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REVIEW

The biochemistry and molecular genetics of host range definition in *Pseudomonas syringae*

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Summary. Many Gram-negative bacterial pathogens of plant and animals harbor a conserved type III protein secretion system for the injection into the eukaryotic cells of host range determinants, or "effectors". In *Pseudomonas syringae*, effectors are the products of avirulence and virulence genes, and the type III secretion apparatus is encoded by *hrp/hrc* genes, which are included in a large pathogenicity island with a tripartite mosaic structure. Individual effectors can have a dual role, depending on the plant host: they can either promote disease or elicit a defence response (hypersensitive response, HR) that diminishes virulence or restricts host range. There is strong evidence to support the belief that effectors act inside host plant cells and interact specifically with the products of plant resistance genes, to elicit the HR, or putative plant "susceptibility genes", to elicit disease. Therefore, the capacity of a *P. syringae* strain to infect a given host, and hence host range, is determined by the sum of the individual activities (either positive or negative) of all the effectors that are injected into a host plant cell. Many *P. syringae* strains also produce other extracellular factors that could be involved in the interaction with the host, although only phytotoxins and certain hormones have been shown so far to increase virulence. Unlike effectors, however, their action does not appear to be host-specific.

Key words: avirulence, effectors, pathogenicity islands (PAIs), toxins, virulence.

Introduction

Plant pathogenic bacteria affect all kinds of plants, including those of agronomic importance such as fruit trees, horticultural plants and ornamentals, causing severe financial losses in the field, greenhouses and during storage. About 1600 bacterial species are known, although only around 100 of them are phytopathogenic (Table 1) (Young *et al.*, 1996; Agrios, 1997). These can cause several kinds of diseases, namely leaf spots and blights, soft rots of fruits, roots, and storage organs, vascular wilts, overgrowths, scabs and cankers (Table 1), suggesting that the pathogenic/virulence strategies they employ are very diverse. Some of the most destructive plant diseases, such as fire blight, soft rots, vascular wilts, or citrus canker, are also produced by bacterial pathogens; their control is

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Table 1. Classification of phytopathogenic bacteria and the diseases they produce[a].

Taxonomical position	Main diseases or types of diseases produced	Main hosts or hosts of agricultural importance
Division: GRACILICUTES - Gram-negative bacteria		
Class: PROTEOBACTERIA		
Family: Enterobacteriaceae		
Genus: *Brenneria*	cankers, watermark disease of willows	Many trees (e.g. oaks, walnut and willows)
Genus: *Erwinia*	fire blight and other wilts soft rots	Pear, apple and other Rosaceae Fleshy vegetables (e.g. potato, endive)
Genus: *Pantoea*	Stewart's wilt of corn, galls, rots and pink-disease of pineapple	Corn, beet, gypsophila, melon, millet and pineapple
Family: Pseudomonadaceae		
Genus: *Acidovorax*	leaf spots	Corn, orchids and watermelon
Burkholderia	wilts, rots and blights	Carnation, gladiolus, mushrooms, onion, rice and wheat
Pseudomonas	leaf spots, blights, vascular wilts, soft rots, cankers, and galls	Many vegetables, fruit trees and cereals; special importance on tomato, bean, and pear
Ralstonia	wilts	Potato, tomato, and nearly 200 other species
Rhizobacter	bacterial gall	Carrot
Rhizomonas	corky root	Lettuce
Xanthomonas	leaf spots, fruit spots, and blights of annual and perennial plants, vascular wilts, and citrus canker.	Many vegetables, trees and cereals; special importance on pepper, tomato, bean, strawberry and citrus
Xylophilus	bacterial necrosis and canker	Grapevine
Family: Rhizobiaceae		
Genus: *Agrobacterium*	crown gall	Most, if not all, dicots; special importance on diverse fruit trees (e.g. peach, almond, apple), a few ornamentals (roses) and grapevine
Family: still unnamed		
Genus: *Xylella*	leaf scorch and dieback	Trees and vine
Division: FIRMICUTES - Gram-positive bacteria		
Class: FIRMIBACTERIA - mostly single-celled bacteria		
Genus: *Bacillus*	rots white stripe	Tubers, seeds, and seedlings Wheat
Clostridium	rots	Tubers
Class: THALLOBACTERIA - branching bacteria		
Genus: *Arthrobacter*	blight	Holly
Clavibacter	wilts	Alfalfa, potato, tomato
Curtobacterium	wilts	Beans
Rhodococcus	fasciation	Sweet pea
Streptomyces	scab	Potato

[a] The diverse genera of symbiotic bacteria, such as *Rhizobium* and *Bradyrhizobium*, are not included in the table since their pathogenicity is mostly a consequence of adverse circumstances that convert in deleterious and otherwise beneficial interaction. Data adapted from Agrios (1997) and Schaad *et al.* (2001)

usually very difficult, and management strategies rely on preventive methods and, when possible, on cultivar resistance. Bacteria are also intensively used as models to study mechanisms of pathogenicity and plant resistance, because of the inherent physiological and genetic simplicity of the prokaryotes. The unexpected realization that plant and animal bacterial pathogens share pathogenicity mechanisms and determinants has also fostered their use as models to study the molecular basis of pathogenicity and host specialization (Mahajan-Miklos *et al.*, 2000). The gram-negative genera *Erwinia*, *Pseudomonas* and *Xanthomonas* include the most common, economically important and most commonly studied species.

The interactions of phytopathogenic bacteria with a plant can lead to one of two different reactions: i) a compatible interaction, seen with susceptible plant hosts and which is characterized by pathogen proliferation and advanced symptom progress, leading to disease development, or ii) an incompatible interaction, with non-host and resistant plants, characterized by a resistance. Often associated with resistance, particularly with high pathogen titers, is a reaction termed the hypersensitive response (HR) (Heath, 2000). The HR is a form of programmed cell death that appears 12 to 36 h after inoculation in the vicinity of the entry point of the pathogen, limiting the multiplication of the pathogen and leading to disease resistance (Heath, 2000; McDowell and Dangl, 2000). Although the HR is microscopically small under natural conditions, it is macroscopically visible and readily assessed in the laboratory when plant tissues are infiltrated with large numbers of phytopathogenic bacteria. Non-pathogens do not grow in plant tissues and are not able to specifically activate the mechanisms leading either to disease or HR, resulting in a so-called null reaction. The accumulated research of the last decades has offered important insights of why bacteria are able to infect some plants and not others; there is still, however, much more to unravel before we can use this information to render target crops immune to economically important pathogens. This review outlines our current knowledge of the mechanisms that operate to define host-range and virulence in one of the most diverse species of phytopathogenic bacteria, *Pseudomonas syringae*. We put emphasis on citing the more recent literature rather than making an exhaustive list of citations, which can be found in other excellent reviews that have focused on particular aspects of the biology and genetics of this and other bacterial pathogens (Rudolph, 1995; Bender *et al.*, 1999; Gabriel, 1999; Collmer *et al.*, 2000; Cornelis and Van Gijsegem, 2000; Preston, 2000; Vivian and Arnold, 2000; Vivian *et al.*, 2001). Throughout the text, we will use the concepts of pathogenicity (the capability of a pathogen to cause disease), virulence (relative capacity to damage the host) and avirulence (the specific inability of a pathogen race to induce disease in a host cultivar with genetic resistance) as recommended (Shaner *et al.*, 1992).

Pseudomonas syringae

Pseudomonas syringae van Hall 1902 is a Gram-negative plant pathogenic bacterium that produces fluorescent pigments in iron-deficient environments and levans in media containing sucrose, is catalase positive and possesses an oxidative metabolism of glucose. The mol% G+C of the DNA is 59-61. The optimum temperature for growth is 25-30°C. All nomenspecies belonging to the group of cytochrome C oxidase-negative and arginine dihydrolase-negative pseudomonads were classified together as *P. syringae*, with the exception of *P. viridiflava* (Palleroni, 1984).

Among phytopathogenic bacteria, the *P. syringae* group appears to be the best adapted for epiphytic growth, defined as an increase of bacteria populations on apparently healthy external parts of the shoot (leaves, buds, pods, etc.) (Hirano and Upper, 2000). Strains of *P. syringae* cause diseases in nearly every cultivated plant and on an unknown number of wild plant species, and they have been classified into some 51 different pathovars depending on their host range (Young *et al.*, 1996). Many of the *P. syringae* pathovars not only are highly specific towards one or few related plant species but also can induce different disease symptoms, such as watersoaking, hypertrophic growth, cankers, chlorosis, and necrosis on leaves, flowers, buds, and twigs. Nonetheless several studies have indicated that the pathovar distinction based on host-range data does not always correlate well with other molecular, biochemical, and physiological typing methods (Mugnai *et al.*, 1994; Arnold *et al.*, 1996; Clerc *et al.*, 1998; dos A. Marques *et al.*, 2000; Yamamoto *et al.*, 2000). The confusing taxonomy

of *P. syringae* has been revised by several authors, and some of the proposals include renaming pathovars *glycinea*, *phaseolicola* and *savastanoi* as pathovars of the new species *P. savastanoi* (Gardan *et al.*, 1992) and the elevation of several other pathovars to the rank of species (Gardan *et al.*, 1999, and references therein). Also, a recent analysis of DNA reassociation rates has allowed the separation of *P. syringae* into nine genomospecies containing one to several pathovars with non-overlapping host ranges (Table 2) (Gardan *et al.*, 1999). To further increase confusion, not all these changes and reclassifications are widely accepted (Schaad *et al.*, 2000). Taking into account the lack of consensus and to avoid ambiguity, we will follow the traditional nomenclature in this review and we will consider *P. savastanoi* and pathovars *glycinea* and *phaseolicola* as pathovars of *P. syringae*. Specialization to different cultivars of a given host species has also allowed the subdivision of pathovars into pathogenic races, which were reported so far in *P. syringae* pvs *glycinea*, *morsprunorum*, *phaseolicola*, *pisi*, *tabaci* and *tomato* (see below).

Determinants involved in phytopathogenic bacteria-plant interactions

Our knowledge of the molecular weapons that a phytopathogenic bacterium uses to direct the plant metabolism to its own benefit has increased dramatically in the last few years (Collmer *et al.*, 2000; Cornelis and Van Gijsegem, 2000; Kjemtrup *et al.*, 2000; Rahme *et al.*, 2000; Vivian and Arnold, 2000). The range of plant species and cultivars infected by plant pathogenic bacteria is determined by the coordinate action of positive factors, which act allowing the pathogen to infect a given plant or increasing its virulence, and the antagonistic action of negative factors, which actively limit host range (Table 3). The ability of most bacterial phytopathogens to elicit the HR in resistant plants and to cause disease in susceptible hosts is in turn controlled by a set of genes known as the *hrp* (pronounced "harp") genes. This cluster of genes constitutes a type III secretion system that forms an infection structure which leads to the injection of pathogen elicitors and pathogenicity and virulence determinants into the plant cell (Fig. 1).

Table 2. Proposed distribution in genomospecies of *Pseudomonas syringae* pathovars and related *Pseudomonas* species[a].

Genomospecies 1

P. syringae
pv. *aptata*
pv. *aceris*
pv. *atrofaciens*
pv. *dysoxyli*
pv. *japonica*
pv. *lapsa*
pv. *panici*
pv. *papulans*
pv. *pisi*

Genomospecies 2

P. amygdali
P. ficuserectae
P. meliae
P. savastanoi
pv. *aesculi*
pv. *ciccaronei*
pv. *dendropanacis*
pv. *eriobotryae*
pv. *glycinea*
pv. *hibisci*
pv. *lachrymans* (2) 1644
pv. *mellea*
pv. *mori*
pv. *morsprunorum* (2) 2116
pv. *myricae*
pv. *phaseolicola*
pv. *photiniae*
pv. *sesami*
pv. *tabaci*
pv. *ulmi*

Genomospecies 3

pv. *antirrhini*
pv. *apii*
pv. *berberidis*
pv. *delphinii*
pv. *lachrymans* (2) 2440
pv. *maculicola*
pv. *morsprunorum* (2) 2351
pv. *passiflorae*
pv. *persicae*
pv. *philadelphi*
pv. *primulae* (2) 11007
pv. *ribicola* (2) 10971
pv. *tomato*
pv. *viburni*

Genomospecies 4

"P. coronafaciens"
pv. *atropurpurea*
pv. *garcae*
pv. *oryza*
pv. *porri* (4)
pv. *striafaciens* (2)
pv. *zizaniae*

Genomospecies 5

pv. *tremae*

Genomospecies 6

P. viridiflava
pv. *primulae* (2) 1660
pv. *ribicola* (2) 2348

Genomospecies 7

pv. *helianthi* (3)
pv. *tagetis* (2)

Genomospecies 8

P. avellanae (4)
pv. *theae* (3)

Genomospecies 9

pv. *cannabina* (3)

[a] Data from Gardan *et al.* (1999). Names starting with pv. correspond to *P. syringae* pathovars. The number of strains examined, if more than one, is shown in parenthesis. In those cases where strains from a given pathovar fall into different genomospecies, the CFBP strain designation number is shown and is underlined for those pathotype strains that were non-pathogenic.

Table 3. Factors produced by *Pseudomonas syringae* and involved in the definition of host range.

Factor	Function	Role	Comments
hrp genes	Secretion of specific molecules to plant cell apoplast, periplasm or cytoplasm	Positive and negative	Necessary for eliciting both disease and HR
Toxins	Alter plant metabolism	Positive: general virulence factors	Found only in *P. syringae* and other related *Pseudomonas*
Hormones (auxins - IAA-, cytokinins ethylene)	Regulate plant growth and organogenesis	Positive: virulence	IAA also increases bacterial fitness
Enzymes (cutinases, pectinases, cellulases, proteases, xilanases, glucosidases and hemicellulases)	Degrade the plant cell wall	Positive?	
Exopolysaccharides (levan, alginate, etc.)	Protect the bacterial cell	Positive?	
Avr genes and *avr-like* genes	Unknown	Positive or negative, depending on the plant host	Regulated or secreted by type III secretion system. Only *avrD* gene product has a known function.

The Hrp system: Type III secretion pathway in bacterial pathogens

Since the early 1980s, independent molecular characterization of genes controlling bacterial pathogenicity by Tn*5* transposon mutagenesis in *P. syringae* pvs *phaseolicola* and *syringae* led to the discovery of an unusual type of mutants. These mutants could not induce disease in their host plants or the HR in non-host plants (Niepold *et al.*, 1985). The mutations causing this phenotype mapped to a chromosomal DNA region that was named the *hrp* region (hypersensitive response and pathogenicity). Since then, *hrp* regions have been described in different phytopathogenic bacterial species, being intensively studied in *Erwinia amylovora* Ea321, *P. syringae* pv. *syringae* 61, *Ralstonia solanacearum* GMI1000 and *X. campestris* pv. *vesicatoria* 85-10 (Alfano and Collmer, 1997; Hueck, 1998; Nguyen *et al.*, 2000).

Within the phytopathogens, comparisons of *hrp* gene sequences, *hrp* arrangements and *hrp* regulatory elements reveal two phylogenetically related groups (Alfano and Collmer, 1997). Group I contains *hrp* regions of *P. syringae* and *E. amylovora*; group II contains those of *R. solanacearum* and *X. campestris* pv. *vesicatoria*. On the other hand, the analysis of the *hrp* cluster sequences surprisingly showed extensive homologies with genes involved in the secretion of virulence factors essential for disease induction in the animal pathogens *Yersinia*, *Shigella* and *Salmonella*, among others (Hueck, 1998; Cornelis and Van Gijsegem, 2000; Nguyen *et al.*, 2000). The nine *hrp* genes that are broadly conserved in plant and animal pathogens have been redesignated as *hrc* (hypersensitive response and conserved) (Bogdanove *et al.*, 1996a), although in this paper we will continue to use the term *hrp* to encompass all of the *hrp*/*hrc* genes. The discrepancy between the phylogeny of the *hrp* clusters and that of their harboring strains provides evidence that *hrp* clusters may have been acquired by horizontal transfer (Alfano and Collmer, 1997; Nguyen *et al.*, 2000).

The near completion of the *hrp* cluster sequences and their functional analysis have revealed that

the *hrp* genes are contained in ca. 22- to 25-kb DNA clusters, with a G+C content significantly lower than that expected for *P. syringae*, and encode 19-28 regulatory and structural proteins that constitute a type III secretion system (He, 1998; Hueck, 1998). This system forms an infection structure which leads to the secretion into the plant host cell of diverse bacterial proteins, including the products of avirulence genes and other proteins involved in pathogenicity and virulence (Fig. 1), all of which were collectively designated as effectors (van Dijk *et al.*, 1999). The analysis of mutants of *P. syringae* pv. *syringae* affected in different *hrp* genes showed that the Hrp secretion system is also required for growth of this pathogen in the phyllosphere (Hirano and Upper, 2000).

In *P. syringae* pv. *syringae* PS61 and B728a and pv. *tomato* DC3000 the *hrp* clusters were shown to be flanked in a tripartite mosaic structure by two DNA regions, named EEL (exchangeable effector locus) and CEL (conserved effector locus), which encode diverse putative effectors (Fig. 2) (Alfano *et al.*, 2000; Collmer *et al.*, 2000). This structure has the hallmarks of the pathogenicity islands (PAIs) found in bacterial animal pathogens (Hacker *et al.*, 1997): i) clustering of many virulence genes; ii) different G+C content than the host strain; iii) absence from non-pathogenic strains; iv) instability, and v) possession of tRNA genes and sequences related to mobile elements. The EELs

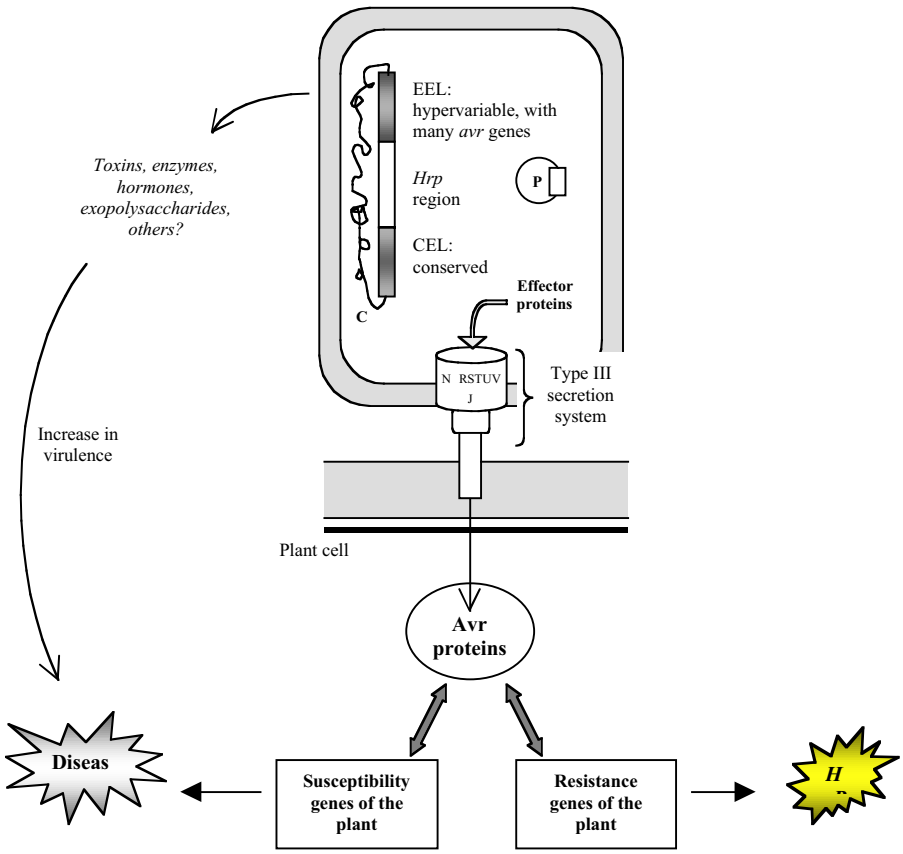

Fig. 1. Putative model to explain bacterial pathogenicity. The Hrp type III secretion system delivers effector proteins (the products of avirulence and virulence genes) into the plant cell; their specific interaction with plant resistance genes and with putative susceptibility genes will determine the production of an HR or disease, and the amount of disease produced. Some other bacterial factors, such as toxins or enzymes, can also participate to increase the virulence. EEL is the Exchangeable Effector Locus and CEL is the Conserved Effector Locus. C, chromosome; P, plasmid.

are regions of dissimilar DNA, 2.5 to 7.3 kb long, that have the same arrangement: they start only 3 nt after the last *hrp* gene, *hrpK*, and end with tRNA^{leu}-*queA*-*tgt* sequences, which are also found in *P. aeruginosa* but not linked to any *hrp* gene. They include from two to six putative effector genes, and the three ORFs (open reading frames) in strain B728a are similar to the known avirulence genes *avrPphC* and *avrPphE*, from *P. syringae* pv. *phaseolicola*, and *avrBsT*, from *Xanthomonas*. EELs also contain sequences related to mobile elements (Alfano *et al.*, 2000), which have also been found surrounding several other effector genes (Kim *et al.*, 1998; Jackson *et al.*, 1999; Arnold *et al.*, 2000; Jackson *et al.*, 2000; Tsiamis *et al.*, 2000) and that can potentially increase their mobility. On the other hand, the CELs are approximately 17 kb long, do not include sequences similar to mobile elements and appear to be highly conserved. Among the ten ORFs found in the CELs, two correspond to the previously described *avrE* locus (Lorang and Keen, 1995) and the harpin gene *hrpW* (Charkowski *et al.*, 1998). The analysis of deletion mutants showed that the EEL had only a minor role in the growth of DC3000 in tomato and was dispensable for the growth of strain B728a on bean, whereas deletion of the CEL completely abolished pathogenicity of DC3000 in tomato plants (Alfano *et al.*, 2000; Collmer *et al.*, 2000).

Proteins secreted by type III secretion systems

The identification of proteins that traverse the Hrp system has been traditionally elusive. On the one hand, proteins secreted by type III systems do not contain signal peptides or any other easily recognizable characteristic. Although it has been proposed that they could posses mRNA-based targeting signals encompassing the first 15 codons of the gene (Anderson *et al.*, 1999), it was recently shown that it is the amphipathic nature of the N-terminus, and not the mRNA base sequence, what determines the type III-dependent secretion of a *Yersinia* effector, and it has been suggested that this could be a universal feature of secreted substrates (Lloyd *et al.*, 2001). On the other hand, it is possible that only a few molecules of each protein are injected into the plant cell and/or that they have a short half-life, which could explain the repeated failure to obtain direct evidence of the translocation of putatively secreted proteins. However, there appear to be two

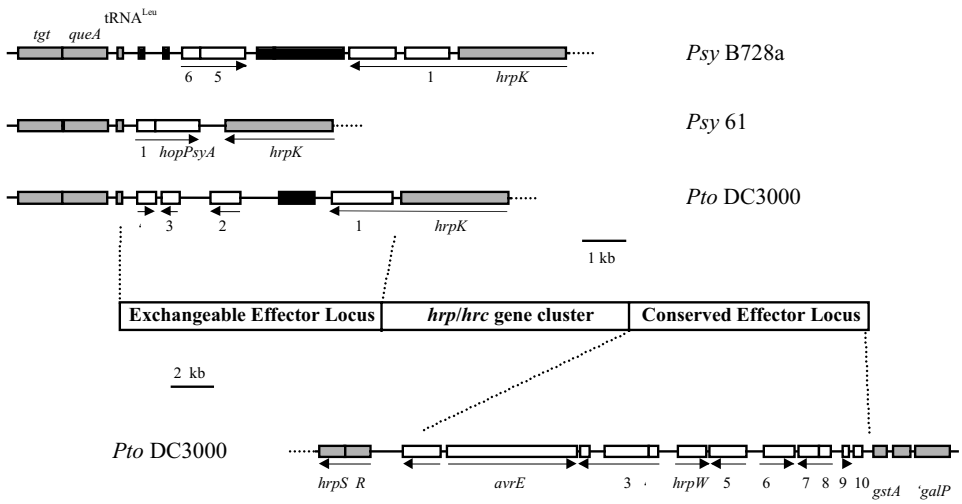

Fig. 2. The tripartite mosaic structure of the Hrp pathogenicity island. The *hrp*/*hrc* cluster contains structural and regulatory genes for the assembly of a type III secretion apparatus, most of which are highly conserved among different *P. syringae* strains. The Exchangeable Effector Locus (or EEL) from *P. syringae* pv. *syringae* B728a (7.3 kb) and 61 (2.5 kb) and from *P. syringae* pv. *tomato* DC3000 (5.9 kb) are shown; they are all flanked by *tgt*-*queA*-tRNA^{leu} and *hrpK* sequences, shown only for strain B728a and denoted by grey boxes. White boxes with numbers indicate open reading frames, which are not conserved between EELs, in spite of identical numbering. Black boxes denote mobile genetic elements. The Conserved Effector Locus (approx. 17 kb) from DC3000 is shown, with bordering genes shown as grey boxes. White boxes denote ORFs (shown with numbers) or previously described genes that belong to the CEL. In all cases, arrows indicate transcriptional units that start with a "*hrp* box". Modified from Alfano *et al.* (2000) and Collmer *et al.* (2000).

classes of proteins that traverse the Hrp secretion system. The first class, exemplified by harpin proteins and HrpA, are secreted in minimal medium in which *hrp* genes are derepressed. The other class of proteins, typified by Avr proteins, appear to be secreted through the Hrp system directly into the plant cell. Due to the important role that Avr proteins play in plant-bacteria interactions, they will be described in another section.

Harpins. Harpins are glycine rich, cysteine-lacking proteins that are secreted in culture when the Hrp system is expressed and that possess heat-stable HR elicitor activity when infiltrated at relatively high concentrations (>0.1 μM) into the leaves of tobacco and several other plants. The products of the *E. amylovora* $hrpN_{Ea}$, *E. chrysanthemi* $hrpN_{Ech}$, *P. syringae* $hrpZ_{Pss}$ and *R. solanacearum popA1* genes were the first bacterial proteins shown to have HR elicitor activity (Alfano and Collmer, 1997). Despite having unifying properties, *Erwinia* and *Pseudomonas* harpin proteins do not appear to be homologous and mutation of their respective genes have very different effects. Harpins have yet to be found in *Xanthomonas* spp., and the primary function of the known harpins is still unclear.

In *P. syringae* two harpin genes have been described: *hrpZ* (He *et al.*, 1993) and *hrpW* (Charkowski *et al.*, 1998), and their gene products were shown to traverse the Hrp secretion pathway and to exhibit HR-eliciting activity when infiltrated into tobacco leaf tissue (He *et al.*, 1993; Charkowski *et al.*, 1998; Lee *et al.*, 2001). In addition, HrpZ also causes HR-associated extracellular medium alkalinization (Hoyos *et al.*, 1996), systemic acquired resistance (Strobel *et al.*, 1996), and expression of plant defence genes (Gopalan *et al.*, 1996), all characteristics of bacteria-induced resistance. However, the amount of HrpZ produced by bacteria *in planta* may not be sufficient for induction of a macroscopic HR (Alfano *et al.*, 1996). The role of harpins in the interaction with the host is unknown: deletion of either *hrpZ* or *hrpW* from *P. syringae* pv. *tomato* had little effect on their HR elicitation activity in tobacco, whereas this activity, but not virulence, was significantly reduced in a *hrpZ hrpW* double mutant (Charkowski *et al.*, 1998). It seems, however that their site of action is the plant cell wall (Collmer, 1998), and HrpZ from several *P. syringae* pathovars was recently found to associate stably with liposomes and synthetic bilayer membranes (Lee *et al.*, 2001). This pore-forming activity may allow nutrient release and/or delivery of virulence factors during bacterial colonization of host plants (Lee *et al.*, 2001).

HrpA and the Hrp pilus. In addition to secreting HrpZ, *P. syringae* pv. *tomato* DC3000 also secretes at least four proteins (EXP-43, EXP-22, HrpW and HrpA) via the Hrp system in culture (Yuan and He, 1996; Charkowski *et al.*, 1998). HrpA is a small, hydrophilic protein encoded by the *hrpA* gene, situated adjacent to the *hrpZ* gene in the same gene operon (Preston *et al.*, 1995). The primary sequence of HrpA shares no homology with any other protein. However, the HrpA protein was found to form a novel pilus-like structure, named Hrp pilus, that is 6-8 nm in diameter, has a length of >2 μm, and is essential for all Hrp phenotypes (Roine *et al.*, 1997b). A non-polar *hrpA* mutant no longer produces the Hrp pilus, does not cause disease, elicit an HR, or support the function of *avr* genes, which are typical phenotypes of *hrp* mutants (Roine *et al.*, 1997b); this suggests that formation of the Hrp pilus is essential for Hrp-controlled plant-*P. syringae* interactions. Highly purified HrpA protein has been shown to self-assemble into pilus-like structures *in vitro*, being morphologically somewhat different from the native Hrp pili (Roine *et al.*, 1997a). Also, immunogold labeling of bacteria-infected *Arabidopsis* leaf tissue with an Hrp pilus antibody further confirmed that HrpA is the major structural protein of the Hrp pilus (Hu *et al.*, 2001). To illustrate the sometimes unpredicted complexity of biological systems, HrpA was shown not only to be essential for the secretion of putative virulence proteins, such as HrpW and AvrPto, but also to participate in the regulation of the type III secretion system (Wei *et al.*, 2000).

Negative factors: restriction of host range by avirulence genes

The only negative factors presently described in phytopathogenic bacteria are encoded by avirulence genes. These genes prevent the infection of cultivars of the plant host that contain matching resistance genes by inducing a specific HR, thereby determining the race structure (Vivian *et al.*, 1997). Also, there are a few documented examples

of their role in shaping the host range at the plant species level, as happens with *avrA* of *R. solanacearum*, which makes some isolates unable to infect pepper, an otherwise susceptible host (Carney and Denny, 1990). The loss of function of an *avr* gene usually leads to the inability to induce the HR in a given plant host, although this does not necessarily imply that the bacterium will gain the capacity to infect that plant, indicating that it is necessary for the concomitant presence of positive factors that confer the pathogenic capacity.

Flor predicted the gene-for-gene hypothesis to explain the genetic basis for elicitation of resistance (Flor, 1971), which was later confirmed (Vivian *et al.*, 1989) by demonstrating the co-segregation of resistance among an F_2 population of pea to both the incompatible *P. syringae* pv. *pisi* race 2 and to the compatible race 1 harbouring the cloned *avrPpiA1* (see Table 7). Basically, the gene-for-gene hypothesis predicts that resistance, or incompatibility, is the result of the specific interaction of the genetic products of single dominant host genes for resistance (*R* genes) and avirulence genes (*avr* genes) for the pathogen. Thus, the *avr* genes impose race-specificity on a pathogen that is otherwise compatible in association with a given plant species. Since then there has been a tremendous increase in our knowledge about the genes and molecular mechanisms that govern such interactions, mainly from studies with phytopathogenic bacteria.

The first cloned avirulence gene, *avrA*, was isolated from *P. syringae* pv. *glycinea* in 1984 (Staskawicz *et al.*, 1984) following a heterologous expression strategy. At present there are more than 30 *avr* genes which have been identified and cloned, mostly from *P. syringae* (Table 4) and *Xanthomonas*, although a few *avr* genes have also been identified in *E. amylovora* and *R. solanacearum* (Vivian *et al.*, 1997; Vivian and Arnold, 2000). The enormous amount of sequence and biological data has not yet provided a general model of their possible mode of action or their general role as host range determinants. Here we will concentrate on the *avr* genes currently described in *P. syringae*, which are summarized on Table 4, with a few references to *avr* genes from other bacterial species.

Structural features

Avirulence genes do not show any obvious general feature at the nucleotide or amino acid sequence level (Vivian *et al.*, 1997), except the *avrBs2* family of *avr* genes from *X. campestris*. The deduced protein products of *avr* genes range from 18 to 100 kDa, but lack substantial sequence similarity to proteins of known biochemical activity or motifs indicative of specific functional domains. Obvious signal sequences indicative of secretion by type II secretion systems are also absent. Most *avr* genes encode for hydrophilic proteins, and only one, *avrD* from *P. syringae* pv. *tomato* and several other pathovars, was found to encode for an enzyme. The *avr* genes generally encode a single ORF, with the exception of *avrEF* (Bogdanove *et al.*, 1998), from *P. syringae* pv. *tomato*, and *avrPphF* (Tsiamis *et al.*, 2000), from *P. syringae* pv. *phaseolicola*, which contain two. In *P. syringae*, expression of *hrp* and *avr* genes is regulated at the transcriptional level by HrpL, an alternative sigma factor which recognizes a conserved promoter sequence (GGAACC-N15/16-CCAC) that has been dubbed as the "*hrp*-box" (Xiao and Hutcheson, 1994). Avirulence genes can be located in large native plasmids or in the chromosome, depending on the particular gene and on the strain (Table 4). In most cases, the G+C content of *avr* genes does not reflect that of the genome, and also many of them are bounded by repetitive elements (Kim *et al.*, 1998; Jackson *et al.*, 1999; Arnold *et al.*, 2000; Jackson *et al.*, 2000; Tsiamis *et al.*, 2000). Additionally, a growing number of *avr* homologues have been found in phylogenetically distant bacterial species. For instance, a homolog of *avrEF* from *P. syringae* pv. *tomato* has been described in *E. amylovora*, named *dspEF*, and both genes are functionally interchangeable (Bogdanove *et al.*, 1998). Also, homologs to *avrRxv* from *X. campestris* pv. *vesicatoria* were found in *E. amylovora* and in the animal pathogens *Salmonella* and *Yersinia* (Bogdanove *et al.*, 1996b; Hardt and Galan, 1997). Taken together, these data strongly support the idea of a probable recent horizontal transfer and frequent exchange of *avr* genes among bacterial pathogens.

The *avrD* gene paradigm

The *avrD* gene was isolated from *P. syringae* pv. *tomato* via conjugation into an isolate of *P. syringae* pv. *glycinea* which was virulent on several soybean cultivars (Kobayashi *et al.*, 1989). In fact, the *avrD* gene function defined a new resistance gene specificity, *Rpg4*, in soybean (Keen, 1992). Sequenc-

Table 4. Avirulence genes cloned from *Pseudomonas syringae* pathovars.

Pathovar source	Name	Genetic location[a]	Virulence function/host[b]	Resistance on/*R* gene	Cloned alleles from pathovar	Reference
glycinea	*avrA*	nd	no	Soybean/*RPG2*	*tomato*	Napoli and Staskawicz, 1987
	avrB	chr	no	Soybean/*RPG1*; *Arabidopsis*/*RPM1-RPS3*		Innes *et al.*, 1993b; Tamaki *et al.*, 1988
	avrC	plasmid	no	Soybean/*RPG3*	*phaseolicola avrPphC*	Tamaki *et al.*, 1988
maculicola	*avrRpm1*	plasmid	yes/*Arabidopsis*	*Arabidopsis*/*RPM1*; pea/*R2*; bean; soybean/*RN1+RN2*	*pisi avrPpiA1*; *maculicola* 2	Dangl *et al.*, 1992; Ritter and Dangl, 1995
phaseolicola	*avrPphA*	nd	no	Bean/*R1*		Shintaku *et al.*, 1989
	avrPphB	chr	no	Bean/*R3*		Jenner *et al.*, 1991
	avrPphC	plasmid	yes/bean	Soybean/*RPG3*	*glycinea avrC*	Tsiamis *et al.*, 2000; Yucel *et al.*, 1994b
	avrPphD	plasmid	no	Pea	*tomato avrPtoD*	Arnold *et al.*, 2001
	avrPphE	chr	no	Bean/*R2*; pea; *Arabidopsis*	*syringae*	Mansfield *et al.*, 1994; Alfano *et al.*, 2000
	avrPphF	plasmid	yes/bean, soybean	Bean/*R1*		Tsiamis *et al.*, 2000
	virPphA	plasmid	yes/bean[c]	Soybean	*glycinea virPgyA*	Jackson *et al.*, 1999
pisi	*avrPpiA1*	chr/plasmid	no	pea/*R2*; *Arabidopsis*/*RPM1*; bean; soybean /*RN1+RN2*	*maculicola avrRpm1*; *maculicola* 2	Dangl *et al.*, 1992; Gibbon *et al.*, 1997
	avrPpiB1	plasmid		pea/*R3*		Cournoyer *et al.*, 1995
	avrPpiC	chr		bean		Fillingham, 1994
	avrPpiD	nd		pea/*R5*		Vivian and Arnold, 2000
	avrPpiG	nd		bean	*syringae*	Alfano *et al.*, 2000; Vivian and Arnold, 2000
	avrRps4 (avrPpiE)	plasmid		*Arabidopsis*/*RPS4*		Hinsch and Staskawicz, 1996
syringae	*hrmA* (*hopPsyA*)	chr	no	tobacco		Collmer *et al.*, 2000; Heu and Hutcheson, 1993
tomato	*avrA*	chr	yes/tomato	soybean/*RPG2*	present in many pathovars	Kobayashi *et al.*, 1989; Lorang *et al.*, 1994
	avrD	plasmid	no	soybean/*RPG4*		Kobayashi *et al.*, 1989; Yucel *et al.*, 1994a
	avrEF	chr	yes/tomato[d]	soybean		Bogdanove *et al.*, 1998; Lorang and Keen, 1995; Lorang *et al.*, 1994
	avrPto	probably chr	yes/tomato[d]	tomato/*PTO*, tobacco		Chang *et al.*, 2000; Salmeron and Staskawicz, 1993
	avrPtoD	plasmid	yes/tomato	bean	*avrPphD*	A. Sesma and J. Murillo, unpublished
	avrRpt2	probably chr	yes/*Arabidopsis*	*Arabidopsis*/*RPS2*; soybean; bean		Chen *et al.*, 2000; Innes *et al.*, 1993a

[a] nd, not determined; chr, chromosomal.
[b] "no" denotes that the gene was shown not to participate in virulence; "yes" means that the gene increased the virulence in the hosts indicated.
[c] Essential for pathogenicity in bean.
[d] The *avr* gene has a virulence function in certain bacterial backgrounds but not in others (see text for details).

ing of *avrD* showed that it could encode a 34 kDa protein that had little similarity to those in the databases. When expressed in several Gram-negative bacteria, including *Escherichia coli*, AvrD directs the production of unusual acyl glycosides called syringolides (Midland *et al.*, 1993). Sequences homologous to the *avrD* gene are found in many isolates of *P. syringae* pv. *glycinea*, although they do not confer an avirulence phenotype but, seemingly, represent silent alleles (Kobayashi *et al.*, 1990; Keith *et al.*, 1997). The presence of *avrD* alleles in several different pathovars of *P. syringae*, often located on large native plasmids (Murillo *et al.*, 1994; Yucel *et al.*, 1994a; Yucel and Keen, 1994), suggests that *avrD* might have a relevant role in the bacterial life cycle. Also it was shown that two distinct homology classes of *avrD* alleles, I and II, occurred in *P. syringae* isolates, and that these alleles differed in the syringolides they directed (Yucel *et al.*, 1994a). An isolate of *P. syringae* pv. *lachrymans* contained two different *avrD* alleles, one in class I and one in class II, with each gene occurring on different indigenous plasmids.

The *avrD* gene cloned from *P. syringae* pv. *tomato* PT23 is the first ORF of a putative operon including five ORFs, and data base searching has identified striking similarities for some of them (Keen *et al.*, 1996). ORF4 shows significant homology to the *Rhizobium nodH* sulfotransferase, ORF2 shows homology with phosphoglyceromutase, and ORF5 shows homology to a *Bacillus* amino transferase. However, none of these identifications have been functionally confirmed.

Two nearly identical γ-lactone structures for the syringolides produced by *avrD* gene product have been proposed, and they have been called syringolide 1 and 2 (Midland *et al.*, 1993). The syringolides are interesting molecules, not only because they elicit the plant HR in hosts with the *Rpg4* gene, but also because they possess a similar structure to different signal molecules from other organisms, such as *Streptomyces*, *Agrobacterium tumefaciens*, *E. carotovora*, and *Vibrio* spp., and are involved in disparate biological processes, such as sporulation and plant infection. The analogies in the structures of these molecules and their roles as signal molecules indicate that their modes of action may be similar.

The failure of other bacterial *avr* genes to generate detectable elicitor activity following overexpression in *E. coli* or other bacteria indicates that the generation of elicitor signals by these genes might be different and not mediated by an enzymatic activity. Thus, generation of the elicitor signal by AvrD is more complex than that of characterized bacterial, viral and fungal *avr* genes, where the primary or processed gene products appear to function as elicitors.

Determination of host range by avirulence genes: race structure in *P. syringae*

The existence of *avr* genes was inferred by the differences in interactions (compatible or incompatible) of individual pathogen strains with a host plant. The race-specific *avr* genes are detected in members of a single pathovar by inoculation to sets of host differentials (host plant lines or cultivars with different resistance genes). An individual pathogen strain might have multiple *avr* genes, and the combination of *avr* genes within a particular strain specifies the physiologic race of the strain. Thus, the *avr* genes impose race-specificity on a pathogen that is otherwise compatible in association with a given plant species.

The first observation of races within *P. syringae* pathovars was reported for *P. syringae* pv. *phaseolicola* when the formerly resistant bean cv. Red Mexican UI3 was attacked by different isolates of this bacteria. Actually, the number of races in pv. *phaseolicola* has been updated to nine, based on five pairs of resistance and *avr* genes (Table 5) (Mansfield *et al.*, 1994; Taylor *et al.*, 1996), although certain isolates from Spain do not fit into any of these and could represent a new race (González *et al.*, 2000).

For the soybean pathogen *P. syringae* pv. *glycinea*, 11 races are currently differentiated (Rudolph, 1995), race 4 being the most aggressive one, since it infects all the cultivars tested (Table 6). *AvrA*, the first avirulence gene characterized, was cloned from a race 6 strain of the soybean pathogen *P. syringae* pv. *glycinea* and, when transferred to other races of *P. syringae* pv. *glycinea*, conferred the ability to elicit an HR only on cultivars of soybean with the *Rpg2* resistance gene (Table 6) (Staskawicz *et al.*, 1984). A recent survey revealed that in every European country studied, race 4 prevailed. Other races occurring in Europe were races 6, 9 and 10. In Yugoslavia, races 4 and 5 were shown to occur, although race 5 may be identical

Table 5. Partly validated model to explain observed interactions between races of *Pseudomonas syringae* pv. *phaseolicola* and cultivars of the host, bean.

						Races / *avr* genes[a]								
						1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
						avrPphF	·	·	·	*avrPphF*	·	*avrPphF*	·	*avrPphF*
						·	*avrPphE*[b]	·	*avrPphE*[b]	*avrPphE*[b]	·	*avrPphE*[b]	·	·
						·	·	*avrPphB*	*avrPphB*	·	·	·	·	·
						·	·	·	·	4	·	·	·	·
Cultivars	Resistance genes[a]					·	5	·	·	·	·	·	5	5
Canadian Wonder	·	·	·	·	·	+	+	+	+	+	+	+	+	+
A52 (ZAA54)	·	·	·	4	·	+	+	+	+	-	+	+	+	+
Tendergreen	·	·	*R3*	·	·	+	+	-	-	+	+	+	+	+
Red Mexican Ul3	*R1*	·	·	4	·	-	+	+	+	-	+	-	+	-
1072	·	*R2*	·	·	·	+	-	+	-	-	+	-	+	+
A53 (ZAA55)	·	·	*R3*	4	·	+	+	-	-	-	+	+	+	+
A43 (ZAA12)	·	*R2*	*R3*	4	5	+	-	-	-	-	+	-	-	-
Guatemala 196-B	*R1*	·	*R3*	4	·	-	+	-	-	-	+	-	+	-

[a] (+), susceptible response; (-), resistant response; (•), gene absent. Avirulence/resistance matching genes: *avrPphF/R1*; *avrPphE/R2*; *avrPphB/R3*. (Adapted from Vivian *et al.* 1997.)

[b] Homologues of *avrPphE* are present in all the 9 races (Stevens *et al.*, 1998), however the gene is functional only as an avirulence gene in the races indicated (2, 4, 5, 7).

to the newly described race 10 (Rudolph, 1995). Races 0 and 1, which have been extensively studied for analysis of avirulence genes (Keen, 1992) do not seem to occur in soybean fields nowadays.

Bacterial blight of pea is caused by *P. syringae* pv. *pisi*. Isolates comprise seven naturally occurring races, which are distinguished by their interactions with eight differential pea cultivars (Table 7) (Bevan *et al.*, 1995; Vivian *et al.*, 1997). The genetic basis for the relationship between races and pea cultivars was explained in terms of gene-for-gene relationships involving between four and six matching gene pairs (Taylor *et al.*, 1989; Bevan *et al.*, 1995). Interestingly, *P. syringae* pv. *pisi* isolates fall into two phylogenetically distinct groups: group I contains isolates from races 1, 5 and 7 and certain isolates from races 3 and 4 (designated races 3B and 4B) whereas group II contained races 2 and 6 and distinct isolates from races 3 and 4 (designated races 3A and 4A) (Arnold *et al.*, 1996; Arnold *et al.*, 1999).

In England, variants of *P. syringae* pv. *morsprunorum* that differed from the typical forms previously described in colony characteristics, gelatinase activity, pathogenicity, and in specialization for different cherry varieties, were designated as race 2 to distinguish them from the typical race 1 isolates. The two races quickly became established as dominant forms on the varieties for which they were pathologically specialized, race 1 on cv. Napoleon and race 2 on cv. Roundel (Rudolph, 1995).

Also, in *P. syringae* pv. *tabaci* two races, race 0 and 1, were identified. Tobacco cultivars containing TL-106-derived resistance were infected only by race 1, but not by race 0 (Rudolph, 1995). Similarly, in *P. syringae* pv. *tomato* two races have been described. Race 1 infected the tomato cv. Ontario 7710 and other cultivars with a similar genetic background in contrast to the less virulent race 0.

Interaction of Avr proteins with plant products

The expression of the avirulence phenotype is, with some exceptions (Knoop *et al.*, 1991), dependent on the concomitant presence in the bacterial cell of an intact *hrp* cluster (He, 1998; Hueck, 1998). Additionally, phenotypic expression in *E. coli* or *P. fluorescens* of seven different *P. syringae avr* genes (*avrA*, *avrB*, *avrC*, *avrPto*, *avrRpm1*, *avrPphB*, and *avrRpt2*) was dependent on the secretion activities of the *hrp* cluster (Gopalan *et al.*, 1996; Pirhonen *et al.*, 1996; Puri *et al.*, 1997). Only when the *hrp* and *avr* genes were expressed together in a single strain

did the transformant acquire the ability to elicit the HR in those plants expressing the corresponding resistance gene. These data suggest that the type III secretion system functions in the translocation of Avr products into the cytoplasm of the host cells in a manner analogous to the translocation of pathogenicity determinants by *Yersinia* and other mammalian pathogens that employ this secretion pathway. The transient expression of Avr determinants *in planta* has generated evidence which strongly supports this hypothesis. One of the first such experiments was the biolistic inoculation of plant leaves with constructs expressing AvrB (that matches the *RPM1* resistance gene product in *Arabidopsis thaliana*) and the reporter gene *gusA* (Gopalan *et al.*, 1996; Leister *et al.*, 1996). The GUS activity was much lower in the *Arabidopsis RPM1* leaves in which the HR elicited by AvrB would have preceded accumulation of the GUS reporter enzyme, showing that the site activity of AvrB is inside the plant cell. Similar indirect evidence supporting interkingdom transport of Avr proteins has been obtained independently for *P. syringae* pv. *tomato* AvrPto (Scofield *et al.*, 1996; Tang *et al.*, 1996; Shan *et al.*, 2000b) and AvrRpt2 (Leister *et al.*, 1996; Guttman and Greenberg, 2001), *P. syringae* pv. *syringae* HrmA (HopPsyA) (Alfano *et al.*, 1997), and *X. campestris* pv. *vesicatoria* AvrBs3 (Van den Ackerveken *et al.*, 1996). Additionally, in the case of AvrPto, a putative intracellular receptor encoded by the tomato disease resistance gene *Pto* has been identified using the yeast two-hybrid system, based on specific binding between AvrPto and Pto in the yeast nucleus (Scofield *et al.*, 1996; Tang *et al.*, 1996; Chang *et al.*, 2001). Previously, the AvrBs3 family of Avr proteins was found to contain functional plant nuclear localization signals (NLSs) that are required for the AvrBs3 family of proteins to trigger the HR, suggesting that some of the members of this protein family not only enter the plant cell, but also enter the plant nucleus (Van den Ackerveken *et al.*, 1996).

All these results support that Hrp-mediated translocation of Avr proteins is necessary before recognition of the proteins occurs. Although we still lack direct evidence, all of the accumulated data strongly suggests that the site of action of most, if not all, of the bacterial *avr* genes is inside the plant cell, and that the role of the Hrp machinery is to accurately direct avirulence proteins to this environment.

Are avirulence genes also virulence determinants?

An as yet unsolved question is why do bacteria harbor *avr* genes given that they are restricting the number of plant hosts that can be successfully infected? The most attractive, and plausible, explanation for this anomaly is that they play a role in bacterial virulence or fitness in susceptible plant

Table 6. Partly validated model to explain observed interactions between races of *Pseudomonas syringae* pv. *glycinea* and cultivars of the host, soybean.

				Races / *avr* genes[a]			
				0	**1**	**4**	**6**
				avrB	*avrB*	·	·
				avrC	·	·	·
Cultivars	Resistance genes			·	·	·	*avrA*
Centennial	·	*Rpg2*	·	+	+	+	-
Flambeau	·	·	*Rpg3*	-	+	+	+
Merit	*Rpg1*	*Rpg2*	·	-	-	+	-
Peking	·	*Rpg2*	*Rpg3*	-	+	+	-
Norchief	*Rpg1*	·	*Rpg3*	-	-	+	+
Harosoy	*Rpg1*	*Rpg2*	·	-	-	+	-
Acme	·	*Rpg2*	*Rpg3*	-	+	+	-

[a] (+), susceptible response; (-), resistant response; (·), gene absent. Avirulence/resistance matching genes: *avrB*/*Rpg1*, *avrA*/*Rpg2*, *avrC*/*Rpg3*; (data from Keen and Buzzell, 1990; Staskawicz *et al.*, 1984; Napoli and Staskawicz, 1987; Staskawicz *et al.*, 1987).

hosts in the absence of the corresponding resistance proteins (see Gabriel, 1999). This hypothesis is gaining growing support with the accumulation of evidence that suggests a role in virulence or pathogenicity for several unrelated *avr* genes, as reviewed below.

Positive factors: virulence and pathogenicity determinants

Recent research has produced compelling evidence that the main positive factors which determine host range, encoding either pathogenicity or virulence in a host-specific way, are avirulence genes (Gabriel, 1999; Collmer *et al.*, 2000; Vivian and Arnold, 2000), and their role will be discussed below. The involvement in virulence of various other factors has been known for a long time, although their activity does not seem to be host-specific. Of these, phytotoxins, which are specifically produced by certain pathovars, generally function as virulence factors and their production results in increased disease severity (Bender *et al.*, 1999). In general, phytotoxins are not required for pathogenicity in *P. syringae*, and they usually contribute to systemic invasion of the host or increase lesion size and *in planta* bacterial populations. Although many *P. syringae* pathovars produce the growth regulator indol-3-acetic acid (Glickmann *et al.*, 1998), this hormone has been shown to participate in virulence only in *P. syringae* pv. *savastanoi*, where it is essential for tumorigenesis (Smidt and Kosuge, 1978; Surico *et al.*, 1985; Iacobellis *et al.*, 1994) and also participates in competitive fitness (Silverstone *et al.*, 1993). High-molecular-weight polysaccharides could also function as virulence factors (Fett and Dunn, 1989; Denny, 1995; Corsaro *et al.*, 2001) although their role, if any, is still in general a matter of controversy; for instance, alginate was reported to contribute to virulence in a strain of *P. syringae* pv. *syringae* (Yu *et al.*, 1999) but not in another strain (Willis *et al.*, 2001). A pectate lyase from *P. syringae* pv. *lachrymans* Pla5 altered the final symptoms in infected cucumber cotyledons (i.e., maceration instead of necrotic lesions), but did not contribute to pathogenicity nor altered host range (Bauer and Collmer, 1997). The role of other extracellular factors, such as other enzymes and hormones, like ethylene, has not been yet fully established for *P. syringae*-plant interactions.

Avirulence genes are specific virulence factors

There are few recognized examples of specific virulence factors, i.e. those that are functional only against one plant species or cultivar, whose func-

Table 7. Partly validated model to explain observed interactions between races of *Pseudomonas syringae* pv. *pisi* and cultivars of the host, pea.

							Races / *avr* genes[a]						
							1	**2**	**3**	**4**	**5**	**6**	**7**
							1	·	·	·	·	·	·
							·	*avrPpiA*	·	·	*avrPpiA*	·	*avrPpiA*
							avrPpiB	·	*avrPpiB*	·	·	·	*avrPphB*
							4	·	·	4	4	·	4
							·	·	·	·	*avrPpiD*	·	·
Cultivars	Resistance genes						6?	·	·	·	6?	·	·
Kelvedon Wonder	·	·	·	·	·	·	+	+	+	+	+	+	+
Early Onward	·	*R2*	·	·	·	·	+	-	+	+	-	+	-
Belinda	·	·	*R3*	·	·	·	-	+	-	+	+	+	-
Hurst Greenshaft	·	·	·	4	·	6?	-	+	+	-	-	+	-
Partridge	·	·	*R3*	4	·	·	-	+	-	-	-	+	-
Sleaford Triumph	·	*R2*	·	4	·	·	-	-	+	-	-	+	-
Vinco	1	*R2*	*R3*	·	*R5*	·	-	-	-	+	-	+	-
Fortune	·	*R2*	*R3*	4	·	·	-	-	-	-	-	+	-

[a] (+), susceptible response; (-), resistant response; (·), gene absent. Avirulence/resistance matching genes: *avrPpiA/R2*; *avrPpiB/R3* and *avrPpiD/R5* (adapted from Vivian *et al.* 1997).

tions were surveyed by mutational studies (Arlat *et al.*, 1991; De Feyter and Gabriel, 1991; Waney *et al.*, 1991). The first such case to be described was *avrBs2* (Kearney and Staskawicz, 1990), from *X. campestris* pv. *vesicatoria*, which was first described as an *avr* gene against pepper cultivars containing the *Bs2* resistance gene. However, strains of *X. campestris* pv. *campestris* and pv. *alfalfae* containing an inactivated version of *avrBs2* were less aggressive than the wild type to susceptible cultivars of pepper and alfalfa, respectively (Kearney and Staskawicz, 1990). Additionally, there is currently a growing list of avirulence genes from *P. syringae* that have been reported to participate in virulence and/or pathogenicity (Table 4).

Genes *avrA*, *avrD*, *avrE* and *avrPto* of *P. syringae* pv. *tomato* PT23 interact with soybean *R* genes. Single or combined marker-exchange mutants in these genes were constructed and tested for virulence on the normal host, tomato, or for resistance in the non-host plant, soybean (Lorang *et al.*, 1994). It was found that mutations in *avrE* lowered the ability of bacteria to grow and cause disease symptoms in tomato, and a similar but less pronounced effect was reported for *avrA*. An *avrE* mutant of *P. syringae* pv. *tomato* DC3000 did not show reduced virulence, suggesting that either DC3000 contains a different *avrE* allele or that the genomic background influences the final phenotype (Lorang and Keen, 1995). A similar phenomenon occurs in the case of *avrPto*. This avirulence gene contributes to the virulence activity of *P. syringae* pv. *tomato* strain T1, supporting more bacterial growth in tomato plants lacking the *Pto* resistance gene, but has no effect on the virulence of strain DC3000 (Chang *et al.*, 2000; Shan *et al.*, 2000a). Analysis of point mutations in AvrPto localized to different regions of the protein identified three motifs which determine virulence on tomato and avirulence on tomato and tobacco (Shan *et al.*, 2000a; Shan *et al.*, 2000b; Chang *et al.*, 2001), suggesting that AvrPto could be recognized by different plant gene products.

Curing of a large plasmid (pAV511, ca. 150 kb) from *P. syringae* pv. *phaseolicola* strain 1449B rendered this strain non-pathogenic in the otherwise susceptible hosts bean and soybean (Jackson *et al.*, 1999). Partial sequencing and mutational analysis of a ca. 30 kb region from this plasmid revealed the presence of a PAI, the first one to be described in a plant pathogen, that included several avirulence genes flanked by sequences homologous to the mobile elements IS*100* from *Yersinia* and Tn*501* from *Pseudomonas aeruginosa* (Jackson *et al.*, 1999). One of the genes, *virPphA*, displayed a dual role in virulence/avirulence since it partially restored virulence towards bean cultivars, and also conferred an HR in certain soybean cultivars, such as Osumi (Jackson *et al.*, 1999). Avirulence gene *avrPphF* was also included in the PAI and likewise displayed diverse phenotypes depending on the plant host (Tsiamis *et al.*, 2000). It was recognised by cvs. containing the cognate resistance gene *R1*, such as the bean cv. Red Mexican, but also partially restored the virulence of cured derivatives of 1449B on bean cv. Tendergreen and on all the soybean cultivars tested (Tsiamis *et al.*, 2000). The importance of the bacterial background on the phenotype conferred by avirulence genes is again illustrated by the fact that *avrPphC*, which is also carried by 1449B on the PAI, was shown to suppress the enhanced HR elicited by *avrPphF* in the bean cv. Canadian Wonder (Tsiamis *et al.*, 2000); therefore, *avrPphC* can also be considered as a virulence factor.

By comparison, the avirulence products that participate in virulence would be predicted to interact specifically with factors in the plant cytoplasm. The first evidence that this could indeed be the case came from an elegant series of experiments showing that *avrRpt2* promotes the virulence of *P. syringae* pv. *tomato* DC3000 from within the plant cell (Chen *et al.*, 2000). It is as yet unknown whether or not these plant factors would be the products of "susceptibility" alleles of plant resistance genes. An exciting recent development was the demonstration that the highly homologous effectors YopJ from *Yersinia pestis* and AvrBsT from *X. campestris* act as cysteine proteases, and that this activity was essential for them to inhibit the host immune response and to induce localized cell death, respectively (Orth *et al.*, 2000). The substrates for YopJ were shown to be ubiquitin-like molecules (Orth *et al.*, 2000), which are highly conserved in all eukaryotic species and modulate a variety of signaling pathways.

Phytotoxins

Phytotoxins are non-enzymatic products of plant pathogens, or the host-pathogen interaction, that directly injure plant cells and influence the course of the disease symptoms in low concentration. Both fungal and bacterial pathogens produce

a number of secondary metabolites that are toxic to plant cells; however, these metabolites may not be important in plant disease. Consequently, phytopathologists have developed criteria for assessing the involvement of toxins in plant disease (Bender *et al.*, 1999): i) reproduction of disease symptoms with the purified toxin; ii) a correlation between toxin yield and pathogenicity; iii) production of the toxin during active growth of the pathogen *in planta* and, iv) reduced virulence or lack of virulence in non-toxigenic strains.

Many *P. syringae* pathovars produce low molecular weight toxins, that are varied in origin and include monocyclic ß-lactam (tabtoxin), sulfodiaminophosphinyl peptide (phaseolotoxin), lipodepsipeptide (e.g., syringomycin and syringopeptins), and polyketide (coronatine) structures (Table 8) (Bender *et al.*, 1999). Although phytotoxins are not required for pathogenicity in *P. syringae*, they generally function as virulence factors and their production usually results in increased disease severity.

Production of coronatine (COR) has been demonstrated in five pathovars of *P. syringae*, i.e. *atropurpurea*, *glycinea*, *maculicola*, *morsprunorum*, and *tomato* (Mitchell, 1982; Wiebe and Campbell, 1993; Cuppels and Ainsworth, 1995). The structure of COR is unusual and has two distinct components: the polyketide coronafacic acid (CFA) and an amino acid derivative, coronamic acid (Ichihara *et al.*, 1977). CFA is also conjugated *in vivo* to produce a variety of compounds, including CFA-Ile, CFA-alloIle, coronofacoylvaline, norcoronatine, CFA-Thr and CFA-Ser, although COR is generally the predominant compound and also the most toxic (Mitchell, 1985; Mitchell and Ford, 1998). The primary symptom elicited by COR is a diffuse chlorosis that can be induced in a wide variety of plant species, although it is also known to induce hypertrophy, to inhibit root elongation, to inhibit the plant defence responses and to stimulate ethylene production in a host-dependent manner (Ferguson *et al.*, 1985; Völksch *et al.*, 1989; Kenyon and Turner, 1992; Mittal and Davis, 1995). Additionally, CFA was shown to delay the onset of the HR on tobacco plants (Budde and Ullrich, 2000). Both COR and CFA share structural and functional homologies with the plant signalling molecule jasmonic acid, although they seem to produce additional biological effects (Feys *et al.*, 1994; Palmer and Bender, 1995; Budde and Ullrich, 2000; Pautot *et al.*, 2001). COR-deficient mutants of *P. syringae* pvs *glycinea*, *maculicola* and *tomato* were shown to reach lower *in planta* populations and/or induce smaller lesions in their plant hosts (Gnanamanickam *et al.*, 1982; Sato *et al.*, 1983; Bender *et al.*, 1987; Mittal and Davis, 1995; Sesma *et al.*, 2001). However, the reduction in virulence observed with *cor*-minus mutants of pv. *tomato* PT23 was shown to be due in part to coronatine and in part to the activity of the CFA biosynthetic gene cluster in coordination with an unlinked DNA region (Sesma *et al.*, 2001). The COR biosynthesis genes are clustered in a 32-kb chromosomal or plasmidic region, that was proposed to constitute a PAI (Alarcón-Chaidez *et al.*, 1999).

Several pathovars of *P. syringae* (*aptata*, *atrofaciens*, *lachrymans* and *syringae*) produce two types of cyclic lipodepsipeptides: the nonapeptides (MW ca. 1200 Da) syringomycins, syringotoxin, syringostatins, and pseudomycins, which exhibit similar biological activity, and syringopeptins (MW ca. 2500 Da), which contain either 22 or 25 amino acid residues (Table 8) (Bender *et al.*, 1999). All the analyzed *P. syringae* pv. *syringae* strains produce both classes of lipodepsipeptides. Syringomycin induces necrosis in plant tissues, and its primary target is the plasma membrane of the host cell, where the amphipathic nature of the toxin molecule promotes its insertion into the lipid bilayers to form pores that are freely permeable to cations (K^+, H^+ and Ca^{2+}), resulting in cell death (Takemoto, 1992). In addition to being phytotoxic, lipodepsinonapeptides also show fungicidal activity against numerous fungi, such as *Rhodotorula pilimanae* (Gross, 1991; Sorensen *et al.*, 1996; Lavermicocca *et al.*, 1997), which has been used as an indicator strain to detect the production of these toxins. It is as yet unknown if this fungicidal activity has any role in favoring the colonization of plant surfaces. The role of syringomycins in the interaction with the plant is underlined by the fact that their production is modulated by phenolic glycosides and sugars which are abundant in different tissues of many host plant species (Mo and Gross, 1991). Syringopeptins exhibit a more pronounced phytotoxicity than syringomycins (Dalla Serra *et al.*, 1999), and they also seem to be more active at uncoupling mitochondrial oxidative phosphorylation (Di Giorgio *et al.*, 1996) and to have a higher pore-forming activity (Agner *et al.*, 2000). Also, syringopeptins have antimicrobial activity against some Gram-positive bacteria and fungi, although

Table 8. Phytotoxins produced by *Pseudomonas syringae* pathovars[a]

Toxin	Producing pathovar(s)	Chemical or biosynthetic origin
Coronatine	*atropurpurea*, *glycinea*, *maculicola*, *morsprunorum*, *tomato*	Polyketide
Persicomycins	*persicae*	Fatty acid
Phaseolotoxin	*actinidiae*, *phaseolicola*, *syringae*	Sulfodiaminophosphinyl peptide
Syringomycins[b]	*aptata*, *atrofaciens*, *syringae*	Lipodepsinonapeptide
Syringopeptins	*syringae*	Lipodepsipeptide
Tabtoxin	*coronafaciens*, *garcae*, *tabaci*	ß-lactam
Tagetitoxin	*tagetis*	Unknown

[a] Data from Bender *et al*. (1999) and Tourte and Manceau (1995); for details see text.
[b] Includes the related toxins syringotoxin, syringostatin and pseudomycin.

their spectrum is different from that of lipodepsinonapeptides, and are produced under the same conditions as syringomycins (Bender *et al*., 1999).

Phaseolotoxin induces chlorosis, and is produced by pvs *actinidiae* and *phaseolicola* (Mitchell, 1976; Tamura *et al*., 1989; Sawada *et al*., 1997; Völksch and Weingart, 1998), although it has also been described in a *P. syringae* pv. *syringae* strain isolated from vetch (*Vicia sativa*) (Tourte and Manceau, 1995). The phaseolotoxin structure consists of a sulfodiaminophosphinyl moiety linked to a tripeptide of ornithine, alanine and homoarginine. Phaseolotoxin is a reversible inhibitor of ornithine carbamoyltransferase (OCTase) and it is hydrolyzed *in planta* by peptidases to produce octicidin, an irreversible inhibitor of the OCTase and the predominant form *in planta*. Inhibition of OCTase causes an accumulation of ornithine and a deficiency in intracellular pools of arginine, leading to chlorosis. Mutants unable to produce this toxin were still pathogenic, but did not move systemically in bean plants (Patil *et al*., 1974).

Tabtoxin is a chlorosis inducing phytotoxin produced by pvs *coronafaciens*, *garcae* and *tabaci*, as well as by other non-classical isolates from pea. Tabtoxin is hydrolyzed to tabtoxinine-ß-lactam by non-specific aminopeptidases from the plant and/or the bacterium. This compound, and not tabtoxin, is responsible for the biological effect of the toxin, the irreversible inhibition of the glutamine synthetase from the plant, leading to the accumulation of toxic concentrations of ammonia which in turn uncouples the carbon and energy fixation components of photosynthesis and destroys the thylakoid membrane of the chloroplast. Tabtoxin has varied effects on the virulence of different *P. syringae* strains: non-producing mutants of pathovars coronafaciens and tabaci only failed to induce chlorosis but still produced necrotic lesions, whilst a mutant of *P. syringae* BR2 was non-pathogenic on its host, bean (Kinscherf *et al*., 1991).

Tagetitoxin production is restricted to *P. syringae* pv. *tagetis*. The proposed chemical structure consists of two fused six-membered heterocyclic rings and it is not related to an amino acid or peptide-like compound. The toxin causes a striking apical chlorosis, as well as necrotic leaf spots, sometimes accompanied by chlorotic haloes. Tagetitoxin acts by inhibiting chloroplast RNA polymerase, having a general role in the repression of chloroplast genes so that only developing leaf tissues are affected (Bender *et al*., 1999).

Concluding remarks

P. syringae cells in the plant apoplast synthesize a sophisticated (type III) secretion apparatus that allows them to deliver a load of avirulence proteins, or effectors, inside cells of the plant host. We do not know how many effectors a given strain has nor how many of them are needed to induce disease in a given host. It is possible, however, that each strain contains a large number of avirulence genes because it is known that the activity of many of them is small and redundant, having additive effects on pathogenicity (see for instance Lorang *et al*., 1994; Jackson *et al*., 1999; Bai *et al*., 2000). Once inside the cell, the individual effectors appear to interact specifically with plant gene products. The interactions of effectors with plant resistance genes could trigger a defence response of variable intensity that could lead to the produc-

tion of a dominant HR, rendering the plant resistant, or to a weak reaction that will reduce the virulence in that particular plant host (Jackson *et al.*, 1999; Tsiamis *et al.*, 2000). Other effectors interact with putative plant "susceptibility genes" and will determine the production of disease, or will increase virulence, in the plant host (Chang *et al.*, 2000; Chen *et al.*, 2001; Tsiamis *et al.*, 2000). Therefore, it is thought that the outcome of the interaction of a *P. syringae* strain with a given plant host, and hence host range, will result from the sum of all the individual effects of the interaction between the effectors with plant resistance or "susceptibility" genes. The identification and cloning of these putative "susceptibility" genes is foreseeable in the near future and will probably open a large avenue of strategies to construct plants that are resistant to pathogens. Many *P. syringae* strains also synthesize various phytotoxins and hormones that have been shown to increase virulence in a non-host specific way. The role of other extracellular factors, such as enzymes and polysaccharides has not yet been fully established.

The fact that all known pathogens contain avirulence genes and that these were shown to be pathogenicity determinants in bacterial plant pathogens, suggests that avirulence genes in fungi, nematodes and viruses could also serve as pathogenicity and virulence determinants in their interactions with certain plant hosts.

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