Review Article

The virulence of *Erwinia amylovora*: molecular genetic perspectives

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The genus *Erwinia* comprises fifteen species of bacteria usually associated with plants as pathogens, saprophytes, or as members of the epiphytic flora (Lelliott & Dickey, 1984). The phytopathogenic *Erwinia* species can be crudely divided into two groups. The first group is comprised of the so-called 'soft-rot' *Erwinia* species which secrete pectin-degrading enzymes and cause rotting, putrefying diseases, particularly of plant storage tissues. This group of *Erwinia* has been extensively studied (Kotoujansky, 1987) and will not be dealt with in this review. The second group is composed of *Erwinia* species incapable of pectate degradation, but which cause necrotic diseases in a number of plant hosts (Seemueller & Beer, 1976). The most-studied species in this second group are *Erwinia amylovora* and *Erwinia stewartii*. This review will focus on the recent progress that has been made in our understanding of the virulence of *E. amylovora*.

Background

E. amylovora was the first bacterium demonstrated to cause disease in plants by a series of experiments performed by Burrill between 1877 and 1883 (Burrill, 1882, 1883). *E. amylovora* is the causative organism of the commercially important disease fireblight, which affects most members of the Pomoideae (apples and pears) as well as a range of ornamental plant species including *Crataegus*, *Cotoneaster* and *Pyracantha* (Eden-Green & Billing, 1974). Originally believed to be indigenous to North America, the bacterium can now be found in Central and South America, Europe, the Middle East and New Zealand (Aldwinkle & Beer, 1979). The route of infection is believed to be primarily via blossom although entry may also occur through wound sites (Eden-Green & Billing, 1974). In the initial stages the infected tissues become water-soaked and under humid

conditions bacterial ooze may seep out. Subsequently, the infected tissue becomes dark and shrivelled, appearing scorched and withered, hence the term fireblight. The pathogenesis of the disease has been investigated at the cellular level over a number of years and involves increasingly severe perturbations to host ultrastructure. Extracellular bacteria induce ion leakage and host cell membrane dysfunction, followed by decompartmentalization, cellular collapse and spreading tissue necrosis (Bachmann, 1913; Nixon, 1927; Youle & Cooper, 1987). The molecular mechanisms underlying these events have for a long time proven elusive, in part due to the fact that *E. amylovora*, in contrast to many plant pathogens, does not secrete any detectable hydrolytic enzymes or easily detected phytotoxic metabolites (Seemueller & Beer, 1976; Cooper *et al.*, 1990). Initial experiments using spontaneous avirulent mutants of *E. amylovora* identified at least two components essential for the pathogenesis of fireblight (Bennet, 1980). Firstly, unencapsulated mutants were avirulent, suggesting a role for extracellular polysaccharide, EPS (Bennet & Billing, 1978). Secondly, fully encapsulated avirulent mutants could also be isolated, indicating that additional factors apart from EPS were essential for the virulence of *E. amylovora* (Bennet, 1980). With the application of the techniques of molecular biology it has been possible to begin to study the plant-microbe interactions in detail and unravel the pathogenicity of this enigmatic plant pathogen.

Bacterial polysaccharides

Four cell-surface polysaccharides have been described in *E. amylovora*. EPS, sometimes referred to as amylovorin (Goodman *et al.*, 1974; Bennet & Billing, 1978; Smith *et al.*, 1990), lipopolysaccharide, LPS (Ray *et al.*, 1986, 1987), a high-molecular-mass levan (Gross *et al.*, 1989) and a low-molecular-mass glucan (A. R. W. Smith personal communication). Since little is known about the possible role of the glucan in the pathogenicity of *E.*

Abbreviations: EPS, extracellular polysaccharide; HR, hypersensitive response.

amylovora, we will concentrate on the other three polysaccharides, which have been studied in more detail.

EPS consists of a repeat unit containing three galactose molecules linked to a glucuronic acid, with a side-chain consisting of a galactose molecule substituted with pyruvate. It has a molecular mass of 50 to 150 MDa (Smith *et al.*, 1990). A number of groups using a variety of transposons have generated mutants of *E. amylovora* altered in their expression of EPS (Steinberger & Beer, 1988; Bellemann *et al.*, 1990; M. J. Coleman & I. S. Roberts, unpublished results). These mutants could be divided into three classes: those that failed to synthesize EPS and were avirulent in apple seedlings; those that synthesized reduced amounts of EPS and were delayed in inducing fireblight symptoms, and those that over-expressed EPS and were unaltered in their virulence (M. J. Coleman & I. S. Roberts, unpublished results).

Transposon insertions that abolished synthesis of EPS were mapped to a single region of the *E. amylovora* chromosome and could be complemented by a cosmid, pEA109, containing a single 15.3 kb *Hind*III fragment of *E. amylovora* chromosomal DNA (F. Bernhard & K. Geider, unpublished results). In addition, pEA109 could complement certain *E. stewartii* mutants defective in EPS biosynthesis, demonstrating common steps in the biosynthesis of polysaccharides in these two *Erwinia* species (Coplin & Cook, 1990). A 29 kb plasmid has been described in *E. amylovora* (Laurent *et al.*, 1989; Falkenstein *et al.*, 1989), and it has been postulated that this plasmid might play a role in the expression of EPS (Falkenstein *et al.*, 1989). However, other groups did not observe any change in EPS expression in plasmid-cured strains of *E. amylovora*, although such strains were unable to synthesize thiamin (Laurent *et al.*, 1989).

The two other groups of transposon mutants expressing either reduced or elevated amounts of EPS are of particular interest since these insertions may define regulatory functions for the expression of EPS. Such regulatory components are likely to play an important role in mediating EPS expression in response to plant stimuli and will be useful in studying the interactions between *E. amylovora* and the infected host plant. Transposon insertions that reduced the expression of EPS were mapped to two distinct regions of the *E. amylovora* chromosome (M. J. Coleman & I. S. Roberts, unpublished results). Surprisingly, these two sets of mutants could be complemented by a single *E. amylovora* gene which, when present on a multicopy plasmid, pISR401, caused overexpression of colanic acid (slime) in *Escherichia coli* (Coleman *et al.*, 1990). In *E. coli* the control of colanic acid biosynthesis is complex, being regulated by both the *lon* gene and three regulatory genes, *rcaA*, *rcaB* and *rcaC* (Gottesman *et al.*, 1985; Brill *et al.*, 1988). The RcsA protein, which is sensitive to the Lon

protease, acts in concert with the RcsB protein to directly stimulate transcription of the colanic acid biosynthesis genes (Stout *et al.*, 1991). In addition, the RcsB and RcsC proteins may comprise a two-component regulatory system, in which the RcsC protein acts as a sensor of environmental stimuli, modifying the RcsB protein to act as an effector to stimulate colanic acid biosynthesis (Stout & Gottesman, 1990). Plasmid pISR401 could complement *rcaA* mutants of *E. coli* (Coleman *et al.*, 1990) and determination of the nucleotide sequence of the *E. amylovora rcaA* gene indicated that it encoded a protein highly homologous to the RcsA proteins of *E. coli* and *Klebsiella pneumoniae* (Coleman *et al.*, 1990; Bernhard *et al.*, 1990). The presence of the *rcaA* gene on a multicopy plasmid stimulated both EPS and levan biosynthesis, indicating that expression of these two polymers is regulated by a common component (Bernhard *et al.*, 1990). Since it is known that EPS expression increases several-fold upon infection of plant material (Bennet & Billing, 1978), analysis of the regulation of the *rcaA* gene may elucidate the mechanisms of signal transduction of plant stimuli and the processes involved in the initial establishment of an *E. amylovora* infection. Gene fusions recently constructed between the *rcaA* gene and the chloramphenicol acetyltransferase reporter gene will soon yield our first information on this problem. It is unclear why the cloned *rcaA* gene is capable of complementing mutants with transposon insertions into genes other than *rcaA* and the nature of the gene products encoded by these genes remains unknown. Suffice it to say that regulation of EPS expression in *E. amylovora* is likely to be as complex, if not more so, than in *E. coli*. Indeed, *E. amylovora* cosmid clones capable of complementing *E. coli rcaB* and *rcaC* mutants have been isolated (M. J. Coleman & I. S. Roberts, unpublished results). Transposon insertion mutants that overexpress EPS may define genes encoding repressors of EPS biosynthesis or may reflect pleiotropic phenotypes due to insertions into the putative *E. amylovora lon* gene.

As yet, we can only speculate on the precise role that EPS plays in the disease process. One possibility is that EPS masks underlying cell-surface proteins from recognition by plant cells, thereby avoiding a plant defence response. Alternatively, the EPS may play a role in water retention or even in producing swelling pressure leading to host tissue collapse (Schouten, 1988). The cloning and detailed analysis of the EPS biosynthesis functions and the ability to generate *E. amylovora* mutants expressing altered forms of EPS should enable this question to be answered.

The structure of *E. amylovora* LPS has been determined and is invariant amongst different isolates of *E. amylovora* (Ray *et al.*, 1986). It consists of a lipid A-linked core oligosaccharide containing 3-deoxy-2-

octulosonic acid (KDO), heptose, glucose and amino sugars linked to a repeating subunit comprised of D-fucose, D-galactose and D-glucose (Ray *et al.*, 1987). Mutants that are specifically defective in LPS biosynthesis have not been isolated, making assignment of the possible role of LPS in the disease process difficult. The spontaneous avirulent mutant S (Bennet & Billing, 1978) can be regarded as a rough mutant of *E. amylovora* lacking any repeating side-chain (Ray *et al.*, 1986). However, this mutant is also unencapsulated and is therefore of little use in studying the role of LPS in fireblight.

Levan is a high-molecular-mass homopolymer of fructose synthesized extracellularly by *E. amylovora* when grown on media with a high sucrose content (Gross *et al.*, 1989). The polymer is synthesized extracellularly by the enzyme levansucrase which is constitutively secreted by *E. amylovora* (Gross *et al.*, 1989). Transposon insertion mutants defective in levan biosynthesis were virulent on pear seedlings but the onset of symptoms was delayed (M. Metzger & K. Geider, personal communication).

Virulence factors other than cell-surface polysaccharides

The second general class of avirulent mutants of *E. amylovora* are fully encapsulated and unaltered in any cell-surface polysaccharides. The existence of such mutants indicates that the virulence of *E. amylovora* is multifactorial. The first such mutant studied in any detail was the spontaneous mutant P66 identified by Billing (Bennet & Billing, 1978; Bennet, 1980). This mutant was not only avirulent but also failed to elicit a necrotic hypersensitive response (HR) when inoculated into a non-susceptible host such as bean or tobacco (Walters *et al.*, 1990). Recently, transposon insertion mutants with the same phenotype as P66 have been isolated (Steinberger & Beer, 1988; Barny *et al.*, 1990). These insertions were localized to a single region of approximately 40 kb of the *E. amylovora* chromosome and cosmid clones capable of complementing these insertions and mutant P66 have been isolated by a number of groups (Steinberger & Beer, 1988; Barny *et al.*, 1990; Walters *et al.*, 1990). This large virulence region has been further subdivided, as described below.

Identification of the *hrp* and *dsp* genes

Laurent and coworkers (Barny *et al.*, 1990) have identified a region of approximately 15 kb, designated *hrp*, essential for both pathogenicity and eliciting a HR; contiguous with this is a second region, insertions into which abolish pathogenicity but have no effect on the ability of *E. amylovora* to elicit a HR. This second region

has been referred to as being disease specific or *dsp*. Little is known about the nature of the products encoded by the genes in these two clusters or their relative interactions in the disease process.

Beer's group isolated a large cosmid, pCPP430, carrying 42 kb of *E. amylovora* chromosomal DNA which complemented all of the Tn5 and Tn10 Hrp⁻ mutants within their laboratory as well as mutant P66 (Beer *et al.*, 1989). Transposon mutagenesis of pCPP430 using TnphoA and Tn5gus showed that the *hrp* gene cluster consisted of at least seven transcriptional units and spanned between 40 and 42 kb. In addition, the *hrp* gene cluster could be divided into two functional regions. Genes encoding functions necessary for eliciting the HR response were scattered along the length of pCPP430, whilst functions specifically involved in the K⁺ efflux/H⁺ influx exchange reaction in tobacco cell suspensions, termed *xrp*, were localized to a 25 kb region of pCPP430 (Beer *et al.*, 1990a).

Interestingly, the introduction of pCPP430 into *E. coli* conferred the ability to elicit a HR (Beer *et al.*, 1989; Wei & Beer, 1990). This indicates that genes encoding factors essential for the pathogenicity of *E. amylovora* are expressed in *E. coli*. However, *E. coli* harbouring cosmid clones carrying only part of this region fail to induce a HR (Walters *et al.*, 1990; Barny *et al.*, 1990). Perhaps more surprising was the observation that certain transposon insertions in pCPP430, which abolish HR when reintroduced onto the chromosome of *E. amylovora*, could be complemented by *E. coli* (Wei & Beer, 1990). The region of functional homology between *E. coli* and *E. amylovora* was located to a 2.9 kb HindIII fragment of pCPP430 encoding functions essential for the rate of development of the HR (Wei & Beer, 1990). The precise nature of the *E. coli* gene capable of complementing insertions in this region is unknown.

Southern hybridization experiments using probes from the *hrp* gene cluster to genomic DNA from other *E. amylovora* isolates of diverse geographical location revealed identical patterns of hybridization, the only polymorphisms being in genomic DNA from *E. amylovora* isolates originating from *Rubus* species (Beer *et al.*, 1990a). The *hrp* gene cluster also hybridized to cosmids encoding the *wts* functions of *Erwinia stewartii* (Beer *et al.*, 1990b). The *wts* functions of *E. stewartii* are responsible for the characteristic water-soaking of leaves that is seen in maize plants infected with *E. stewartii*. In addition, certain Wts⁻ mutants could be complemented by cloned *E. amylovora* *hrp* genes, as could certain Hrp⁻ mutants of *E. amylovora* by cloned *wts* genes in reciprocal experiments (D. Coplin, personal communication). This observation, coupled with the finding that there was homology between the *hrp* gene clusters of *E. amylovora* and *Pseudomonas syringae* strain 61 (Beer *et al.*, 1990a),

indicates that the *hrp* gene cluster may encode functions common to a diverse range of plant pathogens.

Regulation of *hrp* gene expression

Studies using *Tn5gus* mutagenesis of pCPP430 followed by marker-exchange back onto the *E. amylovora* chromosome have allowed the regulation of the expression of the *hrp* gene cluster to be studied in detail. The expression of *hrp* genes was repressed in complex media and enhanced in minimal media. Specifically, ammonium concentrations greater than 50 mM repressed *hrp* gene expression and, as one might predict for a plant pathogen, *hrp* gene expression was greater at pH 5.5 than at pH 7.0. Expression of the *hrp* genes was independent of osmotic pressure (Beer *et al.*, 1990a). The use of MudIIPR13 to generate *lacZ* fusions has shown that both *hrp* and *dsp* genes are temperature-sensitive for expression, being maximally expressed at 25 °C, and are repressed on complex media (Barney *et al.*, 1990). This observation was not surprising since temperature-sensitive expression of virulence genes has been described for other plant pathogens (Alt-Moerbe *et al.*, 1988).

As yet, only one of the *E. amylovora* *hrp* genes has been sequenced. This gene is present on the 2.7 kb *Bam*HI–*Hind*III fragment which was capable of complementing mutant P66. The single open reading frame encoded a protein 43% homologous to the HrpS protein of *P. syringae* (Sneath *et al.*, 1990). The HrpS protein has a predicted molecular mass of 33 500 Da and has significant homology to several prokaryotic regulatory proteins including the NifA and NtrC proteins of *Rhizobium* species, the corresponding proteins from *K. pneumoniae* and the TyrR protein of *E. coli* (Grimm & Panopoulos, 1989). The HrpS protein has the central domain comprising an ATP-binding site, which is conserved within this family of proteins, as well as a C-terminal domain possibly involved in DNA binding. These results, together with other genetic evidence, would tend to suggest that the HrpS protein of *P. syringae* pv. *phaseolicola* is a regulatory protein which mediates expression of other *hrp* genes in response to plant-microbe interactions. The observation that the protein encoded by the 2.7 kb *Bam*HI–*Hind*III fragment of the *E. amylovora* *hrp* gene cluster is homologous to this may suggest a similar role for this protein in mediating *E. amylovora* *hrp* gene expression.

Concluding remarks

In summary, the application of molecular genetics to studying the pathogenicity of *E. amylovora* is already beginning to provide an insight into the microbial

components of the disease process. The generation of transposon insertions has provided well-characterized mutants with single lesions, thereby allowing a more accurate assignment of potential virulence factors. True, we still do not know the role that many of these components play, but as more genes are sequenced and the putative protein sequences analysed a clearer picture should emerge. The role of EPS in disease will be best addressed by the integrated use of both plant tissue culture and biochemical analysis. However, the cloning and analysis of the EPS biosynthesis functions and, more importantly, of regulatory components of EPS biosynthesis will enable plant-microbe interactions to be studied and reveal the processes by which plant-mediated expression of *E. amylovora* genes is achieved. The next few years should hopefully reveal the elusive determinants of pathogenicity and virulence in *E. amylovora*.

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