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Ralstonia solanacearum – a plant pathogen in touch with its host

Timothy P. Denny

Ralstonia solanacearum, one of the world's most important phytopathogenic bacteria, causes lethal wilting diseases of >200 plant species[1]. Its agronomically important hosts include peanut, potato, tomato, tobacco and banana. Although most troublesome in the tropics and subtropics, *R. solanacearum* continues to be a threat in cooler climates, especially on potato. A part-time soil inhabitant, *R. solanacearum* enters plant roots via wounds or where secondary roots emerge, colonizes the root cortex, invades xylem vessels and rapidly spreads throughout the vascular system. Efficient systemic colonization requires production of a high molecular mass extracellular polysaccharide (EPS) and multiple extracellular proteins (EXPs)[2]. Some EXPs, like the enzymes that attack plant cell walls, transit the main

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terminal branch of the general secretory pathway (type II secretion)[3,4] and enhance the rate and severity of wilting. Other EXPs, which have more subtle (and so-far enigmatic) roles in causing disease and eliciting defense responses, are delivered to host cells via a type III secretion system (secreton)[5–7]. Wilting is a result of vascular dysfunction caused by high bacterial cell densities [$>10^{10}$ colony forming units (CFU) per gram fresh weight] and the large amount of EPS these bacteria produce.

Over the past 50 years, extensive fundamental and applied research has established *R. solanacearum* as a model system for studying bacterial pathogenesis of plants[2]. In addition, *R. solanacearum* will be one of the first plant pathogens to have its genome completely sequenced. Extending the cutting-edge research on *R. solanacearum*, Aldon *et al.*[8] recently provided the first example of host-cell-contact-mediated type III secretion by a plant pathogen.

Ins and outs of type III secretion

Numerous phylogenetically diverse bacteria secrete proteins essential for virulence in their animal or plant hosts via conserved type III secretons encoded by large gene clusters within pathogenicity islands[4,7,9]. Type III systems typically contain 11 conserved proteins, some of which assemble into a macromolecular organelle (a needle-like complex and/or pilus) that spans the inner and outer bacterial membranes and through which proteins can move. Secreted effector proteins, which are often unique, are released in the vicinity of target cells or are injected into the host cell plasma membrane or cytosol, and alter host responses in ways that promote pathogen survival, multiplication and colonization. Plant pathogens secrete two types of effectors: harpins and avirulence proteins[7]. The conditions that promote production and secretion of harpins into culture media are known, but the destination of harpins in planta and their role(s) in virulence are uncertain. Strong circumstantial evidence indicates that avirulence proteins are injected into the cytosol of target cells, where they can trigger a defense response or, in some cases, promote disease. However, little is known about how avirulence proteins function in planta, as most do not resemble any protein with a known activity and the quantities injected into the plant cell cytosol are undetectable.

Protein secretion can be controlled either by regulating their movement through a type III secreton or by modulating transcription of genes encoding proteins destined to be secreted. The expression of genes in type III systems is often regulated by one or more environmental parameters, such as temperature, divalent cations (especially calcium), pH, oxygen tension and nutrient availability[4,6,7]. Changes in these parameters are thought to signal to the bacterium that it has moved from a niche outside a host to an inside niche. However, some animal pathogens also appear to have sensing systems that respond to contact with host cells[4,10], which presumably helps to ensure that effector proteins reach their intended destinations. Several reports[11,12] have shown that when *R. solanacearum* is added to a suspension of cultured plant cells it responds by increasing expression of *hrpB*, which encodes an AraC-type transcriptional regulator. HrpB, in turn, enhances production of additional Hrp proteins, among which are the HrpY pilin subunit[13], other proteins comprising the type III secreton, and at least one harpin and two other secreted proteins[14]. Furthermore, sensing of plant cells requires the outer membrane sensor PrhA and its downstream signal transduction cascade[11,12]. These observations suggest that *R. solanacearum* might specifically respond to contact with plant cells.

Contact-mediated expression in *R. solanacearum*

To visualize gene expression in single cells of *R. solanacearum*, Aldon *et al.*[8] engineered a low-copy-number plasmid to produce green fluorescent protein (GFP) from transcriptional fusions of *gfp* to either *hrpB* or *hrpY* promoters, and placed these constructs into wild-type and mutant backgrounds. Many color micrographs show that, for the most part, *hrpB–gfp* and *hrpY–gfp* are highly expressed only when *R. solanacearum* cells are in close proximity to *Arabidopsis*, tobacco, tomato or *Medicago truncatula* cells. Induction must occur rapidly, because fluorescence began to increase after 90 minutes of co-cultivation, during which time the bacteria must adhere, sense the cell, transduce the signal and synthesize functional GFP. These results are comparable with those of Pettersson *et al.*[10], who used a *Yersinia* strain with the *yopE* promoter fused to a bioluminescent reporter cassette to conclude that expression of this effector gene is activated by contact with cultured HeLa cells. *R. solanacearum* factors that are essential for adherence to plant cells have not been identified, because neither PrhA nor the HrpY pilus[13] is required for this initial, and presumably crucial, step.

Unlike animal cells, plant cells have a thick, semi-rigid wall surrounding them that must be the initial point of bacterial contact. Therefore, Aldon *et al.*[8] logically tested isolated plant cell walls for signal activity and found that *hrpB* gene expression was also enhanced by contact with wall fragments, although at a slightly slower rate than by intact cells. Because the cell wall fragments were treated to remove lipids and proteins, these results suggest that the signal might be a pre-formed part of the cell wall polysaccharide matrix. Although the cell wall signal molecule is clearly not host specific, the authors reported only testing four dicot plants and no monocots (and especially no grasses). This could be significant, because the primary cell walls of grasses are substantially different from those of all other flowering plants[15]. Unfortunately, the complexity and heterogeneity of plant cell wall polysaccharides will make characterization of potential signaling molecules challenging.

The nature and the extent of the contact between *R. solanacearum*

and plant cells needed for the induction of *hrpB* was not well defined, because in some micrographs masses of bacteria that were up to 20 μm away from plant cell walls were brightly fluorescent. It is unclear whether all these cases were the result of bacteria being dislodged from the plant cell surface, as the authors suggest. In addition, their evidence that the plant signal is non-diffusible was not convincing, as in this and previous papers, this research group reported only using medium conditioned by prior growth of plant cells and not medium conditioned by co-cultivation of plant cell and bacteria. Only the latter type of conditioned medium restored attachment of some *Agrobacterium tumefaciens* mutants to plant cells[16]. It is also significant that oligosaccharides released from fungal and plant cell walls can act as potent signal molecules[17]. Therefore, it seems possible that induction of *hrpB* could result from a diffusible signal released from plant cell walls by polygalacturonases or endoglucanases secreted by the pathogen.

As expected from previous cocultivation experiments[11,12], *R. solanacearum* requires the PrhA signal cascade to exhibit contact-mediated *hrp* gene expression. Significantly, it does not require a functional type III secreton to respond to the plant cell signal, so in *R. solanacearum* type III secretion is separated from one of the regulatory systems controlling it. This pattern of regulation is different from that found in *Yersinia* species, which coordinately control both protein secretion and expression of effector genes using proteins that transit its type III secreton[4].

An additional virulence regulatory network

Although the Hrp type III secretion system of *R. solanacearum* contributes greatly to pathogenesis, *hrp* mutants retain the ability to invade tomato roots and systemically colonize the vascular system, albeit in greatly reduced numbers[18,19]. Equally important for disease is the Phc confinement-sensing system that controls motility and the production of EPS and multiple cell-wall-degrading EXPs that exit via type II secretion[2]. At the core of this complex regulatory network is PhcA, a LysR-type transcriptional regulator whose activity is modulated by the amount of a unique autoinducer, 3-hydroxy palmitic acid methyl ester, which accumulates in and around cells growing in a confined space. Interestingly, *R. solanacearum* also has a typical quorum-sensing system that responds to acyl homoserine lactone autoinducers (and is itself regulated by the Phc confinement-sensing system), but it is not required for virulence. The nature and significance of possible interactions between the Prh/Hrp and Phc regulatory systems remain uncertain.

Unanswered questions

Research on *R. solanacearum* should continue to be at the forefront of efforts to understand the mechanisms by which plant pathogenic bacteria interact with their hosts. In particular, what we learn about type III secretion systems in this and other plant pathogens can contribute substantially to our overall knowledge of bacterial pathogenesis. Some of the outstanding unanswered questions include: do other plant pathogenic bacteria regulate gene expression by contact with plant cell walls and, if so, do they use a system similar to the PrhA signal transduction pathway? What is the mechanism by which *R. solanacearum* adheres to plant cells and how important is this to subsequent signal recognition by PrhA? What is the chemical identity of the plant cell wall signal recognized by *R. solanacearum* PrhA, and might this compound be released from plant cell walls by bacterial activity? And last, but not least, how do the regulatory responses observed in culture (with or without cultured plant cells) compare with those occurring in planta and exactly how do they contribute to the ability of *R. solanacearum* to be such an accomplished pathogen?

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Ralstonia solanacearum – a plant pathogen in touch with its host: Response

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Dr Denny gives an excellent short overview of the current ideas on how *Ralstonia solanacearum* interacts with its host. However, he suggests that a diffusible molecule could be responsible for the strong induction of the *hrpB* regulatory gene, in contrast to our proposition that the inducer is associated with the plant cell wall. Denny points out that a few clumps of the fluorescent bacteria are also found unattached to plant cells, an observation that might arise if *hrpB* were induced by a diffusible signal released from plant cell walls by degradative enzymes secreted by the pathogen. Two arguments, however, led us to reject this hypothesis: first, the use of a medium conditioned by co-cultivation of plant cells and bacteria does not increase the level of *hrpB* gene transcription compared with a medium conditioned only by plant cells (D. Aldon, unpublished). Second, in the model proposed by Denny, after a long period of co-cultivation the action of pathogen-secreted polygalacturonases or endoglucanases should make this plant signal freely diffusible, thereby leading to the induction of *hrpB* in the majority of bacteria in the medium. However, even after 16 h of co-cultivation, we still observe two distinct bacterial populations: one attached to plant cell surfaces and displaying strong *hrpB* gene expression, and the other non-attached and remaining mostly uninduced[1]. For these reasons, we favor the view that the action of bacterial degradative enzymes on plant cell walls could contribute to make this non-diffusible signal more accessible to attached bacteria rather than making it diffusible. In addition, the loosening of the plant cell wall structure might also facilitate the progression of the Hrp-dependent pilus, which is required for the secretion (and probably the injection into plant cells) of the type-III-dependent effectors[2].

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