4-N-(3-hydroxybutanoyl)-2,4,6-trideoxy-D-glucose. A second major primarily nonglycosidic component (also 40% of the total EPS by weight) contained mannose and glucose. A third minor component contained primarily glucose. The strong aggregation of these components and the unusual sugars present were suggested to be two reasons for the variable EPS composition previously reported for *P. solanacearum* (Orgambide et al., 1991). Based on sugar analysis alone, results of a study of the EPS composition of 17 strains isolated from various hosts in Japan and the U.S.A. indicated that production of the major acidic EPS component described above is common to all strains of *P. solanacearum*, but that strain-dependent variability occurs in the amounts of the various additional EPS components produced (Akiyama et al., 1986b). The molecular weight of unfractionated EPS of *P. solanacearum* was estimated by gel filtration to be 600 kDa (Akiyama et al., 1986a).

P. cepacia is an opportunistic plant, animal and human pathogen. Its primary plant host is onion (*Allium cepa*) on which it causes a rot of the bulb scales (Bradbury, 1986). Several soil, clinical and plant pathogenic strains produced large amounts of EPS on an agar medium with various carbon sources (Sage et al., 1990). The EPS's from selected clinical and soil strains were further analyzed and most contained galactose, glucose, mannose, rhamnose and glucuronic acid (2:1:1:1:1). The EPS from a single strain contained glucose and galactose (3:1). None of the EPS's from the plant pathogenic strains were analyzed and no structural data for any of the EPS's was given (Sage et al., 1990).

An EPS of similar sugar composition as that produced by *P. cepacia*, but different molar ratios, was recently isolated from *P. andropogonis* (Osman and Fett, unpublished). This pathogen has a wide host range causing leaf striping of Gramineae and leaf spots in legumes (Bradbury,

1986). Results to date indicate that the acidic EPS is highly complex, with a repeating unit of eight or nine sugars.

There are no reports on EPS production by the Group III plant pathogenic pseudomonads and Groups IV and V do not contain any plant pathogenic *Pseudomonas* species.

E. *Xanthomonas*

The genus *Xanthomonas* currently contains five species (*albilineans*, *axonopodis*, *campestris*, *fragaria*, *oryzae*) with two others proposed ("graminis" and "populi") (Young et al., 1992). As a group these pathogens primarily cause spotting, streaking or scorching of affected leaves, but also may cause shoot die back, cankers and wilt. *X. campestris* is the most important pathogen and has been further subdivided into 143 pathovars (Young et al., 1992).

Xanthomonads produce an acidic EPS called xanthan gum (Misaki et al., 1962; Sutherland, 1981). Xanthan gum contains glucose, mannose and glucuronic acid (2:2:1) substituted with varying amounts of acetate and pyruvate (Fig. 1) (Jansson et al., 1975). The EPS is of high molecular weight with reported values ranging from 2 to 62 MDa (Kennedy and Bradshaw, 1984). Due to its unique rheological properties xanthan gum is the leading microbial gum used for commercial applications. It is employed as a thickener, stabilizer and suspending agent for a variety of food and nonfood applications (Kennedy and Bradshaw, 1984). The commercial source of xanthan gum is a strain of *X. c. pv. campestris*, causal agent of black rot disease of cruciferous crops.

III. BIOSYNTHESIS AND REGULATION OF EPS PRODUCTION

The study of the biosynthesis of EPS production by plant pathogenic bacteria as well as the regulatory mechanisms involved is still in its infancy. In general, EPS production can be affected by a variety of cultural and environmental factors. Various growth conditions such as level of aeration, temperature, availability and nature of

nitrogen and carbon sources, relative amounts of carbon and nitrogen sources, and availability of certain ions can all influence the amount of EPS formed and its composition, especially the nature and amount of nonsugar substituents (Sutherland, 1982). Regulation of EPS production can be at the level of synthesis and availability of sugar nucleotides and isoprenoid lipid intermediates, and synthesis and activation of the various enzymes involved. There is often competition for precursors between EPS synthesis and synthesis of wall polymers such as lipopolysaccharides (LPS) and teichoic acids. Two excellent reviews on the molecular genetics of EPS production by plant pathogenic bacteria have recently appeared (Coplin and Cook, 1990; Leigh and Coplin, 1992).

A. Homopolysaccharides

The homopolysaccharide levan produced by several plant pathogenic pseudomonads as well as *E. amylovora* is unusual in that its synthesis requires a specific substrate (sucrose) and takes place extracellularly through the action of the enzyme levansucrase. Since levan is synthesized outside of the bacterial cell, its synthesis does not depend on substrate uptake or the availability of sugar nucleotides. Levansucrase (fructanhydrolase) has been isolated and characterized from both *E. amylovora* (Gross et al., 1992) and *P. s. pv. phaseolicola* (Gross and Rudolph, 1987c; Gross and Rollwage, 1987). Production of levansucrase by these two bacteria is constitutive.

In contrast, the synthesis of other homopolysaccharides such as curdlan and cellulose, as well as of heteropolysaccharides, requires that substrates enter the cell and not be solely used for production of energy or cell wall components. Cellulose production by *A. tumefaciens* is constitutive as synthesis can occur during growth in the absence of plant cells and in resting cells. However, the presence of plant cells or extracts induces increased cellulose synthesis. A Mg^{2+} -dependent cellulose synthase (UDP-glucose: 1,4- β -D-glucan glucosyltransferase) is produced and may be membrane-bound (Amikam and Benziman, 1989; Thelen and Delmer, 1986). Bacterial cellulose is formed intracellularly without the

involvement of lipid-linked intermediates and then is excreted through extrusion pores located in the outer membrane. Cellulose synthase is subject to complex regulatory control. A novel cyclic dinucleotide molecule, c-di-GMP, acts as a reversible, allosteric activator. The level of this effector molecule is positively controlled by diguanylate cyclase and negatively controlled by c-di-GMP phosphodiesterase (Amikam and Benziman, 1989; Ross et al., 1991). The genes for cellulose synthesis are most likely chromosomally located (Matthysse et al., 1981). Little is known about the biosynthesis or regulation of curdian production. Based on studies with radiolabelled glucose, synthesis of the polymer was demonstrated to be primarily directly from glucose rather than through triose phosphates (Kai et al., 1993).

B. Heteropolysaccharides

Biosynthesis of succinoglycan by *A. tumefaciens* is via a lipid-linked intermediate. The genes involved are located on the chromosome and not the Ti plasmid (Cangelosi et al., 1987; Staneloni et al., 1984). The defect in an *exoC* mutant which does not produce succinoglycan or cyclic β -1,2-glucan was due to a deficiency in the activity of the enzyme phosphoglucomutase which converts glucose-6-phosphate to glucose-1-phosphate (Uttaro et al., 1990). Glucose-1-phosphate is an intermediate in the synthesis of UDP-glucose. Genes involved in succinoglycan production in *A. radiobacter* are located in at least five distinct genomic regions (Aird et al., 1991). One *exo* mutant of *A. radiobacter* could be corrected by the *R. meliloti* *exoB* gene which encodes UDP-galactose 4-epimerase. On agar media, production of succinoglycan by *A. radiobacter* is unstable and spontaneous mutants which produce reduced amounts of succinoglycan and increased amounts of curdian are readily isolated (Hisamatsu et al., 1977). Instability of succinoglycan formation by plant pathogenic agrobacteria has not been reported.

Studies on the regulation of EPS synthesis by nonpectolytic plant pathogenic *Erwinia* species has benefited from the extensive studies of Gottesman and coworkers on regulation of

synthesis of colanic acid (a capsular EPS) by the related bacterium *E. coli* (for a comprehensive review see Gottesman and Stout, 1991). The synthesis of colanic acid is under complex regulatory control at the transcriptional level. Two positive regulatory proteins, RcsA and RcsB, which increase expression of the capsular polysaccharide synthesis (*cps*) genes, have been identified. The availability of RcsA is usually limited due to degradation by the Lon ATP-dependent protease which acts as a negative regulator. In a second control mechanism, RcsB is the effector component of a two-component environmentally responsive regulatory system with the cytoplasmic membrane spanning sensor component designated RcsC. RcsC may be a phosphorylase/kinase which activates RcsB allowing this activated protein to in turn activate transcription of *cps* genes. RcsA may also interact with RcsB forming a complex which activates *cps* genes. RcsB has extensive sequence homology to AlgR1, a positive regulatory protein involved in alginate production by pseudomonads (see below). The *rscB* gene also appears to be involved in the regulation of cell division (Gervais et al., 1992). Recently, an additional regulatory gene designated *rscF* was cloned and is proposed to promote the phosphorylation of *rscB* leading to enhanced synthesis of colanic acid (Gervais and Drapeau, 1992).

The biosynthesis of EPS by *E. stewartii* and its regulation have been extensively studied by Coplin and associates. The *cps* genes of *E. stewartii* are located on a 10-kb cluster on the chromosome and are arranged in a minimum of five complementation groups (*cpsA* through *cpsE*) (Coplin and Cook, 1990; Leigh and Coplin, 1992). Adjacent to the cluster of genes is the *galE* gene (UDP-galactose-4-epimerase) which provides UDP-sugar precursors for EPS synthesis. Mutagenesis with *TnPhoA* indicated that *cpsB* and *cpsC* encode proteins which are located on the membrane, in the periplasmic space, or are exported out of the cell and may be glycosyltransferases or may have export functions. Based on sequencing and complementation data, regulation of EPS synthesis by *E. stewartii* appears to be very similar to that for colanic acid

production by *E. coli*. Regulatory genes with homology to the *rcsA*, *rcsB* and *rcsC* genes are involved, as well as a protease. The *rcsA* gene from *E. stewartii* can complement *rcsA* mutants of *E. coli*, reversing the defective production of colanic acid. A 7-kb chromosomal gene cluster (*ams*) involved in the production of amylovoran by *E. amylovora* has also been identified based on its ability to confer a mucoid phenotype to *E. stewartii* *cps* mutants (Bernhard et al., 1993). Four of the five *ams* complementation groups were functionally equivalent to the *E. stewartii* *cps* groups. The *ams* clones can restore mucoidy to *E. stewartii* mutants, and the *cps* gene cluster could complement mutations in the *E. amylovora* *ams* genes. The *rcsA* gene from *E. amylovora* and *E. stewartii* has been cloned and sequenced (Bernhard et al., 1990; Chatterjee et al., 1990; Coleman et al., 1990; Poetter and Coplin, 1991). It is highly homologous to the *rscA* gene of *Klebsiella* spp. and can restore colanic acid synthesis to *rscA* mutants of *E. coli*. The RcsA protein is a member of the LuxR family of bacterial activator proteins and may directly bind to *cps* promoters resulting in the activation of EPS synthesis (Poetter and Coplin, 1991). The *rscA* gene is also a positive regulator of levan production by *E. amylovora* (Bernhard et al., 1990) and activates the biosynthesis of structurally distinct EPS's in *Salmonella typhimurium* as well as *E. coli* (Chatterjee et al., 1990).

Studies on the biosynthesis and regulation of EPS production by *P. solanacearum* have until recently been hampered by the lack of structural information. Another difficulty with this pathogen is its propensity to form pleiotropic EPS-deficient mutants in culture. At least three clusters of genes are involved in EPS biosynthesis. By *Tn5* mutagenesis Denny and Baek (1991) identified two regions separated by about 7-kb. Region I contains 2 or more genes and is about 9-kb in size while region II is 2.6-kb and most likely contains a single gene. Class I mutants produce about 95% less EPS on rich and minimal media and 75 to 90% less EPS *in planta* while class II mutants produce nearly wild-type levels of EPS on minimal media and *in planta*, but produce low amounts on rich media. A third unlinked gene cluster containing

seven complementation groups was identified by *Tn3* mutagenesis (Cook and Sequeira, 1991; Kao et al., 1993). Kao and Sequeira (1991) demonstrated that this third, constitutively expressed, gene cluster is also required for LPS synthesis and the complementation units were renamed to *ops* (outer membrane polysaccharide). The complementation unit *opsG* was recently reported to encode enzymes involved in the synthesis of rhamnose (Kao et al., 1993). Four open reading frames were identified which showed homology to the *rfb* operon of *Salmonella typhimurium*. The *rfb* operon encodes the enzymes responsible for LPS O-antigen biosynthesis (Brahmbhatt et al., 1988).

Regulation of EPS production in *P. solanacearum* is dependent on the functioning of a highly complex interacting network (Schell et al., 1992). For full expression of EPS production the products of at least five unlinked regulatory genes (*phcA*, *phcB*, *vsrA*, *vsrB*, and *xpsR*) are required. The *phcA* gene (phenotypic conversion) is a positive regulator which controls not only EPS production but also motility and production of polygalacturonase and endogluconase. The protein PhcA belongs to the LysR group of transcriptional activators and may sense an endogenous volatile signal molecule. A second activator protein is XpsR, while AsrA and VsrB are putative membrane-associated proteins (Leigh and Coplin, 1992). Evidence for a single transcriptional unit being responsible for phenotypic conversion of *P. solanacearum* was also reported by Huang and Sequeira (1990) and in certain strains a small plasmid may be involved (Negishi et al., 1993).

The biosynthesis and regulation of alginate production has been extensively studied using clinical strains of *P. aeruginosa* isolated primarily from the lungs of cystic fibrosis patients where the bacterium causes a serious chronic infection leading to morbidity and death (for a review see May et al., 1991). Genes affecting alginate production are located on three separate chromosomal regions. The biosynthetic pathway from fructose-6-phosphate to GDP-mannuronate in clinical strains of *P. aeruginosa* has been demonstrated and the structural genes cloned. Fructose-6-phosphate is first isomerized to

mannose-6-phosphate by phosphomannose isomerase (*algA*). Mannose-6-phosphate is then converted to mannose-1-phosphate by phosphomannomutase (*algC*) followed by the synthesis of GDP-mannose via GTP and GDP-mannose pyrophosphorylase (*algA*). The encoding of two enzymes which catalyze nonsequential steps of a biosynthetic pathway by a single gene, as does *algA*, is unusual. Finally, GDP-mannose is reduced to GDP-mannuronic acid by GDP-mannose dehydrogenase (*algD*). This last step is thought to be specific for alginate synthesis and the reaction is nonreversible. The *algA* and *algD* genes are located in a cluster of alginate genes around the 34 minute region of the chromosome while *algC* maps at 10 minutes (Zielinski et al., 1991) in a region containing a cluster of alginate regulatory genes (see below). There is no conclusive evidence for the involvement of lipid bound intermediates. A similar pathway appears to be used by the additional pseudomonads known to be capable of alginate synthesis based on results of Southern blot analysis using alginate structural genes cloned from *P. aeruginosa* as probes (Fett et al., 1992; Fiahlo et al., 1990). The subsequent steps of polymerization, export, acetylation of mannuronic acid and epimerization of some of the mannuronic acid residues to guluronic acid are not well defined.

Even though all strains of *P. marginalis* studied to date, as well as many strains of *P. fluorescens*, produce acidic EPS's other than alginate *in vitro* (Fett et al., 1989; Osman and Fett, 1989, 1990, 1993), Southern blot analysis under high stringency conditions using cloned structural alginate genes isolated from *P. aeruginosa* as probes demonstrated that these bacteria do contain chromosomal DNA with homology to *algA*, *algC* and *algD* (Fiahlo et al., 1990). This indicates that alginate production by *P. marginalis* and certain strains of *P. fluorescens* might be under tight regulatory control and that the bacteria may be capable of alginate production under as yet an undefined set of environmental conditions. Alternatively, the alginate structural genes may be nonfunctional. Interestingly, *A. chroococcum* and *A. beijerinckii* simultaneously produce alginate and a second distinct acidic EPS in liquid culture (Cote and Krull, 1988; Likhoshervostov et al., 1991).

The regulation of alginate biosynthesis by the pseudomonads is very complex and several excellent recent reviews are available (Deretic et al., 1991; Govan et al., 1992; May et al., 1991; Ohman and Goldberg, 1990). Almost all the studies have concentrated on *P. aeruginosa* because of its association with cystic fibrosis. Initially, lung-colonizing strains are nonmucoid, but they invariably switch to a mucoid phenotype. When cultured, these alginate-producing strains readily revert to the nonmucoid form. Several environmental factors are known to affect alginate production by pseudomonads. Incubation temperatures lower than those optimal for growth are often optimal for alginate production (Fett et al., 1989; Leitao and Sa-Correia, 1993). High osmolarity, membrane disruption induced by ethanol, nitrogen source and nitrogen or phosphate starvation can also lead to increased alginate production by mucoid strains (May et al., 1991; Singh et al., 1992). Most of these environmental factors are believed to function through the activation of the *algD* promoter. Transcriptional activation of *algD* is strictly required for alginate synthesis in *P. aeruginosa*. The environmental signals are perceived by the positive regulatory proteins AlgR1 and AlgR2 (also referred to as AlgR and AlgQ, respectively). AlgR1 is a response regulator located at 9 minutes on the chromosomal map with homology to members of a class of two-component bacterial sensory transducers including OmpR, NtrC and VirG (Ronson et al., 1987). The phosphorylated form binds to DNA upstream of the *algD* and *algC* promoters and activates transcription of *algC*, *algD* and *algR1* (Zielinski et al., 1992). Gene *algR1* appears to be part of a global regulatory network as it also functions as a transcriptional regulator of the neuraminidase gene *nana* (Cacalano et al., 1992). AlgR2 has been proposed to be a kinase (the sensor component) undergoing autophosphorylation and activating AlgR1 by transfer of the phosphate (Roychoudhury et al., 1992a,b). Phosphorylation of AlgR1 appears to be required for its activity. AlgR3 (also referred to as AlgP) is a histone H1-like DNA binding protein and may also be involved in sensing environmental signals possibly controlling supercoiling of the *algD* promoter

region (Deretic et al., 1992; Kato et al., 1990). The genes *algR2* and *algR3* are constitutively transcribed and all three genes are located at approximately 8 minutes on the *P. aeruginosa* chromosome map. A nearby additional positive regulatory gene (*algB*) which promotes transcription of *algD* and is a member of the NtrC subclass of two-component regulators has also been described (Goldberg and Danke, 1992; Wozniak and Ohman, 1991). A second DNA region involved in the regulation of alginate synthesis is located late on the chromosomal map. This region contains several genes with two tightly-linked genes (*algS* and *algT*) responsible for the switching on and off of alginate production. Gene *algS* appears to be a cis-acting regulator that controls the expression of the trans-active *algT* gene (Flynn and Ohman, 1988). The *algT* gene product positively regulates *algB* transcription (Wozniak and Ohman, 1993). This region also contains a negative regulatory gene (*algN*), which may exert its effects on *algT* (Goldberg et al., 1993), and an additional gene (*algU*) which affects the mucoid phenotype and transcription of *algD* and shows sequence similarity with a sigma factor (Martin et al., 1993).

Alginate-producing pseudomonads other than *P. aeruginosa* may utilize a similar regulatory network, as the presence of gene homologues of *algR1*, *algB* and *algST* have been demonstrated by Southern blot hybridization (Fiahlo et al., 1990; Fett et al., 1992; Goldberg and Fett, unpublished). Interestingly, the transcriptional activator RcsB involved in colanic acid synthesis by *E. coli*, as discussed above, is very similar to AlgR1, having extensive sequence homology, nearly identical molecular weights, activating their own synthesis and exhibiting responsiveness to osmolarity (Gervais et al., 1992). A global regulatory network in *P. aeruginosa* may also include a cyclic AMP-like receptor protein with homology to a CRP-analogue in *X. campestris* (see below) (de Crecy-Ligard et al., 1990; DeVault et al., 1991).

Studies on the biosynthesis of xanthan gum have centered on *X. campestris* pv. *campestris* strain NRRL B-1459, which is the strain used for commercial production of xanthan gum. The genes required for synthesis of the lipid-bound repeating

unit, for polymerization and for export have been located in a contiguous 16-kb cluster (Barrere et al., 1986; Harding et al., 1987; Vanderslice et al., 1989). Twelve open reading frames were found (designated *gumB* through *gumM*) and the region appears to be a single operon. The pentasaccharide repeating unit is built up sequentially on a pyrophosphate lipid carrier in the cytoplasmic membrane (Ielpi et al., 1981a,b, 1993) using the appropriate sugar nucleotides and five specific glycosyltransferases encoded by *gumD*, *gumM*, *gumH*, *gumK*, and *gumI* (Vanderslice et al., 1989). Pyruvate is added to the terminal mannose residue of the pentasaccharide-P-P-lipid with phosphoenolpyruvate as the donor molecule (Ielpi et al., 1981a) via the enzyme ketal pyruvate transferase. The pyruvate transferase gene (*gumL*) has been cloned (Marzocca et al., 1991). Acetate is also added to the internal mannose residue at this stage via acetyl coenzyme A (Ielpi et al., 1983) and acetylase I encoded by *gumF*. A second acetylase is encoded by *gumG* and the enzyme is responsible for acetylation of the terminal mannose when pyruvylation of the outer mannose is blocked (Hassler and Doherty, 1990). The additional genes *gumB*, *gumC*, and *gumE* appear to be involved in polymerization steps and *gumJ* in the final export step (Vanderslice et al., 1989). Hotte et al. (1990) characterized a second large (35.3-kb) gene cluster involved in xanthan gum production, but no gene functions were reported. More recently, a total of seven unlinked chromosomal DNA regions required for xanthan gum production were isolated (Harding et al., 1993). Three of these regions (*xpsIII*, *-IV* and *-VI*) were demonstrated to be involved in the synthesis of sugar nucleotides. These regions are not linked to the genes required for assembly of the lipid-bound repeating unit. The amino acid sequence deduced from the nucleotide sequence of gene *xanB* encoding for the bifunctional enzyme phosphomannose isomerase-GDP-mannose pyrophosphorylase showed 59% homology to AlgA (Koplin et al., 1992).

Regulation of xanthan gum production appears to be highly complex. The instability of xanthan gum production *in vitro* was recognized several years ago (Kidby et al., 1977). A correlation between reduction in xanthan gum

production and appearance of spontaneous chemotactic variant forms has been reported (Kamoun and Kado, 1990). Production is temperature sensitive with maximum production (between 30 and 33°C) above the optimal temperatures for growth (27 to 30°C) (Shu and Yang, 1990). At least two positive regulator genes and one negative regulator gene are involved. A two-component regulatory system affects not only xanthan production but also regulates the synthesis of extracellular enzymes required for pathogenicity (Daniels et al., 1989; Tang et al., 1991). An additional two-component regulatory system is specific for xanthan gum production (Osborn et al., 1990). Southern blot analysis indicated that *X. campestris* contains a homolog of the alginate positive regulatory *algR1* gene (Fiahlo et al., 1990). The negative regulatory gene also coordinately represses extracellular enzyme production (Tang et al., 1990). Xanthan gum production is further regulated by a catabolite gene activator protein, a global regulator of gene expression similar to the catabolite gene activator protein of *E. coli* (de Crecy-Lagard et al., 1990; Dong and Ebright, 1992). This protein also regulates synthesis of protease and polygalacturonate lyase.

IV. ROLE OF EPS IN HOST-PATHOGEN INTERACTIONS

There is much presumptive evidence which indicates that bacterial EPS is important for the full expression of symptoms in several host-pathogen interactions, but is not a determinant of host specificity. Evidence for a role of EPS as a virulence factor includes the demonstration of production of EPS *in planta*, a reduction in symptom formation by naturally occurring and chemical- or transposon-induced EPS-deficient mutants, and induction of typical symptoms by treating plants with pathogen EPS produced *in vitro* or *in planta*. Most EPS's are very hygroscopic consisting of 99% water and bind cations due to their anionic nature (Sutherland, 1990). Thus, their production *in planta* may promote a favorable growth environment early in infection especially in normally air-filled intercellular leaf spaces. Due to their normally high viscosity, EPS's may cause

vascular plugging by xylem-inhabiting pathogens leading to wilting of the host plant. Production of EPS may favor disease development by masking bacterial cell wall elicitors, thus inhibiting recognition and subsequent plant defense reactions such as synthesis of phytoalexins and pathogenesis-related proteins. EPS may also interfere with the agglutination and immobilization of invading bacteria as a defense response mediated by plant lectins. A coating of EPS may protect the bacterial cells from preformed or induced plant antimicrobial compounds. However, the evidence for these various purported roles of EPS in the disease process is not conclusive. Selected examples of the kinds of evidence available in the literature for a role of bacterial EPS in host-pathogen interactions are given below.

A. *Agrobacterium*

A prerequisite for pathogenicity of *A. tumefaciens* and *A. rhizogenes* is the ability to bind to the plant wound site (Matthysse, 1986). The plant and bacterial components involved in the initial attachment have not yet been conclusively established, but EPS's do not appear to be involved. However, based on studies with Tn5-induced cellulose-minus mutants of *A. tumefaciens*, synthesis of cellulose fibrils by agrobacteria appears to mediate secondary bacterial cell aggregation after initial attachment to the wound site (Matthysse, 1983). This aggregation leads to stronger site adhesion and may be important for infection under field conditions, as the cellulose-bound bacteria are more difficult to wash away from wounds. Formation of fibrils by the wild-type pathogen at the surface of mesophyll cells in inoculated tobacco leaves has been demonstrated by the scanning electron microscope. These fibrils were presumed to be of bacterial origin and composed of cellulose based on similar experiments with cellulose-minus mutants (Matthysse, 1983). The nature of the defect(s) in the Tn5-induced cellulose-minus mutants have not yet been reported.

Production of succinoglycan or curdlan during the interaction of *A. tumefaciens* with host plants has not been demonstrated. Ultrastructural studies demonstrated the presence of fibrillar

material surrounding bacterial cells in the xylem vessels of infected grape which was suggested to be EPS (Tarbah and Goodman, 1988). The results of three studies indicate that production of succinoglycan by *A. tumefaciens* may not be required for full symptom expression. Cangelosi et al. (1987) generated mutants (*exo*) of *A. tumefaciens* deficient in succinoglycan formation by Tn5 and chemical mutagenesis. These mutants were complemented by each of five cloned *Rhizobium meliloti* *exo* loci involved in succinoglycan production. The *A. tumefaciens* *exo* mutants were fully virulent on several different plant hosts. Close et al. (1987) also reported that the pleiotropic *ros* mutant strain LBA4301, which did not produce succinoglycan, was not reduced in virulence. This *ros* mutant strain was subsequently used to clone a chromosomal loci (*psdA*) which appears to be a negative regulatory gene for succinoglycan production. Tranconjugants containing extra copies of *psdA* were reduced three to six-fold in succinoglycan production, but were not reduced in virulence (Kamoun et al., 1989). However, none of these studies demonstrated the lack of EPS production by the mutants *in planta* by cytological or direct isolation techniques.

B. *Clavibacter*

Clavibacter michiganensis subsp. *insidiosus*, *michiganensis* and *sepedonicus* colonize the xylem vessels of their host plant and cause wilting. Cells of *C. m.* subsp. *michiganensis* colonizing the xylem of susceptible tomato leaves were found to be surrounded by fibrillar and granular material possibly composed of a mixture of bacterial EPS and host-derived material (Benhamou, 1991). Several other indirect lines of evidence indicate that EPS is a virulence factor for this group of vascular wilt pathogens acting by physically blocking xylem vessels along with possible toxic effects. First, nonmucoid avirulent variants of *C. m.* subsp. *insidiosus* have been reported (Fulkerson, 1960). Nonmucoid strains of *C. m.* subsp. *sepedonicus* which retained their virulence have also been reported, but confirmation of the absence of EPS production *in planta* was not provided (Westra and Slack, 1992). Second, EPS

produced by these bacteria when grown on culture media is capable of inducing wilting of host and nonhost plants in *in vitro* assays. Van Alfen et al. (1987) radiolabelled the three components (molecular weights >5 MDa, 5 MDa, and 22 kDa) of the EPS produced *in vitro* by *C. m.* subsp. *insidiosus* by conjugation with L-(¹⁴C)-lysine and determined their ability to move through alfalfa cuttings. The largest component (> 5 MDa) could not pass through the pit membranes of the stem and accumulated there. The 5 MDa component was able to pass through pit membranes of the stem, but could not pass through leaf traces. The smallest component could pass through all sizes of pit membranes and accumulated in the leaves. Treatments with each of the three components resulted in reduced transpiration of alfalfa cuttings with their relative effectiveness dependent on size; the higher the molecular weight the greater the reduction. EPS directly isolated from plants infected with either subsp. *insidiosus* or *sepedonicus* can induce wilting in *in vitro* assays (Spencer and Gorin, 1961). Third, EPS can be isolated from infected plants (van den Bulk et al., 1991). Apart from reducing transpiration by physical blockage of xylem vessels, it has been proposed that EPS may also induce the formation of tyloses by xylem parenchyma cells which would interfere with water flow (Eichenlaub et al., 1991). In addition, EPS may have direct toxic effects on plant cells as indicated by the inhibition of callus development from protoplasts of the wild tomato species *Lycopersicon peruvianum* by EPS of *C. m.* subsp. *michiganensis* (van den Bulk et al., 1990).

C. *Erwinia*

Studies on the role of EPS in pathogenicity of *Erwinia* species have centered on *E. amylovora* and *E. stewartii*. On its rosaceous plant hosts, *E. amylovora* invades cortical cells resulting in canker formation as well as colonizes the xylem leading to wilting. Spontaneous mutants of *E. amylovora* which appeared nonmucoid on solid media were of reduced virulence (Ayers et al., 1979; Bennett and Billing, 1980). As noted above, *E. amylovora* is capable of producing two distinct EPS's, amylovoran (an acidic heteropolysac-

charide) and levan (a neutral fructan). Bacterial ooze emanating from severely infected shoots of host plants and from the surfaces of inoculated pear slices was found to be of similar composition to amylovoran produced *in vitro* (Bennett and Billing, 1980). Ooze formation may be due to swelling of copious amounts of highly hygroscopic EPS within diseased plant parts (Schouten, 1989). Treatment of shoots with amylovoran leads to non-host specific wilting due to physical blockage of the xylem and not by a direct toxic effect (Beer et al., 1983; Sjulín and Beer, 1978). Ultrastructural studies have supported the plugging of xylem vessels with EPS (Suhayda and Goodman, 1981). More recent studies using EPS-impaired and amylovoran-negative deficient mutants indicate that production of amylovoran is required for full expression of virulence (Bellemann and Geider, 1992; Bernhard et al., 1990; Steinberger and Beer, 1988; Vanneste et al., 1990). That an additional factor(s) is required for disease induction was also indicated by the identification of encapsulated avirulent mutants (Bennett, 1980). In addition, amylovoran itself does not appear to be capable of inducing electrolyte leakage of host cells (Brisset and Paulin, 1992). Both amylovoran and levan induced the production of phenylalanine ammonia-lyase in cultured parsley cells (a nonhost), but not in cultured pear cells (a host), indicating a possible role of these EPS's in host-pathogen specificity (Schwartz and Geider, 1990).

Tn5-generated mutants of *E. amylovora* deficient in production of levan and levansucrase were still fully virulent on pear slices, but gave delayed symptoms on pear seedlings, indicating that levan may be a virulence factor even though its actual presence in diseased plants has not been demonstrated by reisolation and chemical characterization (Geider et al., 1991).

E. stewartii causes a vascular wilt of sweet corn (Stewart's wilt) and leaf blight of field corn. Growth in the xylem of infected sweet corn leads to restriction in water movement and wilting, whereas growth in the leaf intercellular spaces results in the formation of water-soaked lesions. Early studies indicated a relationship between the ability to produce EPS *in vitro* and virulence (Ivanoff et al., 1938). Ultrastructural studies

comparing wild-type and naturally occurring nonmucoid variants also indicated that EPS production *in planta* leads to increased growth and spread in host vascular tissues as well as occlusion of xylem vessels (Braun, 1990). This is in accord with van Alfen's hypothesis (van Alfen, 1982) that the physical pressure produced due to the swelling of hygroscopic EPS may assist in the spread of the bacteria in the vascular system of infected plants. However, production of EPS in infected plants has not yet been confirmed by reisolation and chemical analysis. An earlier purported role for capsular EPS production in inhibiting a host bacterial cell immobilization defence response (Bradshaw-Rouse et al., 1981) was not substantiated by ultrastructural studies as no capsules were formed by the nonmucoid mutants and yet they were not immobilized or agglutinated in the xylem vessels (Braun, 1990).

Genetic evidence for a role of EPS in virulence of *E. stewartii* comes from the studies of Coplin and associates. Nonmucoid strains with mutations in *cps* genes were all unable to cause wilting of corn seedlings and were either completely avirulent on leaves or caused the formation of restricted, necrotic lesions (Coplin and Majerczak, 1990). The mutants produced 5 to 10% of the amount of EPS produced *in vitro* by the wild-type parent on solid media. The inability to cause water-soaked lesions was demonstrated not to be due to a reduced ability for *in planta* growth for all but one of the mutants. The occurrence of mutants which are not defective in EPS production, but are unable to cause wilting or water-soaked lesions, indicates the production of an additional unknown cell-leakage factor required for pathogenicity (Coplin et al., 1992a,b). Inhibition of capsular EPS production in resistant corn plants does not appear to be a defence mechanism as capsules on a virulent strain were noted in both resistant as well as susceptible corn leaves at 24 to 30 hours after inoculation (Braun, 1990).

No information is available on the production of EPS by the soft-rotting pathogens *Erwinia carotovora* subsp. *atroseptica* and subsp. *carotovora*, but ultrastructural observations of cells of subsp. *atroseptica* in rotted potato tuber tissue indicated that capsular EPS may be formed *in*

planta (Lyon et al., 1989). Capsular EPS may protect these pathogens from desiccation in the early stages of infection. In instances where infection occurs through wounds, capsular EPS may protect against wound-induced toxic chemicals and enzymes.

D. *Pseudomonas*

A relationship between EPS production *in vitro* and pathogenicity of the vascular wilt pathogen *P. solanacearum* was noted many years ago for spontaneous nonmucoid variants which are readily formed during culture (Husain and Kelman, 1958; Kelman, 1954). These variants are pleiotropic, exhibiting a variable array of biochemical differences with the wild-type (Brumley and Denny, 1990; Huang and Sequeira, 1990). Denny and associates (Denny and Baek, 1991; Denny et al., 1988) first provided genetic evidence that EPS is a virulence factor for *P. solanacearum*. They reported that a Tn5-induced mutant strain (Aw1-1)(their class I type) which produced only about 5% of the wild-type parent level of EPS on both rich and minimal medium lost the ability to cause wilting of tomato plants. This mutant was also unable to produce EPS *in planta*. In contrast, three additional Tn5 class II mutants were also impaired in the ability to produce EPS on rich media, but were still fully virulent. These anomalous results were later explained by the findings that these class II mutants could produce near wild-type amounts of EPS on minimal media and also produced EPS *in planta*. Conflicting results were initially obtained in similar studies by Sequeira and associates which indicated that Tn5-induced mutants of strain K60 which were impaired in EPS production retained their full virulence when inoculated at high concentrations into tobacco stems (Xu et al., 1990). Further studies by this group with two of these mutants showed they were similar to the class I mutants of Denny and Baek (1991) and that they were impaired in virulence to eggplant and tobacco when lowered inoculum concentrations were employed (Kao et al., 1992). Additional studies by Cook and Sequeira (1991) using Tn3 mutagenesis further supported an important role for EPS in the *in*

planta bacterial growth and virulence of *P. solanacearum*.

EPS may also be a virulence factor for other plant pathogenic pseudomonads. Ultrastructural studies indicate that fibrillar material, which stains with cationic ruthenium red and is most likely alginate, accumulates around bacteria as early as 1 hour after inoculation of susceptible host leaves with *P. syringae* pathovars that induce the formation of water-soaked lesions (Brown and Mansfield, 1988; Drews et al., 1988). In addition, a relationship between the ability of strains of the bean (*Phaseolus vulgaris*) pathogen *P. s. pv. phaseolicola* to produce EPS *in vitro* and their relative virulence has been reported (Gross and Rudolph, 1987a). Production of EPS by *P. syringae* pathovars in leaves of inoculated plants has been confirmed by the isolation of alginate from leaves of susceptible plants showing water-soaked lesions due to infection with *P. s. pvs. phaseolicola, lachrymans* and *tomato* (Fett and Dunn, 1989; Gross and Rudolph, 1987d). Alginate could be isolated from infected leaves before water-soaked lesions were evident. A role for alginate in the formation of water-soaked symptoms was indicated since only a trace amount of alginate was recovered from susceptible bean leaves infected with *P. s. pv. syringae*, a pathogen which induces only necrotic, dry lesions (Fett and Dunn, 1989). The amount of guluronic acid present in the polymer produced in susceptible soybean leaves by the soybean pathogen *P. s. pv. glycinea* was cultivar dependent (Osman et al., 1986).

Levan production *in planta* by pathovars of *P. syringae* appears uncommon (Fett and Dunn, 1989), but a mixture of levan and alginate in approximately equal amounts was extracted from bean leaves infected with one strain of *P. s. pv. phaseolicola* (Gross and Rudolph, 1987d). Available sucrose concentrations in the plant may be too low to support significant amounts of levan production or the bacteria may preferentially produce alginate due to the hygroscopic nature of alginate, but not levan.

Induction of host-specific persistent water-soaking of leaves injected with EPS from *P. syringae* pathovars was claimed when the treated plants were kept under high humidity

(El-Banoby and Rudolph, 1979), but conflicting results for alginates have been obtained (Fett et al., 1986). Stefani and Rudolph (1989) reported that infiltration of bean leaves with alginate preparations from *P. s. pv. phaseolicola* grown *in vitro* caused chlorosis of bean leaves and delayed both the production of typical disease symptoms induced by compatible bacteria and the hypersensitive response induced by incompatible bacteria if infiltration was done 48 hours before inoculation. These effects appear to be the result of lowered bacterial growth and disruption of chloroplasts (Stefani, 1989; Stefani and Bonatti, 1993). Treatment of leaves with a commercial algal alginate preparation did not induce these effects. Both levan and alginate produced by *P. s. pv. glycinea* *in vitro* did not elicit the production of the soybean phytoalexin glyceollin when applied to cut cotyledon surfaces (Fett et al., 1986).

Differential production of EPS by *P. syringae* pathovars in susceptible and resistant plant hosts does not appear to be involved in determining host-pathogen compatibility. Based on ultrastructural studies, acidic EPS appears to be produced within 1 hour after inoculation of bean leaves both with compatible or incompatible races of the bean pathogen *P. s. pv. phaseolicola* and with the incompatible oat (*Avena sativa*) pathogen *P. s. pv. coronofaciens* (Brown and Mansfield, 1988).

Genetic studies designed to substantiate a role of alginate and levan as virulence factors of *P. s. pv. phaseolicola* have been initiated. Initial results using Tn5-induced alginate impaired mutants supports such a role (Neugebauer et al., 1989).

E. *Xanthomonas*

The production of xanthan gum by xanthomonads in infected susceptible plant leaves has been demonstrated both by indirect means (Al-Mousawi et al., 1982; Jones and Fett, 1985; Sutton and Williams, 1970b) and by reisolation and chemical characterization (Sutton and Williams, 1970a). A role for xanthan gum in disease development is suggested by several reports of reduced virulence of nonmucoid variants of

xanthomonads including *X. c. pv. campestris* (Sutton and Williams, 1970b), *X. c. pv. phaseoli* (Corey and Starr, 1957) and *X. c. pv. oryzae* (Goto, 1972) as well as of increased virulence of variants which overproduce xanthan (Kamiunten and Wakimoto, 1982). Production of xanthan gum in infected host leaves has been proposed to be directly responsible for the development of the typical water-soaked lesions caused by many xanthomonads (El-Banoby and Rudolph, 1979). Induction of persistent water-soaking spots on susceptible, but not on resistant, leaf tissue after injection with high concentrations (3 to 10 mg/ml) of xanthan gum was reported for cotton and barley when treated plants were kept under high humidity (Borkar and Verma, 1989; El-Banoby and Rudolph, 1979). However, injection of rice leaves with 1 mg/ml of EPS (presumably xanthan gum) from *X. c. pv. oryzae*, as well as of citrus leaves with 2 mg/ml of commercial xanthan gum, did not lead to increased electrolyte leakage (Goto and Hyodo, 1985; Vidhyasekaran et al., 1989). Addition of xanthan gum to bacterial suspensions at a final concentration of 1 mg/ml increased the rate of growth of *X. c. pv. citri* in citrus leaves and led to more rapid and extensive symptom formation (Goto and Hyodo, 1985; Webb et al., 1986). It also allowed for the production of foliar citrus-canker like symptoms by two pathovars of *X. campestris* which normally do not produce symptoms on citrus (Webb et al., 1986).

Xanthan gum production may also be important for symptom production caused by xylem-inhabiting xanthomonads which cause vascular wilts. Wilting may be induced by physical occlusion of the water movement system or by increased cell membrane permeability (Vidhyasekaran et al., 1989). A reduction in water uptake, an increase in water potential of the stems and wilting of the first leaves were seen by 1 hour after excised rice seedlings were placed in a solution containing 1 mg/ml of EPS (isolated from the rice pathogen *X. c. pv. oryzae*). Concentrations as low as 1 µg/ml of this EPS were able to cause wilting of the excised rice seedlings, but did not adversely affect water movement. It was hypothesized that both physical disruption of water flow and increased membrane permeability due to

toxic low molecular weight breakdown products of the EPS were responsible for wilt induction. Xanthan gum can interact with various plant polysaccharides and these interactions may lead to increased viscosity in infected xylem vessels and to adhesion of the bacteria to internal plant surfaces (Dea and Morris, 1977).

Xanthan gum has been shown to exert additional effects on host physiology. Treatment of soybean cell suspension cultures with xanthan at 50 µg/ml caused large and rapid increases in phenylalanine ammonia-lyase and chalcone synthase (enzymes of the general phenylpropanoid pathway and the flavanoid pathway, respectively), but no subsequent accumulation of the soybean pterocarpin phytoalexin glyceollin (Ebel et al., 1984). Ethylene production was induced in leaves of citrus 1 to 6 hr after injection with 2 mg/ml of EPS produced *in vitro* by *X. c. pv. citri* (Goto and Hyodo, 1985).

The preferential production of xanthan gum in the early stages of infection of cotton by compatible strains of *X. c. pv. malvacearum* was proposed to inhibit the triggering of the hypersensitive response (HR) by precluding the close contact between bacterial and plant cells required for recognition to occur (Zachowski et al., 1989). This conclusion was based on studies where inoculated susceptible cotton cotyledons were kept either under continuous darkness or under a light/dark regime. In the dark, an HR-like reaction occurred by 48 hours postinoculation. Ultrastructural studies using ruthenium red indicated that acidic EPS production was much reduced when cotyledons were kept in the dark. The reduced amount of EPS was proposed to allow for greater bacterial cell to plant cell contact leading to a resistant response. More recently, Pierce et al. (1993) showed that similar amounts of EPS are produced in resistant and susceptible cotton cotyledons by this bacterium during the first 48 h after inoculation, indicating that EPS production *in planta* does not prevent the hypersensitive response from developing.

Studies by Daniels and coworkers (Barrere et al., 1986; Daniels et al., 1989) demonstrated that EPS-deficient mutants of *X. c. pv. campestris* are severely reduced in their ability to cause

disease when inoculated into vein endings (which is the primary route of natural infections), but are not attenuated in virulence when placed into stems or leaf panels. A recent study using chemically-induced mutants of this pathogen indicated that the amount of pyruvate and acetate present on the polymer as well as polymer viscosity may affect virulence due to their effects on the viscocifying ability of xanthan gum *in planta* (Ramirez et al., 1988). Increased viscocifying ability of the xanthan gum produced was correlated with increased virulence of the variant bacteria. Unfortunately, no pathogenicity studies have been done using the well defined (genetically as well as biochemically) mutants of *X. c. pv. campestris* which are known to produce a variety of structurally altered forms of xanthan gum (Hassler and Doherty, 1990). Results of such a study would greatly increase our knowledge of EPS structure-function relationships as they relate to host-pathogen interactions.

A role for xanthan gum at the preinfection stage has also been proposed. Adhesion of *X. c. pv. citri* to wound sites on citrus leaves may be mediated by binding of xanthan gum to a glycoprotein plant agglutinin (Takahashi and Doke, 1985).

V. SUMMARY AND FUTURE PROSPECTS

During the past decade the use of the modern techniques of molecular biology along with improved cytological and biochemical techniques, has lead to a greatly improved understanding of both the nature of EPS's produced by plant pathogenic bacteria and their possible roles in host-pathogen interactions. The structures of several of the EPS's have been elucidated and some are unique. Biochemical evidence to date indicates that individual strains produce only a single acidic EPS. However, genetic studies indicate that silent EPS genes may be present. The structural data taken as a whole indicates that EPS is most likely not a determinant of host-pathogen specificity since xanthomonads as well as *P. syringae* pathovars with different host specificities produce similar acidic EPS's (xanthan gum and alginate, respectively). Differences in the nature

and amounts of non-sugar substituents of the polysaccharides have not yet been demonstrated to relate to host specificity.

EPS production by plant pathogenic bacteria is now recognized to be under highly complex regulatory control at the level of transcription. A variety of environmental conditions affect EPS production, acting through both positive and negative regulatory genes which specifically affect EPS production, as well as through global regulators which affect multiple phenotypes such as synthesis of extracellular enzymes and plant hormones as well as motility and chemotaxis. Similar global regulatory genes appear to be present in plant pathogens of different genera (for instance *algR1* in the group I pseudomonads and *rscB* in the erwinias) and in taxonomically-related non-plant pathogens which synthesize chemically distinct EPS's. To date no specific plant products are known to affect EPS synthesis.

The acidic EPS's produced are normally hygroscopic and form highly viscous aqueous solutions. Undoubtedly, these traits are useful for the bacteria in establishing and maintaining water-soaked lesions on the leaves of plant hosts, contributing to a favorable growth environment as well as to wilting of plant hosts by vascular-inhabiting pathogens. Factors other than EPS are most likely responsible for early plant membrane disruption leading to leakage of water and nutrients.

Even though the use of molecular biology techniques have led to results which strongly implicate EPS as virulence factors for a variety of plant pathogenic bacteria many of the mutants studied so far may be pleiotropic. The generation and testing of non-pleiotropic, genetically- and biochemically-defined mutants is required before this hypothesis is proven. For instance, the generation of mutants of *A. tumefaciens* with a defective cellulose synthase gene or mutants of *P. syringae* pathovars with a defective *algD* gene (deficient in GDP-mannose dehydrogenase) would be very helpful for defining the role of cellulose and alginate, respectively, in the disease process.

Several possible pitfalls in studies on bacterial EPS, leading to erroneous conclusions, have been demonstrated. First, mutants which

appear defective in EPS production based on *in vitro* tests may not be defective *in planta*. Second, EPS impaired mutants may appear unaffected in virulence using a single inoculation technique, but when additional techniques are tested reduced virulence can be demonstrated. Assays for virulence should mimic natural infection routes as closely as possible and it is advisable that more than one type of bioassay be done. Third, the disruption of genes involved in the production of the EPS normally produced *in vitro* may lead to mutants capable of producing a second EPS due to the activation of silent EPS genes. This second EPS may be functionally equivalent to the other during plant-microbe interactions. This was shown for mutants of the plant symbiont *Rhizobium meliloti* defective in the production of succinoglycan (Reuber et al., 1991). Could the succinoglycan impaired mutants of *A. tumefaciens*, which appear unaffected in virulence (Cangelosi et al., 1987), produce a second, functionally equivalent acidic EPS in inoculated plants?

In addition to a probable role as virulence factors, EPS produced by plant pathogenic bacteria are also likely very important in the general ecology of the bacteria, promoting bacterial survival in the soil (Roberson and Firestone, 1992), water and on the surface of plants (Wilson and Lindow, 1993). For instance, xanthan gum was proposed to protect bacteria from the harmful effects of UV light and from desiccation (Ikotun, 1984; Leach et al., 1957; Wilson et al., 1965) and was found to prolong viability in sterile water (Goto and Hyodo, 1985). Bacterial exudates from severely infected plant hosts support long-term viability of bacteria in storage (Wilson et al., 1965). Production of EPS at the leaf surface may also allow for binding of bacteria to plant lectins at wounded leaf tissues, promoting subsequent infection (Takahashi and Doke, 1984, 1985).

The confirmation of these purported roles for bacterial EPS production in plant-bacteria interactions awaits further studies.

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