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Mini review

Autoregulator-dependent control of extracellular polysaccharide production in phytopathogenic bacteria

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Abstract

Extracellular polysaccharides (EPSs) likely provide phytopathogenic bacteria a selective advantage both inside and outside plants. Despite the relatively scant knowledge about EPS biosynthesis in phytopathogenic bacteria, it clearly is a well controlled, complex, energy-intensive process. Unexpectedly, three phytopathogenic bacteria have been found to autoregulate EPS production in response to extracellular signal compounds (pheromones) that they produce. Like many bacteria, *Pantoea stewartii* subsp. *stewartii* produces a *N*-acyl-homoserine lactone (AHL) autoinducer. However, unlike most AHL-dependent autoinduction systems, that in *P. stewartii* subsp. *stewartii* somehow represses EPS production in the absence of autoinducer. Instead of an AHL-dependent system (which it also has), *Ralstonia solanacearum* uses a novel autoregulator identified as 3-hydroxypalmitic acid methyl ester to regulate EPS biosynthesis. A lack of this autoregulator in *R. solanacearum* results in repression of EPS biosynthesis by a complex two-component sensor/response regulator signal cascade. *Xanthomonas campestris* pv. *campestris* has two partially overlapping autoregulatory systems. The autoregulators are incompletely characterized, but one diffusible signal factor (DSF) is thought to be a fatty acid derivative and the other diffusible factor (DF) may be a butyrolactone. The autoregulation pathways in *X. campestris* pv. *campestris* are essentially unknown, but EPS production is controlled by both the DSF and DF systems, whereas production of extracellular enzymes and pigment production are regulated independently. In a confined micro-environment, population density and intercellular concentrations of an autoregulator will increase in parallel, so autoregulation is one way that bacteria can coordinate gene expression to synthesize EPS only at high cell density. However, because there is often limited evidence that it is actually cell density that is being detected, researchers should not assume *a priori* that autoregulation must function for quorum sensing. Some possible reasons for why phytopathogenic bacteria would benefit from delaying EPS production are discussed.

Abbreviations: AHL – *N*-acyl-homoserine lactone; DF – diffusible factor; DSF – diffusible signal factor; EF – extracellular factor; EPS – extracellular polysaccharide; PAME – palmitic acid methyl ester.

Introduction

When conditions are appropriate, many phytopathogenic bacteria produce one or more high molecular mass EPSs. These large polymers may be associated with the bacterial cell as a capsule, be released as fluidal slime, or be present in both forms. The EPSs made by phytopathogenic bacteria are usually heteropolysaccharides, which contain a mixture of sugars precisely arranged in repeating subunits, but some species also produce homopolysaccharides that have only a single type of sugar. Details concerning composition and structure of these EPSs are available in several review articles (Denny, 1995; Leigh and

Coplin, 1992) and more recent papers (Jumel et al., 1997; Nimtz et al., 1996a; Nimtz et al., 1996b).

EPSs may benefit phytopathogenic bacteria in a variety of ways (Denny, 1995; Roberts, 1996). There is now convincing evidence that some pathogens require appropriate amounts of EPS during pathogenesis, because mutants specifically lacking EPSs are reduced in virulence or do not cause disease (Denny, 1995). However, exactly how EPSs benefit bacteria during pathogenesis is still unclear. For example, EPSs might shield bacteria from toxic plant compounds, reduce contact with plant cells to minimize host defense responses, promote multiplication by prolonging water-soaking of tissues, or otherwise aid invasion or systemic colonization. Although the pathogen may not directly benefit, large amounts of EPS also may be a primary cause of wilt symptoms. During saprophytic or epiphytic existence, EPSs may enhance attachment of phytopathogenic bacteria to surfaces, protect cells from desiccation, or help concentrate minerals or nutrients for metabolism.

Despite their many potential benefits, extracellular heteropolysaccharide production is an energy-intensive process requiring a large commitment of cellular resources. Although much is known about the biochemistry of EPS biosynthesis in some bacteria (Harding et al., 1995; Katzen et al., 1998; Roberts, 1996; Vojnov et al., 1998), these processes are, in most cases, less understood in phytopathogenic bacteria. Certainly, many genes are required to encode the biosynthetic machinery that activates sugar monomers, assembles, polymerizes and decorates the subunits, and finally transports the complex polymer to the cell surface (Huang and Schell, 1995; Leigh and Coplin, 1992; Peñaloza-Vázquez et al., 1997; Roberts, 1996). In addition, bacteria must have internal regulatory systems to coordinate the steps in biosynthesis and probably have environmentally-sensitive regulatory systems to ensure that EPS is produced only when conditions are suitable (Leigh and Coplin, 1992).

Recent work has partially characterized the systems that control EPS production in several phytopathogenic bacteria (Kelm et al., 1997; Leigh and Coplin, 1992; Peñaloza-Vázquez et al., 1997; Schell, 1996). Where identified, most of the regulatory elements are members of two-component systems, which consist of a membrane-associated sensor protein (with a variable input domain and a conserved histidine kinase transmitter domain) and a cytoplasmic response regulator protein (with a conserved phosphorylation receiver domain and a variable output domain) (Parkinson and Kofoid, 1992). Although the environmental stimuli controlling EPS production are generally unknown, several phytopathogenic bacteria respond to compounds that they produce and excrete into the extracellular milieu. This review will focus on this intriguing mechanism for regulating EPS production and the implications it has for phytopathogenic bacteria.

The autoinduction paradigm

Although commonly viewed as self-contained entities, bacteria often exhibit social behavior and function as multicellular organisms (Costerton et al., 1995; Kaiser and Losick, 1993; Kaprelyants and Kell, 1996). One way that bacteria coordinately control selected cellular activities is by producing extracellular chemical signals generically known as pheromones (Kell et al., 1995; Stephens, 1986), autoinducers (Fuqua et al., 1996; Meighen, 1991; Nealson, 1977) or autoregulators (Khokhlov, 1991). When other conditions are appropriate, sufficient concentrations of a pheromone trigger altered expression of specific genes in the population of cells that produce them. For example, selected amino acids initiate fruiting body formation in *Myxococcus xanthus* (Dworkin, 1996), whereas oligopeptides stimulate competence for genetic transformation and sporulation in *Bacillus subtilis* (Solomon et al., 1996), and activate conjugation by *Enterococcus faecalis* (Clewell, 1993). In *Streptomyces* spp., a γ-butyrolactone derivative controls aerial hyphae formation, sporulation, and antibiotic production (Horinouchi and Beppu, 1994; Stephens, 1986).

More recently, it was discovered that many bacteria produce AHL autoinducers (Cha et al., 1998; Fuqua et al., 1996; Salmond et al., 1995). The known AHLs all have the same homoserine lactone ring, but they differ in the length and modification of the *N*-acyl chain. Depending on the bacterial species, AHLs regulate functions as diverse as bioluminescence, production of exoenzymes and antibiotics, rhamnolipid and capsular biosynthesis, and conjugal plasmid transfer (Fuqua et al., 1996; Salmond et al., 1995). Because AHLs are thought to diffuse freely across the bacterial envelope (Kaplan and Greenberg, 1985), concentrations inside and outside the cell will be similar when a population is within a confined space. Thus,

intracellular AHL concentrations sufficient to trigger a response will occur only when intercellular concentrations increase after a population increases. This method of gene regulation is now commonly called 'quorum sensing' (Fuqua et al., 1996; Fuqua and Greenberg, 1998; Swift et al., 1996), since it appears that cells may use an AHL-dependent system to detect their population density.

In most Gram-negative bacteria, synthesis of and response to AHLs require homologues of the *V. fischeri luxI* and *luxR* genes, respectively (Fuqua et al., 1996). Based on three biochemical studies (Jiang et al., 1998; Moré et al., 1996; Schaefer et al., 1996), LuxI homologues are synthases that produce AHLs from *S*-adenosylmethionine and *N*-acylated acyl carrier protein (acyl-ACP) substrates. LuxR homologues are generally transcriptional regulators that respond to sufficient quantities of one or more AHLs by activating transcription of target promoters (often including the *luxI* homologue). Figure 1 illustrates the prototypical AHL-dependent autoinduction system. Since *luxR* homologues or other components of AHL-dependent expression systems are often influenced by additional regulatory pathways (Flavier et al., 1997a, 1998; Fuqua et al., 1996; Pierson, III et al., 1998; Seed et al., 1995), they are not always affected simply by cell density (and thus the system is not purely quorum sensing).

Autoregulator-dependent control of EPS production

Autoregulator-dependent control of gene expression has only recently been examined in phytopathogenic bacteria. Surprisingly, three pathogen species provide the first examples of autoregulators having an essential role in regulating EPS production. Furthermore, both the autoregulators and their cognate regulatory networks are different in each pathogen.

Pantoea stewartii *subsp.* stewartii

Pantoea stewartii subsp. *stewartii* (synonym *Erwinia stewartii*), is the causal agent of Stewart's wilt and leaf blight of sweet corn. When inoculated into a leaf of a susceptible variety, either mechanically or by its natural insect vector, wild-type bacteria colonize intercellular spaces in leaves resulting in water-soaked lesions. Bacteria also colonize the xylem, where they move systemically and cause necrosis and wilting (Braun, 1990). This pathogen is not known to make either phytotoxins or plant cell wall-degrading enzymes (Braun, 1990; Denny, 1995).

The EPS made by *P. stewartii* subsp. *stewartii* is called stewartan, which is present as a capsule in culture (Leigh and Coplin, 1992) and in planta (Braun, 1990), but also appears as slime when a readily metabolized

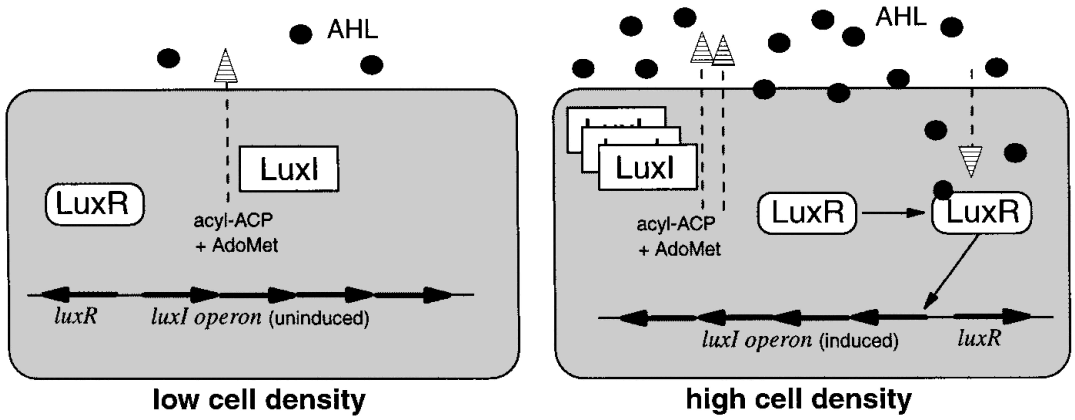

Figure 1. Model of the LuxR–LuxI autoinduction system in *V. fischeri*. LuxI is a synthase that produces AHL (●) from *N*-acylated-acyl carrier protein (acyl-ACP) and *S*-adenosylmethionine (AdoMet). AHL diffuses freely in and out of bacterial cells, so intracellular concentrations are high only when there is a high cell density in a confined space. When LuxR binds AHL it activates transcription of the *luxI* operon (which also has genes that encode for bioluminescence). Some components of the regulatory circuit were omitted for clarity.

sugar is present. The biosynthetic pathway for stewartan is encoded by the *cps* gene cluster, which is similar to the loci for biosynthesis of colanic acid in *Escherichia coli* and amylovoran in *Erwinia amylovora* (Denny, 1995; Leigh and Coplin, 1992). When infiltrated into corn leaf mesophyll, *cps* mutants are almost avirulent or cause smaller lesions than the wild type (Coplin and Majerczak, 1990; Dolph et al., 1988). A spontaneous EPS^- mutant did not move systemically despite multiplying normally in the xylem near the site of infection (Braun, 1990).

Until recently, regulation of EPS biosynthesis in *P. stewartii* subsp. *stewartii* was thought to be very similar to that in *E. coli*, where either RcsA or RcsB can activate transcription of *cps* genes under appropriate conditions (Gottesman, 1995). The primary activator is RscB, which is part of a two-component system along with RcsC, whose function is modulated by RcsA. Recent results, however, show that in *P. stewartii* subsp. *stewartii* production of stewartan is also controlled by an AHL-dependent regulatory system (Beck von Bodman et al., 1998; Beck von Bodman and Farrand, 1995).

When screening phytopathogenic bacteria for production of AHLs, Beck von Bodman and Farrand (1995) observed that *P. stewartii* subsp. *stewartii* culture supernatants contained significant autoinducer activity. The autoinducer was determined to be 3-oxohexanoyl homoserine lactone (OHHL), the same AHL as made by *V. fischeri*. Cloning and DNA sequence analysis revealed that the locus essential for OHHL production has both *luxI* and *luxR* homologues, designated *esaI* and *esaR*, respectively (Beck von Bodman et al., 1998). Inactivation of *esaI* in *P. stewartii* subsp. *stewartii* eliminates production of both OHHL and stewartan, and the mutant is essentially nonpathogenic. As would be predicted for an AHL-dependent system, tests showed that stewartan production per wild-type cell rapidly increases 10-fold in the two to three generations after a batch culture exceeds 1×10^8 cells ml^{-1} (i.e., during late exponential phase) (Beck von Bodman et al., 1998). Addition of exogenous OHHL to a batch culture only reduces the cell density at which stewartan production begins to increase to 5×10^7 cells ml^{-1} (Beck von Bodman et al., 1998), so OHHL is not the only factor required to increase *cps* gene expression, and the system is not purely quorum dependent.

Despite its many similarities to the system in *V. fischeri*, some features of the AHL-dependent regulatory system controlling stewartan synthesis in *P. stewartii* subsp. *stewartii* (Figure 2) are unusual. First, expression of *esaI* and production of OHHL are not regulated by EsaR (Beck von Bodman et al., 1998; Beck von Bodman and Farrand, 1995), so there is no positive feedback loop for AHL production. In fact, production of OHHL appears to be constitutive because it increases linearly with cell density (Beck von Bodman et al., 1998). Second, EsaR represses expression of *esaR* (Beck von Bodman and Farrand, 1995). Third, inactivation of *esaR* results in high-level stewartan production independent of cell density, presumably due to constitutive expression of *cps* genes. This contrasts

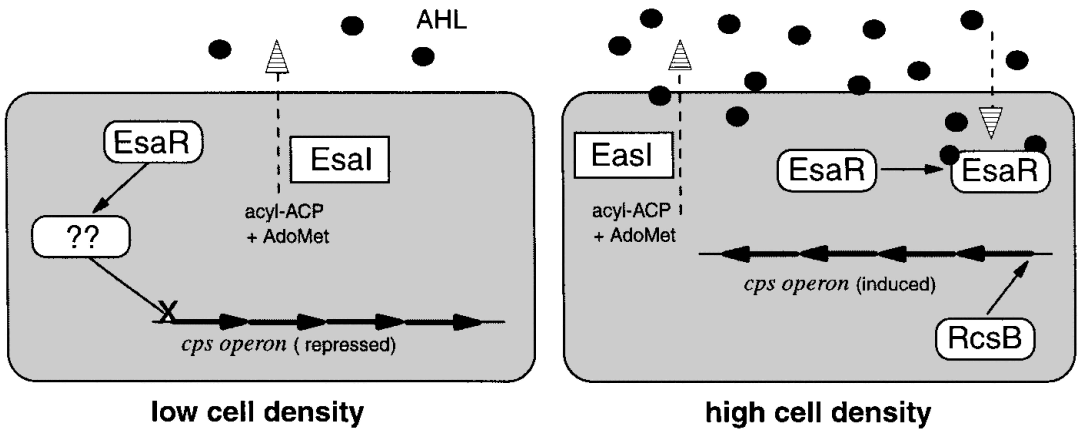

Figure 2. Model of the AHL-dependent autoregulatory system in *P. stewartii* subsp. *stewartii*. This system works similarly to that in *V. fischeri*, except that at low cell density, in the absence of AHL, EsaR represses expression of the *cps* operon and at high cell density the increased AHL concentration relieves repression. RcsB is part of the two-component system that induces *cps* expression.

with the reduced expression of AHL-regulated genes that occurs in most bacteria when their *luxR* homologue is inactivated. These results suggest that EsaR is part of a system that represses expression of *cps* in the absence of OHHL, which occurs in an *esaI* mutant or when cell density is low. Although EsaR behaves genetically as a repressor, it is not known whether it functions biochemically to repress transcription of the *cps* locus directly or functions indirectly as part of a larger regulatory network that contains a true transcriptional repressor.

Strains that do not produce stewartan, either due to mutation of *cps* genes or *esaI*, are almost nonpathogenic (Beck von Bodman et al., 1998; Beck von Bodman and Farrand, 1995; Coplin and Majerczak, 1990; Dolph et al., 1988). Therefore, it is surprising that *esaR* mutants, which constitutively produce stewartan, cause fewer lesions than the wild type and do not induce systemic wilting when 10^6 cells were inoculated into stem wounds (Beck von Bodman et al., 1998). However, EsaR mutants are as virulent as the wild type when $>10^7$ cells were inoculated into wounds, or when applied to unwounded leaves to assess the ability to incite water soaking.

Ralstonia solanacearum

Ralstonia solanacearum (synonyms: *Pseudomonas solanacearum*, *Burkholderia solanacearum*) causes lethal wilting of several hundred plant species, including important crops such as potato, tobacco, peanut, and banana. Normally a soil-borne pathogen, *R. solanacearum* enters plant roots via wounds or sites of secondary root emergence and initially colonizes intercellular spaces in the root cortex. Bacteria soon move into the vascular cylinder, penetrate the xylem vessels, and then rapidly spread upward into the stem. The onset of wilting in tomato plants is correlated with reduced water uptake, probably due to general vascular dysfunction caused by high bacterial cell densities ($>10^9$ cells/g plant tissue) and production of copious amounts of EPS (Denny, 1995; Schell, 1996). Extracellular enzymes are produced by *R. solanacearum* in culture and in planta, and enhance the rate of wilting (Allen et al., 1997; Huang and Allen, 1997; Schell, 1996).

The primary EPS made by *R. solanacearum* is an acidic, high molecular mass heteropolysaccharide (Orgambide et al., 1991) known simply as EPS1. Large quantities of EPS1 are released as slime when cells are cultured on rich media or in planta. Recent research indicates that 10–20% of the material that reacts with an EPS1-specific monoclonal antibody is tightly bound to wild-type cells (McGarvey et al., 1998); whether this material is a capsule of EPS1 or another form of this polysaccharide has not been studied. The 16-kb *eps* gene cluster likely encodes functions specific for biosynthesis of EPS1 because (i) partial DNA sequence analysis identified seven *eps* genes similar to those required for heteropolysaccharide synthesis in other bacteria and (ii) inactivation of *eps* genes reduced production of EPS1 to $\leq$2% of normal (Huang and Schell, 1995; Denny and Baek, 1991). EPS1 is the single most important virulence factor of *R. solanacearum*, since *eps* mutants are severely reduced in systemic colonization of tomato plants when inoculated via unwounded roots (Saile et al., 1997) and do not cause typical wilt symptoms even when introduced directly into stem wounds (Denny and Baek, 1991; Denny, 1995).

A complex regulatory network in *R. solanacearum* controls expression of the *eps* locus and genes encoding extracellular enzymes. Central to this network is PhcA, a LysR-type transcriptional regulator that has global effects (Brumbley et al., 1993; Schell, 1996). Inactivation of *phcA* reduces transcription of the *eps* locus and genes encoding some extracellular enzymes. Normal expression of *eps* also requires a pair of two-component systems (VsrA/D and VsrB/C) and an unusual 'signal integrator' protein (XpsR) (Huang et al., 1995; Schell, 1996); the signals recognized by these additional network components in *R. solanacearum* have not been identified.

Autoregulation of EPS1 production in *R. solanacearum* was first observed while characterizing nonmucoid mutants generated by random transposon mutagenesis (Clough et al., 1994). Similar to a *phcA* mutant, the transposon mutant AW1-83 produces neither EPS1 nor several extracellular enzymes. Unlike a *phcA* mutant, however, AW1-83 produces EPS1 when streaked adjacent to the wild-type parent or an *eps* mutant. This suggested that the wild type makes an extracellular factor (EF) required for EPS1 production, and that the transposon insertion in AW1-83 (in a new locus designated *phcB*) inactivates this function. The EF recognized by AW1-83 is made by *R. solanacearum* on both minimal and rich media, and restores production of both EPS1 and several extracellular enzymes. All 80 *R. solanacearum* strains in a diverse collection produced EF, whereas out of 58 strains representing 20 species in 10 genera, only a few strains of *Agrobacterium tumefaciens* were weakly EF-positive

(Flavier et al., 1997b). Unlike any other known bacterial autoregulators, EF is active both in solution and in the vapor phase.

EF activity was purified from *R. solanacearum* culture supernatants and identified as 3-hydroxypalmitic acid methyl ester (3-OH PAME) (Flavier et al., 1997b). Authentic 3-OH PAME and the purified EF fully restore expression of *eps* and production of two other virulence factors in a *phcB* mutant at $\leq$5 nM, and increase expression of *eps* when delivered via the vapor phase. Although initial analysis of the predicted PhcB amino acid sequence did not reveal any homologues in the databases (Clough et al., 1997a), later analysis revealed the motif typical of small-molecule *S*-adenosylmethionine-dependent methyltransferases (Flavier et al., 1997b). Therefore, it seems likely that PhcB catalyzes synthesis of 3-OH PAME from a naturally occurring intermediate in the fatty acid biosynthetic pathway.

Clough et al. (1997b) determined that expression of several PhcA-regulated genes occurs preferentially late in the exponential phase. For example, in a wild type strain expression of *eps* is low at cell densities $<10^7$ cells ml^{-1}, but subsequently increases >50-fold during the next four generations (Clough et al., 1997b; Flavier et al., 1997b). In contrast, inactivation of *phcB* results in *eps* expression remaining low at cell densities $>10^9$ cells ml^{-1}. Addition of excess 3-OH PAME to cultures containing $<10^5$ cells ml^{-1} has no immediate effect, but subsequently reduces by 5-fold the cell density at which expression begins to increase in both *phcB* and wild-type backgrounds (Flavier et al., 1997b). Simultaneous determination of *eps* expression and the concentration of extracellular 3-OH PAME revealed that 3-OH PAME exceeds a half-saturating concentration at about the time *eps* expression is activated (Flavier et al., 1997b). Thus, 3-OH PAME appears to be essential for triggering *eps* expression, and its pattern and rate of accumulation are consistent with it being an intercellular autoregulatory signal. However, because 3-OH PAME does not immediately induce gene expression when added to cultures at low cell density, other factors must also control initiation of gene expression, and autoregulation in *R. solanacearum* is not solely cell density dependent.

DNA sequence and genetic analyses of the region immediately downstream of *phcB* revealed the presence of two genes, *phcS* and *phcR*, which are involved in sensing and responding to 3-OH PAME (Clough et al., 1997a). PhcS is predicted to be a histidine kinase sensor, whereas PhcR is similar to response regulators. However, PhcR is atypical, because its putative output domain resembles the histidine kinase domain of a sensor protein. PhcS and PhcR appear to work together to repress production of PhcA-regulated virulence factors in the absence of 3-OH PAME. How PhcS and PhcR function biochemically is still unclear. One model consistent with the data (Figure 3) is that, when the extracellular concentration of 3-OH PAME is low, PhcS phosphorylates PhcR, which in turn reduces expression

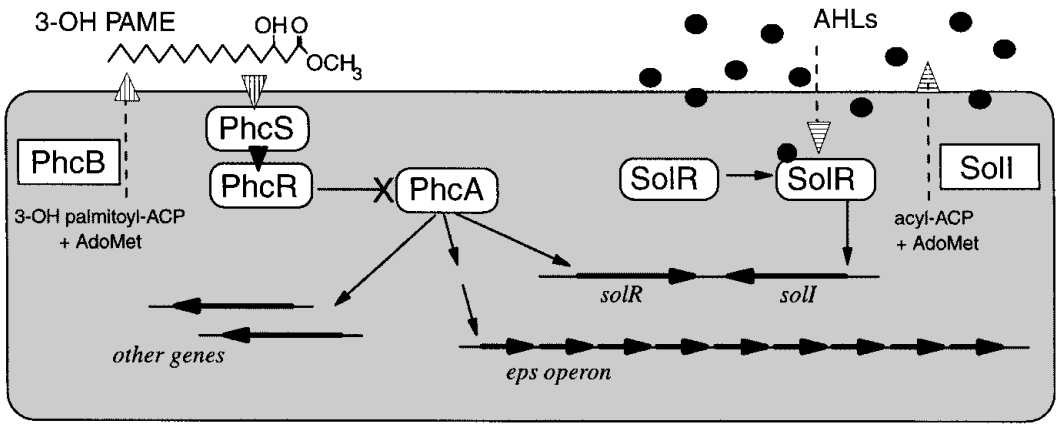

Figure 3. Model of the two autoregulatory systems in *R. solanacearum*. The AHL-dependent system is analogous to that in *V. fischeri*; it does not regulate *eps* expression. PhcB likely synthesizes the 3-OH PAME autoregulator from an *N*-acylated-acyl carrier protein (3-OH palmitoyl-ACP) and *S*-adenosylmethionine (AdoMet). PhcS and PhcR constitute a two-component system that represses expression of *phcA* or function of PhcA in the absence of 3-OH PAME. PhcA is a global transcriptional regulator that controls expression of *eps* and other genes (only some of which are shown).

of *phcA* or the function of PhcA. Since PhcR does not appear to be a DNA-binding protein, it may work via an unidentified component of the signal cascade. When the 3-OH PAME concentration exceeds a threshold, it likely interferes with the phosphotransfer functions of PhcS, which relieves repression by PhcR and results in increased function of PhcA.

Subsequent study of AHL production in *R. solanacearum* discovered a typical AHL-dependent autoinduction system consisting of *luxR* and *luxI* homologues, designated *solR* and *solI*, respectively (Flavier et al., 1997a). Specific elimination of AHL production by mutation of the *solI* AHL synthase has no effect on production of EPS1 or other virulence factors, and does not alter virulence of *R. solanacearum* on tomato (Flavier et al., 1997a). Although the function of this AHL-dependent autoregulatory system is currently unknown, it is unusual because expression of *solR* (and hence *solI*) is regulated by the 3-OH PAME-dependent system via PhcA. Thus, just like production of EPS1 and other PhcA-regulated traits, production of AHLs is greatly reduced in *phcB* mutants.

Xanthomonas campestris *pv.* campestris

In black rot of crucifers, caused by *Xanthomonas campestris* pv. *campestris*, bacteria normally enter hydathodes at the leaf margins and then multiply and spread within the xylem vessels (Dow and Daniels, 1994; Sutton and Williams, 1970). Obstruction of vessels (probably by bacterial EPS) is soon followed by vein blackening, the first visible symptom, and disorganization of the vascular tissues. The resulting water stress in distal portions of the leaf results in chlorosis, wilt, and death of tissues in the typical V-shaped pattern. Recent research has highlighted the importance of using more natural methods to introduce bacteria into the hydathodes when assessing strains for their ability to colonize plant tissues and cause disease (Denny, 1995; Dow and Daniels, 1994; Hugouvieux et al., 1998; Poplawsky and Chun, 1998). Inoculation of wounded vessels at the leaf margins with low numbers of bacteria also gives useful results (Chou et al., 1997).

The EPS produced by *X. campestris* pv. *campestris* and other xanthomonads is called xanthan (or xanthan gum). Many strains produce copious amounts of xanthan slime when they are cultured in medium with a high C : N ratio, and this EPS has a variety of commercial applications (Harding et al., 1995; Lo et al., 1997). *X. campestris* pv. *campestris* also produces a variety of extracellular enzymes that contribute to virulence (Dow and Daniels, 1994). Up to seven loci may be required for production of polysaccharides by *X. campestris* pv. *campestris*, but the *gum* locus appears to be the only one specific for synthesis of xanthan. DNA sequence analysis predicts that the *gum* locus contains 12 open reading frames (*gumB* to *gumH*). The likely function of many Gum proteins in xanthan biosynthesis has been determined (Katzen et al., 1996; Katzen et al., 1998; Vojnov et al., 1998). Recent reports indicate that defined *gum* mutants are less virulent than the wild type, because they caused smaller lesions that developed more slowly (Chou et al., 1997; Katzen et al., 1998). One *gum* mutant multiplies poorly when infiltrated into the leaf mesophyll (Newman et al., 1994; Vojnov et al., 1998), and is non-pathogenic when applied to hydathodes or wounded veins at the leaf margin (M.J. Daniels, pers. comm.)

Control of xanthan production is clearly complex, but since most research has focused on optimizing industrial production, surprisingly little is known about the genetics of regulation (Harding et al., 1995; Katzen et al., 1996). The *gum* locus is primarily expressed from a single promoter upstream of *gumB*, but there is evidence for internal promoters with lower activity (Katzen et al., 1996; Vojnov et al., 1998). Eight genes at the *rpf* locus positively regulate production of both xanthan and multiple extracellular enzymes (Dow and Daniels, 1994; Tang et al., 1991). Mutations in *rpf* genes usually reduce production of these factors 10-fold and significantly reduce virulence (Poplawsky et al., 1998; Tang et al., 1991). Results to date indicate that *rpfC* and *rpfG* encode a two-component system (Dow and Daniels, 1994), *rpfA* encodes an aconitase (Wilson et al., 1998), and *rpfB* and *rpfF* are part of an autoregulator system (Barber et al., 1997) (see below). There is also a negative regulator, which when inactivated results in overproduction of xanthan (Tang et al., 1990). Several other regulatory systems have also been identified that affect production of EPS and either extracellular enzymes or other traits (Dow and Daniels, 1994). Details concerning signal transduction within these regulatory pathways are lacking, and no regulatory system specific for xanthan biosynthesis has been described.

The diffusible signal factor (DSF) autoinduction system

Two autoregulator systems in *X. campestris* pv. *campestris* affect production of xanthan and either

extracellular enzymes or pigment production (Poplawsky et al., 1998). One system was discovered by Barber et al. (1997) who observed enhanced extracellular protease production by an *rpfF* mutant when it is cultured adjacent to the wild type, suggesting that *X. campestris* pv. *campestris* produces a diffusible extracellular factor that stimulates gene expression. Additional tests using *rpf* mutants showed that insertions in *rpfB* and *rpfF* greatly reduce or eliminate production of the DSF, respectively. The DSF can be extracted from culture supernatants with ethyl acetate, and when added to broth cultures of an *rpfF* mutant it restores production of endoglucanase and polygalacturonate lyase enzymes (Barber et al., 1997); DSF also restores xanthan production by this mutant (Poplawsky et al., 1998). DSF activity in culture medium increases sharply in early stationary phase, and then returns to an almost undetectable level after an additional 5 h incubation. Although the transient accumulation of DSF is unlike that reported for other autoinduction systems (Chun et al., 1997; Flavier et al., 1997b; Fuqua et al., 1996; Meighen, 1991; Nealson, 1977), a similar phenomenon was recently reported for production of an autoregulator (possibly an AHL) by *E. coli* (Surette and Bassler, 1998).

Five of six *X. campestris* pv. *campestris* strains tested produce DSF activity, but production varies among selected strains of nine other pathovars and one other *Xanthomonas* species (Barber et al., 1997). The structure of the DSF is still undetermined, but it likely is a fatty acid derivative because (i) DNA sequence analysis of *rpfF* and *rpfB* suggest that they probably are involved in fatty acid metabolism, and (ii) acid-hydrolysed lipid preparations from *X. campestris* pv. *campestris* cells contain DSF-like activity. High concentrations of C10- and C12-fatty acids also are active, but fatty acid methyl esters (including 3-OH forms) are not. There is no evidence for production of AHLs by the strain used in this research (M.J. Daniels, pers. comm.). Barber et al. (1997) hypothesized that RpfF and RpfB act on existing lipid pools to create one or more fatty-acyl CoA derivatives, which are then conjugated to an unknown component and released extracellularly as DSF. Extracellular DSF might subsequently be taken up by the cells (explaining the reduction in its extracellular concentration) and the fatty acid moiety recycled to create more intracellular signal.

As expected for autoregulator-dependent traits, production of xanthan and extracellular enzymes by *X. campestris* pv. *campestris* is cell density-related in batch cultures of *X. campestris* pv. *campestris* (Barber et al., 1997; Harding et al., 1995; Lo et al., 1997). For example, endoglucanase accumulated during late exponential and early stationary phases, and expression of the *prtA* protease gene was highest in early stationary phase. Although DSF accumulation and expression of *prtA* increase in parallel, addition of DSF to exponentially growing cultures does not significantly affect the timing of *prtA* expression (Barber et al., 1997). Comparable experiments with the *gum* locus have not been reported. DSF is clearly not the sole determinant for timing of enzyme synthesis, and there is no evidence that the DSF system is a mechanism for quorum sensing in *X. campestris* pv. *campestris* (Barber et al., 1997).

The diffusible factor (DF) autoinduction system

The second autoinduction system was found by Poplawsky and Chun (1997a) while studying the *pig* locus that encodes for xanthomonadin pigment production in *X. campestris* pv. *campestris*. They observed that *pigB* mutants produce on average 5-fold less pigment and 4-fold less xanthan than the wild-type parent, but that these mutants appear normal when streaked adjacent to the wild-type parent and other *pig* mutants. Therefore, the wild type appears to produce a diffusible factor (DF) that is greatly reduced or eliminated by inactivation of *pigB*. Preliminary results suggest that one of the two open reading frames at the *pigB* locus encodes a regulatory protein, and that another *pig* transcriptional unit and an unlinked locus are also part of the DF autoinduction system (Poplawsky and Chun, 1997b) (A.R. Poplawsky, pers. comm.).

Although *pigB* mutants are fully virulent when injected into leaf mid-veins (Poplawsky and Chun, 1997a, 1998), they cause significantly fewer lesions than the wild type after being misted onto the surface of cauliflower leaves (Chun et al., 1997; Poplawsky and Chun, 1998). Reduced virulence might be due to the inability of *pigB* mutants to establish a large population on leaf surfaces, but Poplawsky and Chun (1998) prefer the hypothesis that they are defective in an 'infection specific process' in the hydathodes. The role of DF, EPS, and/or pigmentation in these host–pathogen interactions is unclear, but preliminary results suggest that pigment production is not required for epiphytic survival (A.R. Poplawsky, pers. comm.).

DF activity is produced by nearly all the strains of six *Xanthomonas* species tested, but not by selected strains of *Erwinia carotovora* or *R. solanacearum* (Chun et al., 1997; Poplawsky and Chun, 1997a). No

AHL autoinducer activity was detected using a special *Agrobacterium tumefaciens* reporter strain (Shaw et al., 1997) that is responsive to most of the known AHLs (Chun et al., 1997; Poplawsky and Chun, 1997a). DF activity accumulates in culture medium during batch culture, reaching a plateau in stationary phase, and can be extracted from acidified (pH 3.5) culture supernatants using ethyl acetate (Chun et al., 1997). The DF activity passes through an ultrafilter with a 500-Da cutoff, is stable between pH 3 and 8, and is not destroyed by autoclaving for 30 min. Simple HPLC/MS analysis suggested a chemical formula of $C_9H_{14}O_3$, and Chun et al. (1997) proposed that the DF might be a butyrolactone.

Despite the uncertainty regarding their chemical structures, DF is clearly different from DSF (Poplawsky et al., 1998). First, in side-by-side comparisons, *pigB* and *rpfF* mutants are distinctly different in production of pigment and extracellular enzymes. Specifically, absence of DF reduces pigmentation but not endoglucanase activity, whereas absence of DSF has the opposite effect. Second, DF and DSF cannot be substituted for one another. Third, the two autoregulators are chemically different, since DSF migrates faster ($R_f = 0.5$) than DF ($R_f = 0.2$) on thin-layer chromatography plates.

Although the DF and DSF autoinduction systems regulate pigment and endoglucanase production independently, both have a role in regulating xanthan production (Poplawsky et al., 1998). The systems overlap to at least some extent because DSF activity (measured by restoration of endoglucanase activity in an *rpfF* mutant) increases in a *pigB* mutant. In addition, further increasing the level of DSF by introducing additional copies of *rpfF* and *rpfB* into a *pigB* mutant restores xanthan production despite the lack of DF. In contrast, introduction of *pigB* into an *rpfF* mutant has no effect on xanthan production. A model of how these systems might interact is shown in Figure 4.

The involvement of DF in a cell density-dependent phenomenon was not reported by Poplawsky and co-workers (Chun et al., 1997; Poplawsky and Chun, 1997a; Poplawsky et al., 1998), who call it a pheromone. Likewise, Barber et al. (1997) avoided calling DSF an autoregulator, going so far as to say 'DSF does not behave autoinductively' because it does not stimulate its own production (unlike most AHL-dependent systems). However, Fuqua et al. (1996) noted that positive feedback of autoregulator production is 'not in principle essential for quorum-dependent

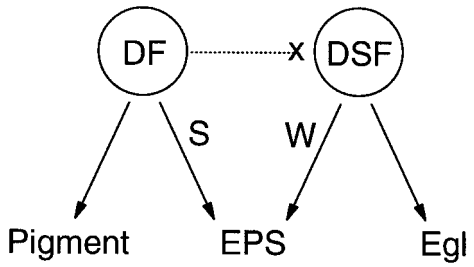

Figure 4. Hypothetical model of the DF and DSF autoregulatory systems in *X. campestris* pv. *campestris*. DF and DSF independently regulate pigment production and endoglucanase activity (Egl), respectively. The two systems appear to co-regulate EPS production, but DF has a stronger effect (S) than the weak effect (W) of DSF. Over production of DF appears to reduce production of DSF.

(viz. autoinduction) gene expression.' In fact, as noted above, for many bacteria autoregulator-dependent regulation is not solely cell density-dependent. Therefore, until evidence is presented to the contrary, I consider both DF and DSF to be autoregulators.

Ecological implications of autoregulation

When contemplating the role of autoregulation in bacteria, one fundamental consideration is how the external environment affects the concentration of the autoregulatory compound. Probably the most important parameter is the rate at which the compound diffuses into or is diluted by the surrounding medium. For bacteria in a confined micro-environment the intercellular autoregulator concentration will be most affected by the rate of diffusion away from the bacterial cell. In contrast, in an open environment the rate of dilution by medium moving past the bacteria is likely to be more important. These rates are difficult to predict for most bacteria, which exist in complex and discontinuous environments. Additional factors affecting intercellular autoregulator concentration are how rapidly the compound is degraded spontaneously or enzymatically, or is taken up by heterologous organisms. Other organisms may also produce similar compounds that are antagonists of the autoregulator or replace the autoregulator. For phytopathogenic bacteria, these considerations are likely to be more important on plant surfaces, where the pathogen must coexist with many other microorganisms, than after infection when the pathogen is virtually alone inside the host plant.

Another major consideration is whether autoinduction is actually used by cells as a mechanism to sense population density (i.e., quorum sensing). This is a difficult question to answer, and often, autoregulatory systems have been described as quorum sensing simply because this is a convenient, popular term. Part of the problem is that, as noted by Kell et al. (1995), autoregulators are by definition secreted by producer cells, so their actions necessarily exhibit a cell density dependence (provided they are not acted upon by external factors as noted above). This is particularly true in batch cultures, where it is difficult to separate growth phase from culture cell density. Consequently, finding that gene expression per cell increases in late exponential or early stationary phases in culture is insufficient to conclude that cell-density is the critical feature being sensed rather than another change that occurs when the culture ages.

How does one demonstrate true quorum sensing? One approach is to show that cell density has an effect that is independent of growth phase or nutritional status. For example, when *V. fischeri* is grown in continuous culture in a chemostat, nutritionally limited cells at low cell density are not luminescent, whereas similar cells at high cell density are luminescent (Rosson and Nealson, 1981). Unfortunately, similar experiments have not been reported for other bacteria. For fruiting body formation by *M. xanthus* (Dworkin, 1996; Kuspa et al., 1992), and conjugation by *A. tumefaciens* (Fuqua and Winans, 1996) true cell density dependence was observed because low and high densities of otherwise comparable cells exhibit distinctly different behaviors. Additional evidence for quorum sensing is the rapid stimulation of target gene expression by the addition of exogenous autoregulator to a culture at low cell density. Here again, *V. fischeri* and *M. xanthus* provide the best examples of this type of behavior (Kuspa et al., 1992; Rosson and Nealson, 1981). However, as noted above, control of gene expression by multiple factors may prevent a rapid response when an autoregulator is added at low cell densities, so the absence of precocious gene induction does not rule out an autoregulatory system having a role in quorum sensing. Therefore, in the absence of definitive data regarding the role of an autoregulator, researchers should keep an open mind as to the possible functions of such systems.

A third major consideration when considering the role of autoregulation is whether the phenomenon observed in culture also occurs when bacteria are in natural habitats. Given the novelty of this line of research, it is not surprising that there is little experimental evidence for autoregulation in bacteria during their association with plants (Pierson et al., 1998). One example that suggests autoregulation occurs in a natural setting is the regulated antibiotic production in *Pseudomonas aureofaciens*, a biocontrol organism. Wood et al. (1997) observed that expression of the phenazine biosynthetic locus in cells growing on sterile wheat roots requires AHL synthesis, but they did not report the relationship between gene expression and cell density or growth phase. Recently, Kang et al. (1999) observed that, very similar to results in culture, expression per cell of the *eps* biosynthetic locus in *R. solanacearum* increased dramatically as the bacterial population in tomato plants increased during pathogenesis. That *esaR* mutants of *P. stewartii* subsp. *stewartii*, which constitutively produce stewartan, are reduced in virulence (Beck von Bodman et al., 1998) also suggests that regulated production of EPSs in planta is similar to that observed in culture (Beck von Bodman et al., 1998). If these results are representative, then it is likely that autoregulation is important for plant-associated bacteria both outside and inside plants.

Several ideas have been proposed for why phytopathogenic bacteria might benefit from regulating production of EPSs and other extracellular molecules (enzymes and antibiotics) so that they are not produced at low cell density. One time during their 'life cycle' when phytopathogenic bacteria will be at low density is outside a host plant, either in the soil (perhaps in plant debris) or as an epiphyte on intact plant surfaces. Although locally high cell densities may occur in microcolonies or biofilms, because these are open and relatively nutrient-poor environments, at least initially autoregulator concentrations likely will be insufficient to activate target genes. This is especially true for a volatile autoregulator like 3-OH PAME. Under these circumstances, phytopathogenic bacteria may exhibit a low-virulence phenotype adapted for soil survival, saprophytic or epiphytic existence, and/or early parasitic activities. This scenario is especially attractive for *R. solanacearum*, where autoregulation is part of a global network controlling production not only of EPS1, extracellular proteins, and AHLs, but other traits such as motility and salt tolerance. Similar arguments could be made for epiphytic survival of *X. campestris* pv. *campestris*, since the DF and DSF systems regulate multiple traits.

Phytopathogenic bacteria are also at low cell density inside host plants during a latent infection or the

early stages of pathogenesis. For pathogens not to produce virulence factors like plant cell wall-degrading enzymes soon after infecting a plant seems counterintuitive, but there are several plausible reasons. One possibility is that virulence factors are not needed initially because freely available nutrients are sufficient to support low numbers of bacteria; however, as cell density increases, resource depletion would require liberation of fresh nutrients by factors such as cell wall-degrading enzymes (Dow and Daniels, 1994). Barber et al. (1997) proposed this scenario for *X. campestris* pv. *campestris*, since they observed DSF accumulation in cultures during early stationary phase but had no evidence for quorum sensing. An alternative hypothesis, originally proposed by Pirhonen et al. (1993) for the soft-rot pathogen *Erwinia carotovora*, is that cell wall-degrading enzymes can release elicitors that trigger plant defense reactions (Palva et al., 1993), so producing them early in pathogenesis would be disadvantageous for bacteria. However, once bacteria multiply to a high density, probably with the aid of pathogenicity factors secreted by a *hrp* type III secretion system (Lindgren, 1997), then coordinated production of enzymes would contribute to an irresistible 'mob' attack on the plant (Alfano and Collmer, 1996).

Reasons for reduced EPS production early in pathogenesis are easier to conceptualize. One possibility is that bacteria producing relatively little EPS adhere more easily or more firmly to plant cells, which may be essential for the proper functioning of *hrp* type III secretions systems that export pathogenicity and avirulence proteins (Alfano and Collmer, 1996; Lindgren, 1997). It is also possible that adherence enables the pathogen to establish microcolonies or biofilms (Costerton et al., 1995) within plant tissues that would promote bacterial growth independent of *hrp* functions. Production of EPS by bacteria in areas of high cell density later in pathogenesis could enhance movement of bacteria by physically disrupting plant tissues (e.g., after hydration) or by helping to release cells from microcolonies so that they can colonize distant sites. In this latter scenario, the pathogen would alternate between low- and high-virulence phenotypes multiple times during pathogenesis. In contrast, excessive production of EPS early in pathogenesis might interfere with movement of bacteria within tissues. For *P. stewartii* subsp. *stewartii*, it was suggested that premature production of an EPS capsule might make the pathogen too large to move easily through xylem pit membranes (Beck von Bodman et al., 1998).

Perspectives

During the last decade it has become apparent that autoregulation is commonly used by microbes to control expression of a variety of genes. Although autoregulation of EPS production in phytopathogenic bacteria has some similarities with systems in other bacteria, the differences are more noteworthy. One difference is that no other Gram-negative bacteria have been reported to autoregulate EPS production. Since EPS was not known to be autoregulated in phytopathogenic bacteria before the serendipitous discoveries described above, additional research may reveal that other EPS-producing bacteria behave similarly. On the other hand, because three disparate phytopathogenic bacteria autoregulate EPS production, there may be aspects of the pathogen–plant relationship that select for this process. It will be interesting to see if fluorescent pseudomonads autoregulate EPS and whether plant pathogenic species differ from nonpathogenic species.

A second difference is that, despite widespread occurrence of AHL-dependent autoregulation, only one of the three phytopathogenic bacteria control EPS production using such a system. Instead, four different systems were identified, at least one of which uses a novel autoregulator (3-OH PAME). Rather than being unique, it seems likely that autoregulatory systems in phytopathogenic bacteria are only the first examples of the variety of systems that will be found in Gram-negative bacteria. If the plant pathogens can serve as a guide for examining other bacteria, then it may be significant that all the autoregulators are either known or thought to be fatty acid derivatives (i.e., have acyl moieties). If not simple coincidence, then the most likely explanation is that the nonpolar acyl side chains allow these molecules to more readily diffuse through bacterial membranes.

A third difference is that, in contrast to most AHL-dependent autoregulatory systems, in both *P. stewartii* subsp. *stewartii* and *R. solanacearum*, EPS biosynthetic genes appear to be repressed in the absence of the autoregulator. It is not known whether the DF and DSF systems regulating EPS in *X. campestris* operate the same way. The use of autoregulators to derepress rather than induce gene expression might be biologically significant, since spontaneous mutations that inactivate the autoregulatory system would not prevent EPS production. For example, *P. stewartii* subsp. *stewartii* EsaR mutants that constitutively produce stewartan, although

somewhat less virulent than the wild type, remain highly pathogenic (Beck von Bodman et al., 1998). In contrast, if these pathogens had autoregulatory systems that induced EPS production, then inactivation of the system would result in greatly reduced production of EPS and, consequently, greatly reduced virulence.

The discovery that expression of virulence factors in phytopathogenic bacteria is responsive to extracellular signals has potentially important consequences. If methods could be devised that alter the environment to eliminate signal production or interfere with signal perception, then we should be able to reduce pathogen virulence. Commonly, however, the critical signals that regulate virulence are unknown. Therefore, the identification of extracellular autoregulators of virulence in phytopathogenic bacteria is significant, because it provides obvious targets for this line of attack. It will be interesting to see if this potential weakness can be exploited to improve disease control.

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