Pathogen profile

Pseudomonas syringae pv. *phaseolicola*: from 'has bean' to supermodel

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SUMMARY

Pseudomonas syringae pv. *phaseolicola* causes halo blight of the common bean, *Phaseolus vulgaris*, worldwide and remains difficult to control. Races of the pathogen cause either disease symptoms or a resistant hypersensitive response on a series of differentially reacting bean cultivars. The molecular genetics of the interaction between *P. syringae* pv. *phaseolicola* and bean, and the evolution of bacterial virulence, have been investigated in depth and this research has led to important discoveries in the field of plant–microbe interactions. In this review, we discuss several of the areas of study that chart the rise of *P. syringae* pv. *phaseolicola* from a common pathogen of bean plants to a molecular plant–pathogen supermodel bacterium.

Taxonomy: Bacteria; Proteobacteria, gamma subdivision; order *Pseudomonadales*; family *Pseudomonadaceae*; genus *Pseudomonas*; species *Pseudomonas syringae*; Genomospecies 2; pathogenic variety *phaseolicola*.

Microbiological properties: Gram-negative, aerobic, motile, rod-shaped, 1.5 µm long, 0.7–1.2 µm in diameter, at least one polar flagellum, optimal temperatures for growth of 25–30 °C, oxidase negative, arginine dihydrolase negative, levan positive and elicits the hypersensitive response on tobacco.

Host range: Major bacterial disease of common bean (*Phaseolus vulgaris*) in temperate regions and above medium altitudes in the tropics. Natural infections have been recorded on several other legume species, including all members of the tribe Phaseoleae with the exception of *Desmodium* spp. and *Pisum sativum*.

Disease symptoms: Water-soaked lesions on leaves, pods, stems or petioles, that quickly develop greenish-yellow haloes on leaves at temperatures of less than 23 °C. Infected seeds may be symptomless, or have wrinkled or buttery-yellow patches on the

seed coat. Seedling infection is recognized by general chlorosis, stunting and distortion of growth.

Epidemiology: Seed borne and disseminated from exudation by water-splash and wind occurring during rainfall. Bacteria invade through wounds and natural openings (notably stomata). Weedy and cultivated alternative hosts may also harbour the bacterium.

Disease control: Some measure of control is achieved with copper formulations and streptomycin. Pathogen-free seed and resistant cultivars are recommended.

Useful websites: *Pseudomonas*–plant interaction <http://www.pseudomonas-syringae.org/>; PseudoDB <http://xbase.bham.ac.uk/pseudodb/>; Plant Associated and Environmental Microbes Database (PAMDB) <http://genome.ppws.vt.edu/cgi-bin/MLST/home.pl>; *PseudoMLSA* Database <http://www.uib.es/microbiologiaBD/Welcome.html>

INTRODUCTION

Pseudomonas syringae pathovar *phaseolicola* causes halo blight of the common bean, *Phaseolus vulgaris*, and the disease in leaves is classically recognized by the presence of water-soaked lesions surrounded by haloes (Fig. 1) (Murillo *et al.*, 2010). Halo blight disease was characterized by Burkholder (1926) and the isolated bacterium was initially named *Phytomonas medicaginis* variant *phaseolicola*, before being renamed *Pseudomonas medicaginis* variant *phaseolicola* (Dowson, 1943) and, subsequently, *P. syringae* pathovar *phaseolicola* (Young *et al.*, 1978). The final renaming grouped the bean pathogen with the many other plant-pathogenic strains also prefixed *Pseudomonas syringae*.

Pseudomonas syringae pv. *phaseolicola* persists on dry bean seeds and becomes active after their germination (Burkholder, 1930). It can survive as both a parasite within plant tissue and as

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Fig. 1 Symptoms produced by *Pseudomonas syringae* pv. *phaseolicola* on *Phaseolus vulgaris*. Disease symptoms observed in the field on bean leaves (A) and bean pods (B). Symptoms on artificially inoculated bean leaves, 72 h after incubation, appear as disease (C), characterized by water-soaked necrotic lesions surrounded by yellow haloes, and hypersensitive response (HR) (D), characterized by tissue collapse and browning of the inoculated area. (E) and (F) demonstrate the disease response and HR, respectively, on artificially inoculated bean pods. Photographs in (A) courtesy of Jesus Murillo and in (B) courtesy of John Taylor.

an epiphyte. The disease is spread within the crop by rain splash, plant-to-plant contact, irrigation water and field workers, and favours cool (less than 25 °C) moist conditions. It is prevalent worldwide, and causes most devastation in developing countries (Taylor *et al.*, 1996a). Halo blight is considered to be such a serious disease that US law (Idaho State) demands that any crops found to contain *P. syringae* pv. *phaseolicola* must be destroyed. Up to 43% reductions in total yield have been reported and further losses occur owing to the poor quality of infected pods (Prosen *et al.*, 1993). Halo blight is controlled mainly by sowing pathogen-free seed, the use of resistant varieties and through crop rotation (Taylor *et al.*, 1996b).

The molecular genetics of *P. syringae* pv. *phaseolicola* have been studied extensively, leading to discoveries of general impact on our knowledge of microbial pathogenicity and plant defences. For example, the first characterization of the *hrp* genes that encode the Type III secretion system (T3SS), which delivers proteins directly into plant cells, was achieved using the halo blight bacterium. More recently, comparative analysis of the whole-genome sequences of *P. syringae* pv. *phaseolicola* and other *P. syringae* pathovars has greatly advanced our understanding of the evolution of host specificity. Here, we review the lessons learned that have led to the bean pathogen becoming a supermodel species for research on plant-pathogen interactions.

RACE STRUCTURE AND GENE-FOR-GENE INTERACTIONS

Bacterial pathogens manipulate their hosts by delivering effector proteins into the plant cell cytoplasm through the conserved T3SS (Casper-Lindley *et al.*, 2002). Pioneering research by Panopolous and co-workers identified genes encoding the T3SS in *P. syringae* pv. *phaseolicola* as a region of the chromosome required for the induction of the hypersensitive reaction (HR) and pathogenicity (*hrp*) (Lindgren *et al.*, 1986). Plants have evolved resistance proteins (R) that recognize a subset of effectors, termed 'avirulence' (Avr) proteins, that trigger a defensive HR, generating an antimicrobial environment and leading to the restriction of bacterial colonization (Jones and Dangl, 2006). The gene-for-gene interaction between Avr and R proteins has led to the establishment of race structures within *P. syringae* pathovars, including *P. syringae* pv. *phaseolicola*.

Taylor *et al.* (1996a, b) carried out an exceptionally thorough study of *P. syringae* pv. *phaseolicola* and its host plant. They collected 893 isolates from Africa and other bean-growing areas, and used 175 to differentiate pathotypes. Nine races were identified on the basis of their interactions with eight bean cultivars and five pairs of *avr* and *R* genes (Table 1). A number of races (1, 2, 5, 6 and 7) were distributed worldwide, with race 6 predominant. Other races were found mainly in Africa: races 3 and 4 in

Table 1 Race differentiation in *Pseudomonas syringae* pv. *phaseolicola*.

Different cultivars	Resistance alleles (R1–R5)†					Races/avirulence genes (A1–A5)*								
						1	2	3	4	5	6	7	8	9
	1	2	3	4	5	1	2	3	4	5	6	7	8	9
Canadian Wonder	.	.	.	.	.	+	+	+	+	+	+	+	+	+
52 (ZAA54)	.	.	.	4	.	+	+	+	+	–	+	+	+	+
Tendergreen	.	.	3	.	.	+	+	–	–	+	+	+	+	+
Red Mexican U13	1	.	.	4	.	–	+	+	+	–	+	–	+	–
1072	.	2	.	.	.	+	–	+	–	–	+	–	+	+
A53 (ZAA55)	.	.	3	4	.	+	+	–	–	–	+	+	+	+
A43 (ZAA12)	.	2	3	4	5	+	–	–	–	–	+	–	–	–
Guatemala 196-B	1	.	3	4	.	–	+	–	–	–	+	–	+	–

Adapted from Taylor *et al.* (1996a, b).*The avirulence genes A1, A2 and A3 have been cloned as *avrPphF*, *avrPphE* and *avrPphB*, respectively.†Current nomenclature used for the *R* genes is *Pse-1* to *Pse-4*, and *pse-5* (because this was found to be recessive), as described by Miklas *et al.* (2009).

East/Central Africa and races 8 and 9 in southern Africa (Taylor *et al.*, 1996a). In total, 1048 bean accessions were also collected from Africa and the Americas, and evaluated for their resistance to *P. syringae* pv. *phaseolicola*. Race-specific *R* genes were detected in 49.4% of accessions with the following gene frequencies: *R1* (10.3%), *R2* (0.3%), *R3* (25.0%), *R4* (35.0%) and *R5* (0.2%) (Taylor *et al.*, 1996b). Corresponding *avr* gene frequencies were calculated as: *A1* (27.4%), *A2* (23.9%), *A3* (23.2%), *A4* (3.5%) and *A5* (18.7%). In general, the higher the *R* gene frequency, the lower the matching *avr* gene frequency, suggesting that, where an *R* gene is widely deployed, the races of the pathogen carrying the corresponding *avr* gene are suppressed. Taylor *et al.* also found some isolates that could not be assigned to any of the nine races, results confirmed by Rico *et al.* (2003) and Rivas *et al.* (2005). Three of the predicted *P. syringae* pv. *phaseolicola* *avr* genes have been cloned: *avrPphB* (*A3*), *avrPphE* (*A2*) and *avrPphF* (*A1*) (Jenner *et al.*, 1991; Mansfield *et al.*, 1994; Tsiamis *et al.*, 2000). Additional *avr* genes, *avrPphC* and *avrPphD*, were cloned on the basis of their contribution towards hypersensitive resistance in the nonhost plants, soybean and pea, respectively (Mansfield, 2009). *AvrPphB* triggers the HR in *Arabidopsis thaliana* accessions with the matching *RP55* resistance gene (Shao *et al.*, 2003).

HYPERSENSITIVE RESPONSE AND PATHOGENICITY GENES

Research using *P. syringae* pv. *phaseolicola* has shaped our understanding of the T3SS. *hrp* mutants were first identified in the bean pathogen using Tn5 insertions (Lindgren *et al.*, 1986; Niepold *et al.*, 1985). The mutants lost pathogenicity to bean as well as the ability to elicit HR in tobacco. *hrp* genes have sub-

sequently been shown to be conserved in other *P. syringae* pathovars, and are clustered on a pathogenicity island (PAI) that comprises at least six operons (Alfano *et al.*, 2000).

The transcriptional regulation of components of the T3SS and effectors in *P. syringae* pv. *phaseolicola* has been studied in some detail (Thwaites *et al.*, 2004). After induction in minimal medium, quantitative real-time polymerase chain reaction (PCR) was used to analyse the transcription of three effector genes, *avrPphE*, *avrPphF* and *virPphA*, together with several *hrp* genes, including the first gene of each *hrp* operon and the regulators *hrpRS* and *hrpL*. A number of interesting observations were made, including that *hrpL* was rapidly induced, confirming its role as the key regulator of *hrp* gene expression. Further analysis comparing *in vitro* and *in planta* regulation of the same genes showed that transcript accumulation within the first 2 h after inoculation was considerably higher in the plant than *in vitro*, indicating that plant cell wall contact may enhance the transcription of T3SS and effector genes regulated by HrpL. Recently, Ortiz-Martin *et al.* (2010a, b) have described the positive and negative regulation of the Hrp region in strain 1448A. They analysed the roles of HrpL, HrpA and GacA in the positive regulation of T3SS, and Lon and HrpV in its negative regulation. Importantly, they showed that HrpL acts as a general regulator of gene expression, as it not only up-regulates non-T3SS-related virulence determinants, but also down-regulates flagellar function.

To identify additional genes involved in the regulation of the T3SS and effector genes, Deng *et al.* (2009) screened for mutants with reduced induction of luciferase-based reporter genes constructed with promoters of *hrpL* and the effector gene *avrPto*. Forty-six mutants were selected after screening approximately 28 000 transposon insertions. Most of the genes disrupted were

found to encode metabolic enzymes, but three encoded regulatory functions. Further work revealed that the novel regulatory gene *aefR*_{NPS3121} controlled both quorum-sensing and T3SS genes, leading to the suggestion that *P. syringae* pv. *phaseolicola* may regulate T3SS gene expression in response to cell density.

Harpins are a group of secreted effector proteins that travel through the T3SS in plant-pathogenic bacteria (Li *et al.*, 2002). The *P. syringae* harpin, HrpZ1, encoded by *hrpZ* located within the *hrp* cluster, may operate as a 'helper' protein by facilitating the movement of effectors through the Hrp pilus. HrpZ1 also forms ion-conducting pores in membranes, and therefore may play a role in the delivery of effector proteins during infection (Lee *et al.*, 2001). HrpZ1 is also recognized by plant cells as a pathogen-associated molecular pattern (PAMP) triggering immune responses (Chisholm *et al.*, 2006). Recent work by Engelhardt *et al.* (2009) using HrpZ1 from *P. syringae* pv. *phaseolicola* has dissected the two properties of the protein and shown that ion pore formation and immune activation have different structural requirements. They showed that a C-terminal peptide fragment retains the ability to activate plant immunity, whereas ion pore formation requires intact HrpZ1.

Further analysis of the *hrp* cluster has focused on *hrcN*, encoding an ATPase that is related to the *F*₁-ATPase β subunit and catalyses protein translocation through the T3SS (Müller *et al.*, 2006; Pozidis *et al.*, 2003). The action of the HrcN ATPase is essential for export from the T3SS. The ATPase binds to and forms clusters in the bacterial inner member and can be extracted as a dodecamer (Pozidis *et al.*, 2003). Müller *et al.* (2006) used cryo-electron microscopy to a resolution of 1.6 nm to reveal the three-dimensional structure of the dodecameric form of the T3SS ATPase of *P. syringae* pv. *phaseolicola*, and also proposed a model for the incorporation of the ATPase at the base of the secretion apparatus.

On the basis of their ability to utilize the T3SS pathway, effectors have been designated Hop, defining *Hrp* outer proteins (Alfano and Collmer, 1997). Lindeberg *et al.* (2005) proposed nomenclatural guidelines for type III Hop effector proteins in *P. syringae* to overcome the lack of an established systematic naming procedure. For example, AvrPphB, AvrPphE and AvrPphF are now also known as HopAR1, HopX and HopF1, respectively.

ROLE OF EFFECTORS IN PATHOGENICITY

Initial work on the determinants of pathogenicity in *P. syringae* used techniques such as transposon mutagenesis and heterologous library screening. The transposon insertion mutants isolated typically proved to be knockouts of *hrp* genes. Library screening, by the exchange of genomes between races, identified a number of *avr* genes, but no positive determinants of virulence (Mansfield, 2009). In 1999, a study was published that concentrated on

the removal of plasmids from the bacterium and the examination of subsequent effects on pathogenicity (Jackson *et al.*, 1999). A 154-kb plasmid (pAV511) was cured from *P. syringae* pv. *phaseolicola* strain 1449B by incompatibility with a cloned plasmid origin of replication. The resulting strain, RW60, lost virulence towards bean, causing the HR in previously susceptible cultivars.

Cosmid clones spanning a 30-kb region were found to restore virulence by complementation. This region contained a number of previously identified *avr* genes, including *avrD*, *avrB2* and *avrPphF*. Further sequence analysis revealed four potential virulence (*vir*) genes that were predicted to encode hydrophilic proteins and shared the *hrp*-box promoter motif indicating regulation by HrpL and, therefore, T3SS delivery. One gene achieved partial restoration of virulence when cloned on its own and was therefore designated *virPphA* (*hopAB1*). Interestingly, in soybean, *virPphA* acts as an *avr* gene controlling the expression of a rapid, cultivar-specific HR. The proximity of several *avr* and *vir* genes in the region that restored virulence led the authors to conclude that they had identified a plasmid-borne PAI. An additional interesting aspect of this work was that the loss of the plasmid led to the uncovering of a previously suppressed HR. This suggested that gene(s) encoding proteins able to trigger the HR are present elsewhere in the *P. syringae* pv. *phaseolicola* chromosome, but the functions of the plasmid-borne virulence genes were epistatic to the avirulence phenotype.

Subsequently, homologues of *virPphA* have been identified, namely *virPphA*_{Pgy}, and *virPphA*_{PSV} from *P. savastanoi* pathovars *glycinea* and *savastanoi*, respectively, and *avrPtoB* from *P. syringae* pv. *tomato* (Jackson *et al.*, 2002). Sequencing showed that the *virPphA*_{Pgy} homologue had a 48-bp central deletion in the open reading frame (ORF) compared with *virPphA* and *virPphA*_{PSV}, but otherwise all three of these alleles had >98% identity at the DNA level. By contrast, *AvrPtoB* from *P. syringae* pv. *tomato* was predicted to have only 51% amino acid similarity with *VirPphA*. However, *AvrPtoB* and *VirPphA* have been shown to share a C-terminal E3 ubiquitin ligase domain (Abramovitch *et al.*, 2006). Each of the *virPphA* homologues was introduced into *P. syringae* pv. *phaseolicola* strain RW60, and all were shown to restore virulence to bean.

A second gene found on plasmid pAV511, *avrPphF*, was also shown to be capable of restoring virulence to *P. syringae* pv. *phaseolicola* strain RW60 in bean cv. Tendergreen. However, if this strain (RW60 containing *avrPphF*) was inoculated onto the universally susceptible bean cv. Canadian Wonder, HR was observed (Tsiamis *et al.*, 2000). Moreover, another gene from pAV511, the soybean avirulence gene *avrPphC*, was found to block the activity of *avrPphF* in Canadian Wonder, but not in bean cv. Red Mexican. This work first revealed the complex web of interactions between *avr* and *vir* functions, highlighting the now commonly recorded suppression of the HR by effector proteins.

EFFECTOR EVOLUTION AND MOBILITY

The effector gene *avrPphB* (*hopAR1*) was cloned by Jenner *et al.* (1991). *avrPphB* is chromosomally located and comprises an 801-bp ORF that is predicted to encode a cytoplasmic protein. The gene has a limited distribution, being present in races 3 and 4 of *P. syringae* pv. *phaseolicola*, but not in the numerous other *P. syringae* pathovars examined. The extensive study of *avrPphB* has provided interesting insights into effector evolution and function.

A variant of race 3 strain 1302A, named RJ3, exhibited an extended host range in cultivars of bean, and was found to have lost from the chromosome a large, 106-kb, region that contained *avrPphB* (Jackson *et al.*, 2000; Pitman *et al.*, 2005). The excised region shared similarity with genomic islands (GIs) found in other bacterial pathogens of animals as well as plants (Hacker and Carniel, 2001; Hacker and Kaper, 2000; van der Meer and Senthilo, 2003), and was designated PPHGI-1.

Pitman *et al.* (2005) examined the dynamics of PPHGI-1 loss during infection and showed that, with repeated passaging of 1302A through resistant cv. Tendergreen, the GI was quickly lost from the bacterial population. For example, after just three 1-week passages, 75% of bacterial colonies recovered from the plants caused disease symptoms, i.e. had lost *avrPphB*, whereas the remainder, as seen in the original 1302A, activated a rapid HR. This work showed how rapidly bacteria can evolve to overcome host resistance when interacting with the stressful environment of a resistant host plant (Arnold *et al.*, 2007). PPHGI-1 is transferred between strains of *P. syringae* pv. *phaseolicola* (Lovell *et al.*, 2009). Co-inoculation of donor 1302A and recipient 1448A strains into bean leaves led to the acquisition of an intact copy of PPHGI-1 in the chromosome of 1448A. It was also shown that the mechanism behind the transfer was transformation, because heat-treated cells or DNA extracts could also be used as donors. This process did not take place *in vitro* unless media were supplemented with apoplastic fluid extracted from bean leaves.

PPHGI-1 becomes a circular episome when it excises from the *P. syringae* pv. *phaseolicola* chromosome (Pitman *et al.*, 2005) and, more recently, the circular form was found to be capable of independent replication (Lovell *et al.*, 2011). When it is in the circular form, the expression of *avrPphB* is greatly reduced, leading to speculation that this may be a mechanism to prevent recognition of the Avr protein by the plant, and therefore allow time for the island to be reintegrated into the chromosome or transferred to another cell (Godfrey *et al.*, 2011).

Deletion of *avrPphF* (*hopF1*) has also been implicated in the increased virulence of strains to cultivars carrying the matching *R1* resistance allele (Table 1). *avrPphF* is located on the 154-kb plasmid, pAV511, in *P. syringae* pv. *phaseolicola* strain 1449B (Jackson *et al.*, 1999). The PAI is generally well conserved in

strains of *P. syringae* pv. *phaseolicola*, but *avrPphF* is sometimes lost as a result of large 9.5-kb or 10.5-kb deletions. The deletions are probably mediated by a chimeric mobile element composed of two novel insertion sequences, designated IS_{Psy16} and IS_{Psy17}, that define the left border of each deletion (Rivas *et al.*, 2005).

Small-scale changes have also been shown to occur in an effector sequence that lead to the avoidance of the plant's surveillance machinery. The *P. syringae* pv. *phaseolicola* effector gene *avrPphE* (*hopX1*) is a good example. *avrPphE* was initially cloned from *P. syringae* pv. *phaseolicola* race 4 strain 1302A (Mansfield *et al.*, 1994). Homologues of *avrPphE* are present in all nine races of *P. syringae* pv. *phaseolicola*, but *avr* phenotypes are only observed in races 2, 4, 5 and 7 (Stevens *et al.*, 1998). Comparison of the sequence of the *avrPphE* alleles from the nine races of *P. syringae* pv. *phaseolicola* revealed single base-pair substitutions in races 1, 3, 6 and 9, which lead to single amino acid changes in the predicted translation product and abolish *avr* activity. The *avrPphE* allele from race 8 has a 104-bp insertion with a 12-bp inverted repeat at its borders. This insertion is predicted to produce a protein that is 60 amino acids longer than the wild-type and fails to induce the HR. Questions remain concerning the function of the *AvrPphE* variants that do not elicit the HR. Why are they maintained in the genome? Do they have a function in pathogenicity, or in another aspect of the *P. syringae* pv. *phaseolicola* life cycle?

FUNCTION OF EFFECTORS

Perhaps the most topical area of research into microbial pathogenicity concerns the biochemical functions of effector proteins. These studies have highlighted the clear similarities between mechanisms of pathogenicity in bacterial pathogens of both plants and animals. Whereas the primary amino acid sequence for most effector genes provides little insight into their biochemical function, structural analysis by crystallography has proved to be more rewarding.

The autoproteolytic form of *AvrPphB* was the first reported crystal structure of a type III effector protein from a plant pathogen (Zhu *et al.*, 2004). The protein is composed of a central antiparallel β -sheet, with α -helices packing on both sides to form a two-lobe structure. The core resembles papain-like cysteine proteases. *AvrPphB* and the *Yersinia pestis* effector YopT are now considered to define a family of 19 proteases found in pathogens of plants and animals (Shao *et al.*, 2002). Their proteolytic activities are dependent on the invariant amino acids C139, H258 and D274. *AvrPphB* was shown to undergo autoproteolytic cleavage that required the C/H/D residues. This cleavage converted the wild-type protein from a 35-kDa into a 28-kDa form. These results were in line with earlier work of Puri *et al.* (1997), who showed that *AvrPphB* is processed in both

P. syringae pv. *phaseolicola* and *Escherichia coli* to give a 28-kDa protein. The protease activity of YopT cleaves the post-translationally modified Rho GTPases near their carboxyl termini, releasing them from the cell membrane. In animal cells, this activity leads to disruption of the actin cytoskeleton.

Tain *et al.* (2009) proposed that AvrPphB, like YopT, might target microfilaments in the host plant. They found that, in *A. thaliana*, actin-depolymerizing factor (AtADF4) was specifically required for resistance triggered by AvrPphB and that loss of AtADF4 conferred enhanced susceptibility to *P. syringae* expressing AvrPphB. How AtADF4 mediates AvrPphB-triggered resistance remains unknown, but the authors put forward several hypotheses. For example, AtADF4 may play a role in the transport of AvrPphB and the plant's RPS5 protein within or away from the plasma membrane following initial recognition, or the factor may be involved directly in defence signal transduction (Tain *et al.*, 2009).

There have been interesting developments in the discovery of the targets of protease activity in plant cells, which may indicate virulence functions for AvrPphB. Cleavage of PBS1, a protein kinase found in *Arabidopsis*, is required for RPS5-mediated resistance (Shao *et al.*, 2003). More recently, Zhang *et al.* (2010), investigating the possible virulence functions of AvrPphB in plants lacking RPS5, found that the effector inhibits PAMP-triggered immunity by cleaving cytoplasmic kinases in addition to PBS1. The kinase targets appear to function as integrators of immune signalling originating from several receptors, including the flagellin receptor FLS2.

The crystal structures of the gene products of both ORF1 and ORF2 of *avrPphF* have also been determined (Singer *et al.*, 2004). Both of these ORFs are required for virulence on susceptible bean cultivars, as well as for the initiation of the HR on resistant plants. ORF1 was found to be very similar to type III chaperones from bacterial pathogens of animals. The ORF2 protein is novel, having a 'mushroom'-like structure containing 'head' and 'stalk' subdomains. Although the 'head' structure possesses limited structural homology to the catalytic domain of bacterial ADP-ribosyltransferases, no enzyme activity could be detected in standard assays. A number of predicted surface-

exposed residues from ORF2 were mutated and shown to be important for its virulence activity in bean, as well as for recognition by the R1 resistance gene product (Singer *et al.*, 2004).

COMPARATIVE GENOMICS AND EFFECTOR REPERTOIRES

The genome sequence of *P. syringae* pv. *phaseolicola* strain 1448A was published in 2005 (Joardar *et al.*, 2005). The 6.5-Mbp genome is split between one circular chromosome and two plasmids (Table 2) which, together, are predicted to encode 5353 ORFs (Joardar *et al.*, 2005). Two other *P. syringae* genomes were published around the same time: *P. syringae* pv. *tomato* strain DC3000, which is pathogenic on tomato and *A. thaliana* (Buell *et al.*, 2003), and *P. syringae* pv. *syringae* strain B728a, the cause of brown spot on bean (Feil *et al.*, 2005).

Extensive comparisons have been made between *P. syringae* pv. *phaseolicola* 1448A and *P. syringae* pv. *tomato* DC3000 (Joardar *et al.*, 2005). In summary, the two strains were shown to be generally similar, sharing 83% of their ORFs, including high conservation for genes required for basic physiological and metabolic functions. Joardar *et al.* (2005) also compared the genome sequences of *P. syringae* pv. *phaseolicola* 1448A and *P. syringae* pv. *tomato* DC3000 with *P. aeruginosa* PAO1, *P. putida* KT2440 and *P. fluorescens* Pf-5 to identify the core *Pseudomonas* genome. They showed that a total of 3567 ORFs (67%) were present in all four species, 365 (7%) were specific to *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *tomato*, and 392 (7%) were specific to *P. syringae* pv. *phaseolicola* 1448A. Investigation of the function of the species- and pathovar-specific ORFs should lead to a greater understanding of their roles in host specificity.

Mining of genome sequences has been used to try to identify all of the effector proteins secreted by different *P. syringae* pathovars, as reviewed by Lindeberg *et al.* (2006). Chang *et al.* (2005) predicted 19 T3SS proteins in *P. syringae* pv. *phaseolicola* 1448A using a differential fluorescence induction method for the high-throughput discovery of HrpL-regulated genes. They confirmed these candidates using a functional secretion assay employing the COOH-terminus of AvrRpt2 as a reporter for deliv-

Table 2 General features of the *Pseudomonas syringae* pv. *phaseolicola* 1448A genome.

Feature	Chromosome	Plasmid p1448A-A	Plasmid p1448A-B	Characterization of selected ORFs	Number of ORFs (%)
Size (bp)	5 928 787	131 950	51 711	Transport and binding proteins	728 (14)
G + C (%)	58.0	54.1	56	Cellular processes	708 (13)
Coding regions (%)	87.0	78.0	84.9	Regulatory role	535 (10)
No. of ORFs	5144	149	60	Mobile genetic elements	256 (5)
Mean ORF length (bp)	1007	660	719	Hypothetical proteins	224 (4)
No. of ORFs with annotated function (%)	3492 (68)	97 (62)	37 (62)	Conserved hypothetical proteins	822 (15)
				Proteins of unknown function	681 (13)

ORF, open reading frame.

Adapted from Joardar *et al.* (2005).

ery of the proteins into plants containing the cognate R protein RPS2 (Guttman *et al.*, 2002). Bioinformatic approaches have also been used to scan the genome sequences for features common to effector genes, e.g. screening for the consensus *hrp*-box motif, 5'-GGAACCNA-N13-14CCACNNA-3', which is a promoter sequence found upstream of effectors that are known to be regulated by *hrpL*, and also T3SS secretion signals. Using these parameters, Vencato *et al.* (2006) identified 27 high-probability candidates in *P. syringae* pv. *phaseolicola* 1448A.

To identify the complete complement of *P. syringae* pv. *phaseolicola* 1448A effectors, Zumaquero *et al.* (2010) analysed the translocation and/or expression of the 22 experimentally validated effectors plus other candidates to bring the confirmed effector inventory to 27. They also produced single- and double-knockout mutants of the majority of effectors, and used competitive index (CI) assays, comparing the multiplication of different strains within the same inoculated leaf, to investigate the individual contribution of effectors to virulence. For example, AvrB4-1 and AvrB4-2, which differ by three amino acids, were shown to be fully redundant and made a quantitative contribution to Pph1448A virulence in bean. The highly discriminatory CI assay may allow the contribution of individual effector genes to be revealed despite a background of functional redundancy.

PHASEOLOTOXIN

The name halo blight comes from the characteristic chlorotic halo that surrounds water-soaked lesions on a plant infected with *P. syringae* pv. *phaseolicola*. The chlorotic halo results from the action of phaseolotoxin, which is a nonspecific phytotoxin. Phaseolotoxin inhibits ornithine carbamoyl transferase, a critical enzyme in the urea cycle (Moore *et al.*, 1984). The production of phaseolotoxin is dependent on temperature, with 18–20 °C being optimal and no detectable amounts of the toxin being present at 30 °C (Nüske and Fritsche, 1989). Transcription profiling has shown that the optimum expression of phaseolotoxin not only requires a low temperature, but also specific plant components present in leaf and apoplast (Hernández-Morales *et al.*, 2009). The genes involved in phaseolotoxin biosynthesis are organized into the *argK-tox* gene cluster, which includes five transcriptional units (Aguilera *et al.*, 2007; Peet *et al.*, 1986). *argK* encodes a phaseolotoxin-resistant ornithyl carbamoyl transferase (Mosqueda *et al.*, 1990). The *argK-tox* gene cluster comprises 23 genes, is flanked by insertion sequences and transposases and is located in a highly conserved 38-kb DNA fragment with the characteristics of a GI (Aguilera *et al.*, 2007; Genka *et al.*, 2006). The horizontal transfer of the cluster has never been demonstrated functionally, but its conservation in both *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *actinidiae* indicates that it has been inherited by horizontal transfer (Sawada *et al.*, 2002).

Significantly, the importance of phaseolotoxin in the bean pathogen was questioned in a study carried out in Castilla y León County, Spain (Rico *et al.*, 2003). From a collection of 138 *P. syringae* pv. *phaseolicola* isolates taken from plants in commercial fields, 95 did not produce phaseolotoxin *in vitro*, as determined by an *E. coli* inhibition bioassay. Ninety-four of the isolates also failed to produce the expected PCR amplification product from primers designed to ORF6 of the phaseolotoxin gene cluster. This was the first report of the widespread occurrence of Tox⁻ isolates in the field, and has been confirmed by San José *et al.* (2010). Prior to this, it was considered that Tox⁻ strains were of little or no epidemiological significance.

PSEUDOMONAS SYRINGAE PV. PHASEOLICOLA AS A PROBE FOR DETERMINANTS OF NONHOST RESISTANCE

Strains of *P. syringae* pv. *phaseolicola* have been used to probe mechanisms of nonhost resistance in *Arabidopsis*. Interestingly, despite encoding numerous effectors (Block *et al.*, 2008; Cunnac *et al.*, 2009; Joardar *et al.*, 2005), *P. syringae* pv. *phaseolicola* typically does not cause the HR in *Arabidopsis* accessions, although ectopic expression of *avr* genes will, in some cases, activate the programmed cell death response. Microscopy of defence responses was carried out by Soylu *et al.* (2005), who examined the HR induced by *P. syringae* pv. *phaseolicola* strain 1448A harbouring the *avr* genes *avrPpiA* and *avrPphB* (matching *R* genes *RPM1* and *RPS5*, respectively; Grant *et al.*, 1995; Simonich and Innes, 1995), as well as basal, cell wall-associated defences. It has been argued that the response of *Arabidopsis* to PAMPs from *P. syringae* pv. *phaseolicola*, notably flagellin, may be so rapid that it blocks the activation of the HR, perhaps because of the prevention of delivery of effector proteins (Forsyth *et al.*, 2010).

Lu *et al.* (2001) used *P. syringae* pv. *phaseolicola* strain NPS3121 to screen mutants of the *A. thaliana* Columbia (Col-0) accession for increased susceptibility to colonization. Their detailed study resulted in the cloning of the nonhost resistance 1 (*NHO1*) gene that was found to encode a glycerol kinase (Kang *et al.*, 2003). Expression of *NHO1* is increased rapidly after bacterial challenge, but the precise role of the glycerol kinase, as a component of the mechanism of defence, has not been resolved (He *et al.*, 2006).

Using single and double mutants with disruptive insertions in known defence genes, Ham *et al.* (2007) examined their roles in resistance to *P. syringae* pv. *phaseolicola* strain NPS3121. Their results showed that mutations in individual genes, *pmr4*, *pad4* and *npr1*, did not affect the ability of *P. syringae* pv. *phaseolicola* to multiply in Col-0 leaves, but the double mutant *pad4/pmr4* allowed enhanced colonization, particularly if NPS3121 expressed the effector HopM1. They concluded that nonhost

resistance was a 'multilayered' process controlled by several genes regulating different signalling pathways and mechanisms of resistance.

Expression of the effector AvrPtoB in the RW60 strain, derived from *P. syringae* pv. *phaseolicola* strain 1449A (Jackson *et al.*, 1999), also reduces resistance particularly in the *Arabidopsis* Wassilewskija (Ws) accession but not in Niedersenz (Nd; de Torres *et al.*, 2006). In contrast to Nd or Col, Ws was found to be weakly susceptible to *P. syringae* pv. *phaseolicola* strain 1448A (Forsyth *et al.*, 2010). The strong resistance of Nd was attributed to the presence of *FLS2* encoding the flagellin receptor (which is absent from Ws) and a second gene that was mapped to chromosome 1. Flagellin from *P. syringae* pv. *phaseolicola* seems to be very effective in eliciting resistance in *Arabidopsis* and, although other potential sensors may contribute to defence activation (for example, the receptor-like protein ATRLP30; Wang *et al.*, 2008), the receptor *FLS2* represents the major barrier to infection.

DISEASE CONTROL AND CONCLUDING REMARKS

Taylor *et al.* (1979) highlighted the importance of using disease-free seed for the control of outbreaks of halo blight, pointing out that there are no bacteriocidal sprays of lasting value available for control once the disease has become established. Taylor *et al.* have also shown the importance of the selection of varieties with resistance to the prevalent pathotype of *P. syringae* pv. *phaseolicola* (Taylor *et al.*, 1996a, b). The current knowledge of race structure has allowed some *avr* genes to be genotyped using PCR-based methods and provides a reliable indication of the likely success of certain cultivars. Unfortunately, although some genotypes appear to show reduced susceptibility, there are no varieties of bean highly resistant to the most prevalent race 6 (Terán *et al.*, 2009).

Clean seed is essential and molecular probes have been developed to allow the detection of seed-borne inoculum. One approach has been to use PCR or serological protocols that detect the phaseolotoxin gene cluster or its products. However, reports of Tox⁻ strains in diseased crops have brought into question the practice of targeting phaseolotoxin as a biomarker (Rico *et al.*, 2003; San José *et al.*, 2010). An alternative method has recently been reported using a TaqMan probe and primer set based on a site-specific recombinase gene (Cho *et al.*, 2009). Li *et al.* (2009) have developed a loop-mediated isothermal amplification (LAMP) protocol for rapid and specific characterization and identification of *P. syringae* pv. *phaseolicola* from other pathovars of *P. syringae* and other plant-associated bacteria. The additional methods should allow the rapid detection of all *P. syringae* pv. *phaseolicola* pathotypes. The use of molecular probes is therefore already having an impact on disease control strategies.

Where next for the 'supermodel'? We suggest that the value of *P. syringae* pv. *phaseolicola* as a model Gram-negative pathogen will continue to be realized in the following five areas of basic understanding of plant-pathogen interactions.

1. The contribution of effectors to host specificity will become more apparent from genomic analyses coupled with functional assessment of the targets of individual effectors. Analysis of the variation in virulence of *P. syringae* pv. *phaseolicola* to diverse legumes should prove particularly informative.
2. The dissection of plant defences will be achieved using effectors from the halo blight bacterium that have defined roles in the suppression of *R* gene-based defences and also PAMP-triggered immunity.
3. The involvement of effectors in harnessing nutrients from the invaded plant should become more apparent, particularly through research on the prolonged biotrophic infections that are able to develop in pods (Harper *et al.*, 1987).
4. The unexpected plasticity of genomes, as revealed from experiments on the dynamics of PPHGI-1 (Lovell *et al.*, 2011), should be examined in depth using high-throughput sequencing platforms. It would be intriguing to understand the frequency of occurrence of genome rearrangements and the contribution they make to the evolution of new strains of the pathogen.
5. An understanding of the spatial dynamics of genome rearrangement within the infected plant will become possible using the fluorescent tagging strategies developed by Godfrey *et al.* (2011). Bacteria tagged with fluorescent proteins will also be invaluable in attempts to develop mathematical models of effector delivery and plant responses occurring during colonization.

These are exciting times for molecular plant pathology and there is little doubt that research using the halo blight pathogen will lead to further advances in our understanding of microbial pathogenicity and plant defences. It is to be hoped that the more basic research ongoing that uses *P. syringae* pv. *phaseolicola* as a model bacterial pathogen will have spin-offs in control, particularly in countries in which beans represent part of the staple diet.

ACKNOWLEDGEMENTS

We are indebted to Jesus Murillo for his advice in preparing this review. We acknowledge support from the Biotechnology and Biological Sciences Research Council (BBSRC) for most of our work on the molecular genetics of the halo blight pathogen.

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