

INVITED REVIEW  
**BACTERIAL EFFECTOR GENES AND THEIR ROLE  
IN HOST-PATHOGEN INTERACTIONS**

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**INTRODUCTION**

Bacterial pathogens often show great discrimination among potential host plants with regard to their abilities to cause disease. The basis of this race/cultivar specificity has been shown to reside in the matching of specific resistance (*R*) genes in the plant with determinants called avirulence (*avr*) genes in the pathogen: recognition in this way between host and pathogen enables the plant to react defensively to limit the invasion of the pathogen (Keen, 1990).

Recent advances have established that the recognition between host and pathogen occurs inside plant cells (Gopalan *et al.*, 1996; Leister *et al.*, 1996; Scofield *et al.*, 1996; Tang *et al.*, 1996; Van den Ackerveken *et al.*, 1996). A specialised bacterial secretion system, the type III Hrp protein secretion system, specified by a set of *hrp* genes (hypersensitive response and pathogenicity), is required both for signalling between the pathogen and the plant involving matching *avr/R* genes and also for pathogenicity (Hueck, 1998). There is circumstantial evidence that *avr* gene products are transferred into plant cells by the Hrp system and by implication other proteins that are required for pathogenicity. The term effector protein has been suggested to encompass all proteins that traverse the Hrp pathway into the plant, irrespective of whether they function as elicitors or as virulence factors (van Dijk *et al.*, 1999).

A range of effector genes and their products have been shown to depend for their effects *in planta* on a functional Hrp pathway and these include the products of *avr vir* (virulence) and *hop* (Hrp-dependent outer protein) genes (Alfano and Collmer, 1996; Mudgett and Staskawicz, 1998). This review will seek to present our current knowledge of these effector genes among the Gram-negative genera, *Pseudomonas*, *Xanthomonas*, *Ralstonia* and *Erwinia* and to consider their probable role(s) in the disease process.

*Key words:* avirulence, *Erwinia*, *Pseudomonas*, *Ralstonia*, *Xanthomonas*.

**A BRIEF LOOK AT EARLY APPROACHES TO THE  
INVESTIGATION OF BACTERIAL PATHOGENESIS**

**AVIRULENCE GENES.** The first *avr* gene to be isolated was from the soybean (*Glycine max*) pathogen, *P. syringae* pv. *glycinea* and was designated *avrA* (Staskawicz *et al.*, 1984). The same investigators also searched for cloned DNA that might confer 'virulence' as opposed to avirulence, but failed to identify any such genes and thus for the first time produced evidence to support the notion of specificity residing in the incompatible combination of *avr/R* genes. Subsequently, a number of *avr* genes were identified, but the demonstration of co-segregation of resistance among an F<sub>2</sub> population of pea to both *P. syringae* pv. *pisi* race 2 and to a race 1 strain harbouring the cloned *avrPpiA1* gene, was the first confirmation of the genetic basis of matching genes (Vivian *et al.*, 1989). At about the same time, transfer of cloned DNA from one pathovar to another, showed that recognition of *avr* genes could also occur with non-host plants. A gene *avrRxv* cloned from *Xanthomonas campestris* pv. *vesicatoria*, a pathogen of tomato (*Lycopersicon esculentum*), on transfer to *X. campestris* pv. *phaseoli* elicited a differential reaction in bean cultivars, leading to the identification of a partially dominant resistance gene in bean, *Rxv* (Whalen *et al.*, 1988). Subsequently, a functionally homologous resistance gene was identified in a tomato line (Whalen *et al.*, 1993).

Genes previously identified as cultivar-specific for their normal hosts, were also shown to confer avirulence in a number of other non-host plants. Thus, *avrPphB*, when transferred to *P. syringae* pv. *pisi*, was shown to confer avirulence toward all differential pea cultivars, while *avrPpiA* in *P. syringae* pv. *phaseolicola* showed a pattern of differential interaction with bean that was unlike any known race of the homologous pathogen (Fillingham *et al.*, 1992). The *P. syringae* pv. *tomato* strain DC3000 carrying *avrPphB* was shown to interact

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with *RPS5*-bearing accessions of *Arabidopsis thaliana* (Simonich and Innes, 1995).

It may be that such apparent differences in the levels at which specificity operates reflect the availability of routine screening systems for particular host-pathogen combinations rather than being due to fundamental differences in the nature of the genes themselves.

**THE HYPERSENSITIVE REACTION.** The resistant reaction seen in incompatible gene-for-gene interactions with these pathogens is termed the hypersensitive reaction (HR) and was first described by Klement (1963). It is a form of programmed cell death (Dangl *et al.*, 1996), having counterparts in apoptosis during pathogenic responses in animals (Wyllie, 1995; Dangl *et al.*, 1996) and at a gross level can exhibit phenotypically distinct appearances, implying subtle modulations of the processes involved (Jackson *et al.*, 1999; Tsiamis *et al.*, 2000).

**HRP GENES.** A number of laboratories have sought to investigate the basis of pathogenicity through mutation of the pathogen, followed by screening on compatible combinations of host and pathogen. In all cases, this approach resulted in the isolation of a class of mutants that came to be known as *hrp* (pronounced 'harp') mutants, the designation reflecting their pleiotropic nature, which results in loss of HR towards non-host plants such as tobacco and of pathogenicity towards their natural host (Lindgren *et al.*, 1986). Similar clusters of *hrp* genes were subsequently studied in fine detail from *Ralstonia solanacearum* (Van Gijsegem *et al.*, 1995), *Erwinia amylovora* (Wei and Beer, 1995), *P. syringae* pv. *syringae* (Huang *et al.*, 1995; Preston *et al.*, 1995), *X. campestris* pv. *vesicatoria* (Bonas *et al.*, 1991) as well as the original *P. syringae* pv. *phaseolicola* (Grimm *et al.*, 1995).

### THE TYPE III PROTEIN SECRETION SYSTEM

It is now clear that a number of these genes is highly conserved not only among plant pathogens, but also among a range of animal pathogens from *Yersinia* to *Shigella* and these have been redesignated *brc* (for HR conserved) genes, which are responsible for a protein secretion system, the so-called type III system (Bogdanove *et al.*, 1996a). In general these gene clusters are chromosomally located in plant pathogens, but in *R. solanacearum* they are located on a giant plasmid of about 2100 kb (Boucher *et al.*, 1986; 1988; C.A. Boucher, personal communication), while in *E. herbicola* pv. *gypsophilae* they are located on a 150 kb plasmid,

pPATH (Nizan *et al.*, 1997).

The *hrp* clusters of plant pathogens fall into two groups, based on sequence conservation: in group I are the genes from *E. amylovora* (Bogdanove *et al.*, 1996b), *E. chrysanthemi* (Bauer *et al.*, 1994; Kim *et al.*, 1998) and *P. syringae* and in group II those from *R. solanacearum* and *X. campestris*. Regulation of the expression of the Hrp pathway differs between groups I and II, although most seem to be induced *in vitro* by conditions of low nutrient status (Bonas, 1994). In group I, HrpL, a member of the extracytoplasmic function (ECF) subfamily of sigma factors, controls expression (Xiao *et al.*, 1994; Wei and Beer, 1995), while in group II, the regulatory proteins HrpX and HrpG in *X. campestris* and HrpB in *R. solanacearum* are thought to act as sensor and response regulator proteins, respectively, for a two component AraC-type sensor system (Genin *et al.*, 1992; Wengelnik and Bonas, 1996; Wengelnik *et al.*, 1996b). A putative promoter motif, the PIP (plant inducible promoter) box has been postulated to function upstream of some *hrp* and *avr* genes (Fenselau and Bonas, 1995), but since genes such as *avrBs3* and *hrpA* lack PIP boxes (Knoop *et al.*, 1991; Wengelnik and Bonas, 1996), some alternative mode of regulation must be involved for these genes. Interestingly, *avrRxv*, which has a perfect PIP-box, was found to be constitutively expressed *in vitro*, irrespective of the nutritional status of the medium, also *in planta* at high levels and to be unaffected by disruption of the PIP-box sequence (Ciesiolka *et al.*, 1999). A novel regulatory pathway in *R. solanacearum* involves PrhA, which may act as a receptor for plant-specific signals to regulate *hrp* gene expression (Marenda *et al.*, 1998).

In *X. campestris* pv. *vesicatoria*, the protein HrpA1 has been localised to the bacterial outer membrane. It is essential for pathogenicity and the induction of the HR. From its relatedness to the pIV protein of filamentous bacteriophages, it has been suggested that it may play a role in the structure of the export channel for type III secretion (Wengelnik *et al.*, 1996a). Recently it has been shown that the Hrp protein secretion in *P. syringae* involves the production of a filamentous pilus, composed mainly of the structural protein HrpA and dependent on *brcC* and *hrpS* for its production under conditions similar to those required for *hrp* gene induction (Roine *et al.*, 1997; Taira *et al.*, 1999). Thus, the Hrp system in plant pathogens appears to be specialised to deliver secreted proteins, probably by way of this pilus, directly into the cytosol of plant cells. At present much of the evidence is circumstantial and will be presented briefly below.

## HARPINS

At the time of their discovery there was considerable excitement about a novel class of proteins that could elicit an HR in plant hosts. The first of these proteins, which are all heat-stable, glycine-rich, cysteine-lacking peptides, was isolated from *E. amylovora* and mutants in the *hrpN* gene failed to elicit an HR in the non-host, tobacco or to cause disease in pear fruit (Wei *et al.*, 1992; He *et al.*, 1994). Harpins are a class of secreted protein, not involved structurally in the bacterial pilus, that appear to be directed to the plant cell wall, rather than being effector molecules that are translocated inside plant cells. Similar genes, *hrpZ* in *P. syringae* pv. *syringae* 61 (He *et al.*, 1993), and *hrpN* in *E. chrysanthemi* were identified (Bauer *et al.*, 1995) and their products shown to be dependent on the *hrp* cluster for their secretion (Huang *et al.*, 1995; Charkowski *et al.*, 1997). The elicitor activity of HrpZ was found to reside in multiple regions of the protein (Alfano *et al.*, 1996). Recently, a novel second harpin, HrpW, containing a C-terminal domain that is homologous to a unique class of pectate lysases, has been identified in *E. amylovora* (Gaudriault *et al.*, 1998; Kim and Beer, 1998). It has been suggested that the C-terminal sequences may be involved in targeting this elicitor to the plant cell wall. Galan and Collmer (1999) have suggested that these gene products may assist the delivery of Avr proteins across the plant cell wall.

## GENE-FOR-GENE MATCHING

Resistance genes often comprise complex loci, involving additional copies of the gene (Jones, 1996), but it has recently been shown that for the resistance gene *RPM1*, a single copy of the gene is present in *Arabidopsis* and two separate copies in *Brassica napus*, while the recessive alleles are deletions of the gene from the corresponding chromosomal sites (Grant *et al.*, 1998).

Bacteria on the other hand are haploid and in consequence either do or do not carry a functional *avr* gene. The quadratic check (Table 1) shows that specificity resides in the matching *avr/R* gene combination, whereas any other interaction results in disease. For example, the current race structure of *P. syringae* pv. *pisii* and pea (Table 2) can be explained in terms of 5 or 6 matching gene pairs. Evidence has so far been obtained by classical genetics for the *R* genes present in each pea cultivar and three (of the six postulated) matching *avr* genes cloned. Vivian and Mansfield (1993) proposed a uniform naming system for *avr*

genes, the designations reflecting the pathovar from which the gene was obtained as well as the matching resistance gene. Thus for *P. syringae* pv. *pisii*, *avrPpiA1.R2* is the first gene (*A*) isolated and the first allele (*1*) and matches the *R2* gene in pea; the remaining genes being designated *avrPpiB1.R3* and *avrPpiD1.R5* (Dangl *et al.*, 1992; Cournoyer *et al.*, 1995; D.L. Arnold, C. Gunn, A. Vivian, unpublished).

Pathovars do not always correspond to single defined genomic groups, suggesting that phylogenetically distinct ancestors have evolved in parallel to cause phenotypically similar diseases in the same host plant. Two genomic groups have been identified for *P. syringae* pv. *pisii*. The use of random short sequence primers with polymerase chain reaction (RAPD-PCR) amplification enabled Arnold *et al.* (1996) to develop specific PCR primers for the identification of *P. syringae* pv. *pisii*. Two sets of primers were found to amplify mutually exclusive fragments from races of the pathovar, indicating that it was comprised of two genomic groups, group I containing races 1, 3B, 4B, 5 and 7 and group II containing races 2, 3A, 4A and 6. The specificities shown by races 3 and 4 are therefore determined in each race by strains from different genomic groups (Arnold *et al.*, 1996).

**Table 1.** Quadratic check.

Pathogen genotype	Host genotype	
	<i>RR</i> or <i>Rr</i>	<i>rr</i>
<i>avr</i> gene present	–	+
<i>avr</i> gene absent*	+	+

*R/r*: resistance; *avr*: avirulence; +: compatible (disease); –: incompatible (resistant).

\* Would also include mutations in *avr* genes, which can be regarded as inactive alleles, resulting in compatibility in gene-for gene combinations.

## TYPES OF AVIRULENCE GENE

A number of recent reviews have dealt with types of *avr* gene and their likely roles in the interaction with plant hosts (Dangl, 1994; Leach and White, 1996; Bonas and Van den Ackerveken, 1997; Van den Ackerveken and Bonas, 1997; Vivian and Gibbon, 1997; Vivian *et al.*, 1997; Mudgett and Staskawicz, 1998; Bonas and Van den Ackerveken, 1999). There are broadly two groups of *avr* gene among phytopathogenic bacteria, those that resemble *avrBs3* and those that

**Table 2.** Gene-for-gene relationships between pea cultivars and races of *P. syringae* pv. *pisii* (based on Bevan *et al.*, 1995).

Cultivar	Resistance genes						Races/avirulence genes						
							1	2	3*	4*	5	6	7
							1	.	.	.	.	.	.
							.	2	.	.	2	.	2
							3	.	3	.	.	.	3
							4	.	.	4	4	.	4
							.	.	.	.	5	.	.
							6?	.	.	.	6?	.	.
Kelvedon Wonder	.	.	.	.	.	.	+	+	+	+	+	+	+
Early Onward	.	2	.	.	.	.	+	-	+	+	-	+	-
Belinda	.	.	3	.	.	.	-	+	-	+	+	+	-
Hurst Greenshaft	.	.	.	4	.	6?	-	+	+	-	-	+	-
Partridge	.	.	3	4	.	.	-	+	-	-	-	+	-
Sleaford Triumph	.	2	.	4	.	.	-	-	+	-	-	+	-
Vinco	1	2	3	.	5	.	-	-	-	+	-	+	-
Fortune	.	2	3	4	.	.	-	-	-	-	-	+	-

\* Races 3 and 4 are further subdivided into genomic groups which do not differ in their respective host specificity. Thus races 3A and 4A are in genomic group II and races 3B and 4B are in genomic group I (Arnold *et al.*, 1996).

+: susceptible response; -: resistance response; ?: gene probably present; .: gene absent.

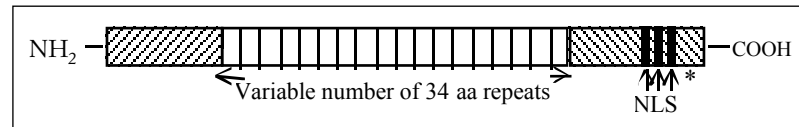
do not (Vivian and Gibbon, 1997). The *avrBs3*-like genes have been found in a several *X. campestris* pathovars and have very distinct structural features, which include between 15.5 and 25 repeats of a 102 bp sequence, specifying 34 amino acid repeats located centrally within the open reading frame (ORF) of the gene (Fig. 1; Table 3). The repeat sequences are highly conserved and variation within the sequences is limited to about 5 amino acid positions within a single repeat (Bonas *et al.*, 1989). Deletion of the repeated sequences in *avrBs3* resulted in changes of specificity (both loss and gain of recognition) in the host plants, pepper and tomato (Herbers *et al.*, 1992). The genes, *avrB6* and *pthA* from *X. campestris* pv. *malvacearum*, *avrBs3* from *X. campestris* pv. *vesicatoria* and the gene *avrXa10* from *X. oryzae* pv. *oryzae* have been shown to contain in their C-terminal regions, nuclear localisation signals, at least one of which was functionally active (Yang and Gabriel, 1995a; Van den Ackerveken *et al.*, 1996; Zhu *et al.*, 1998). Recently, a transcriptional

activation domain has been identified in AvrXa10, which can be functionally replaced by the transcriptional activation domain from Herpes simplex virus protein, VP16: such domains typically comprise acidic or large hydrophobic amino acids and in this case there was little sequence identity between the two. Nevertheless, mutational studies showed that transcriptional activation in yeast and the avirulence phenotype in rice were coincident (Zhu *et al.*, 1999). These features imply that this family of genes may target their gene products to the nucleus of the plant cell and could function to modify the control of gene expression in the host (Bonas and Van den Ackerveken, 1999; Gabriel, 1999).

The remaining *avr* genes comprise the second group (Table 4) and tend to be relatively small (about 18 to 40 kDa) proteins, which are hydrophilic in nature and probably occupy the cytosol of the bacterium. They have been found mainly in *P. syringae* but also include examples from *X. campestris* and *R. solanacearum*.

**Fig. 1.** Structural features of the AvrBs3 family of effector proteins.

NLS: nuclear localisation signals; \*: transcription activation domain.

**Table 3.** *avrBs3*-like avirulence genes.

Bacterium	Gene	Host plant	R gene	Peptide (kDa)	Repeats*	Location	Accession number	Reference
<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>	<i>avrBs3</i>	pepper	<i>Bs3</i>	122	17.5	plasmid	X16130	Bonas <i>et al.</i> , 1989
	<i>avrBsP</i>	tomato	nd	nd	nd	plasmid	--	Canteros <i>et al.</i> , 1991
	<i>avrBs3-2</i>	tomato	nd	122	17.5	plasmid	X68781	Bonas <i>et al.</i> , 1993
<i>pv. malvacearum</i>	<i>avrBn</i>	cotton	nd	nd	nd	chromosomal	--	Gabriel <i>et al.</i> , 1986
	<i>avrb6</i>	cotton	<i>B1</i>	108	13.5	plasmid	L06634	De Feyter <i>et al.</i> , 1993
	<i>avrB4</i>	cotton	<i>B1, B4</i>	nd	19	plasmid	--	De Feyter <i>et al.</i> , 1993
	<i>avrb7</i>	cotton	nd	nd	19	plasmid	--	De Feyter <i>et al.</i> , 1993
	<i>avrB1n</i>	cotton	nd	nd	21	plasmid	--	De Feyter <i>et al.</i> , 1993
	<i>avrB101</i>	cotton	nd	nd	nd	plasmid	--	De Feyter <i>et al.</i> , 1993
	<i>avrB102</i>	cotton	<i>B1</i>	nd	18	plasmid	--	De Feyter <i>et al.</i> , 1993
	<i>avrB103</i>	cotton	nd	nd	nd	chromosomal	--	Yang <i>et al.</i> , 1996
	<i>avrB104</i>	cotton	nd	nd	nd	chromosomal	--	Yang <i>et al.</i> , 1996
	<i>avrB5</i>	cotton	nd	nd	nd	nd	--	Yang <i>et al.</i> , 1996
<i>pv. citri</i>	<i>pthA</i>	bean/cotton	nd	122	17.5	nd	U28802	Yang and Gabriel, 1995b
<i>X. oryzae</i> pv. <i>oryzae</i>	<i>avrxa5</i>	rice	<i>xa-5</i>	nd	nd	nd	--	Hopkins <i>et al.</i> , 1992
	<i>avrXa7</i>	rice	<i>Xa-7</i>	nd	25	nd	--	Hopkins <i>et al.</i> , 1992
	<i>avrXa10</i>	rice	<i>Xa-10</i>	116	15.5	chromosomal	J03710	Hopkins <i>et al.</i> , 1992

\* number of 102 bp direct repeats in the central region of the gene.  
nd: not determined; --: not available.

Some of these genes are much larger, such as *avrA* (100 kDa) from *P. syringae* pv. *glycinea*, *avrBs2* (80kDa) from *X. campestris* pv. *vesicatoria* and *avrXca* from *X. campestris* pv. *raphani* (67 kDa).

Another gene, designated *brmA* and associated with a *hrp* cluster, was originally thought to code for a regulator that modulated the HR (Heu and Hutcheson, 1993). It is now clear that the product of this gene has no regulatory function, but it does behave like an avirulence protein (Alfano *et al.*, 1997) and is secreted by the type III system: in consequence, van Dijk *et al.* (1999) have recently proposed that the gene be renamed *hopPsyA*, to reflect the role of its product as an effector protein.

(For a more detailed account of the genes shown in Tables 3 and 4, see Vivian *et al.*, 1997).

Many of these *avr* genes show a G + C ratio between 40.0 and 57.6%, which is below the normal range for the host bacterium: that is 59.0-61.0% for *P. syringae* (Palleroni, 1984) and 63.5-69.2% for *X. campestris* (Bradbury, 1984). This has been taken to indicate that the genes have been acquired from some low G + C ancestor (Barinaga, 1996; Alfano and Collmer, 1997). However, this hypothesis is far from proven and it is conceivable that these distinctive features may have some functional significance that ensures the maintenance of the lower ratios. For example, if one examines

organisms such as *Salmonella typhimurium*, where the average genomic G + C content is 51.0-53.0%, which is not dissimilar to many *avr* genes, two pathogenicity islands (PAI) have G + C contents of 44.6 and 47.0%, respectively (Shea *et al.*, 1996). Within phytopathogenic PAIs there is considerable variation in G + C ratios for different ORFs, for example, the *hrpA* gene in *P. syringae* pv. *syringae* and *P. syringae* pv. *tomato* has a high G + C content and shows low conservation of sequence in comparison with other genes within the *hrp* cluster (Preston *et al.*, 1995).

### GENOMIC CONTEXT AND SEQUENCES FLANKING EFFECTOR GENES

The location of effector genes within the bacterial genome is receiving increasing attention in view of the insights we might gain from these studies into the past evolution of bacterial plant pathogens and their potential for adaptation to changing agricultural practices, seen as epidemic outbreaks, often involving novel races or strains. The regions immediately flanking a number of *avr* genes were examined and found to contain a number of features with potential significance for gene mobility.

The genes, *avrA*, *avrB* and *avrC* from *P. syringae* pv. *glycinea*, *avrPpbC*, *avrRpm1* from *P. syringae* pv. *maculicola* and *avrPpiA* are flanked by sequences resembling insertion sequences (IS51, IS801 and IS1240) and transposable elements (Tn501), which could have been involved in their horizontal transfer between pathovars (Kim *et al.*, 1998). At least four members of the *avrBs3* family have been shown to be associated with a 62 bp sequence: inverted repeat copies flank both *avrBs3* and *avrBs3-2*, which are located on different plasmids in the pepper pathogen, and it is likely that this is a feature of this group of genes (Bonas *et al.*, 1993; De Feyter *et al.*, 1993; Yang and Gabriel, 1995b).

The *avrPpiA* gene is almost unique in being located on plasmids in races 5 and 7 of *Ppi*, but being chromosomal in race 2 (Gibbon *et al.*, 1997). Analysis of the chromosomal race 2 allele, showed it to be present in a region of 8.5 kb of DNA, bounded by slightly imperfect direct 7 bp repeat sequence (CCAGCT/AT), which was deemed to be indicative of horizontal acquisition of this region (Arnold *et al.*, 1999). Also present were sequences resembling *rulAB* genes, usually found on plasmids (Sundin and Murillo, 1999): the *rulB* homologue was disrupted by a 4.5 kbp region, including *avrPpiA* and ORFs resembling bacteriophage and transposase

genes, suggestive of a further insertion within this region (Arnold *et al.*, 2000).

The gene *avrD* has been found in several pathovars of *P. syringae* and is unusual for an *avr* gene, in that the products of these genes appear to have an enzymatic function, resulting in the production of low molecular weight syringolides, which act as elicitors (Yucel *et al.*, 1994a, b). Not all of the alleles detected appear to function in this way, as none of those present in the 7 races of *P. syringae* pv. *glycinea* acted as *avr* genes in soybean nor produced syringolides (Kobayashi *et al.*, 1990b; Keith *et al.*, 1997). However, this may not exclude their function in some other way in the interaction with the plant, particularly since they appear to be widely maintained in plant pathogens and evidence for their occurrence in the soft rot pathogen, *E. carotovora* has been obtained by hybridisation analysis (Hanekamp *et al.*, 1997). A novel insertion sequence, IS1240 close to *avrD* on the B plasmid in *P. syringae* pv. *tomato*, may reflect its potential for mobility within and between genomes (Hanekamp *et al.*, 1997).

Alfano and Collmer (1996) proposed that the spread and variable distribution of effector genes among strains of a given taxon could result from two driving forces. Changes in the plant target receptors would reduce the parasitic ability of the pathogen, while recognition by a plant *R* gene of a virulence effector would abolish the value of the virulence gene. The consequences would be a repetitive process, resulting in the evolution of multiple virulence genes in a successful pathogen and a concomitant redundancy of function. This model has derived considerable support from the study of regions flanking effector genes and also from the observation that it has been possible to identify virulence genes only after removal (by plasmid curing) of substantial regions of DNA proposed to represent a PAI (Jackson *et al.*, 1999).

It is also clear that the regions flanking the *hrp* clusters in *P. syringae* pathovars are important locations for effector genes. In *P. syringae* pv. *phaseolicola*, the gene, *avrPpbE* was found adjacent to the left hand end (*hrpK*) of the *hrp* gene cluster and in a location corresponding to that of *hopPsyA* (previously designated *brmA*) in *P. syringae* pv. *syringae* (Mansfield *et al.*, 1994). At the opposite end of the *hrp* cluster in strain DC3000, the genes *avrE* and *avrF* were detected in two strains of *P. syringae* pv. *tomato* (Lorang and Keen, 1995; Bogdanove *et al.*, 1998). Recent analysis of the entire *hrp* region, including the flanking sequences in two strains of *P. syringae* pv. *syringae* and *P. syringae* pv. *tomato* DC3000, has revealed a tripartite mosaic structure for this part of the *P. syringae* genome (Alfano *et al.*, 2000).

An exchangeable effector locus (EEL) has been identified to the left of *hrpK*, in which the DNA sequences diverge for regions varying from 2.5 kb to 7.3 kb until sequence similarity was restored near a tRNA<sup>Leu</sup> gene. Homologues of effector genes *avrPphC*, *avrPphE*, *avrBst* plus three other ORFs were present in the *P. syringae* pv. *syringae* B728a strain, while *hopPsyA* and a novel ORF were in *P. syringae* pv. *syringae* 61. DC3000 contains four ORFs plus a homologue of TnpA1 from *P. stutzeri*. The G + C content of all these ORFs was lower than that of *hrpK*, a feature typical of effector genes.

At the opposite end of the cluster, beyond *hrpR*, a conserved effector locus (CEL) was identified: in B728a and DC3000, representing two different pathovars, the first seven ORFs were arranged identically and shared 78% sequence identity. A deletion in the EEL of DC3000 reduced fitness, as indicated by lowered *in planta* growth of the pathogen, while a large deletion in CEL abolished pathogenicity in tomato (Alfano *et al.*, 2000). Thus, it appears that there are discrete locations in the genomes of plant pathogens for positioning and exchange of (probably host-specific) packages of effector genes.

#### PLASMID-BORNE VERSUS CHROMOSOMAL

The bacterial genome in *Pseudomonas* is comprised of a single circular chromosome and usually one or more plasmids. Most *avr* genes that have been described are either found exclusively on plasmids or are chromosomal (Tables 3 and 4), but a small number are found in both locations, with consequences for their gene dosage, due to the generally higher copy number of most plasmids. The *avrD* gene has generally been found to be located on plasmids in *P. syringae* pv. *tomato*, *P. syringae* pv. *lachrymans* and *P. syringae* pv. *phaseolicola* and in all races of *P. syringae* pv. *glycinea*, except race 1 (Kobayashi *et al.*, 1990b; Yucel *et al.*, 1994a; Keith *et al.*, 1997). In *P. syringae* pv. *pisi* and *P. syringae* pv. *maculicola* the homologous genes *avrPpiA* and *avrRpm1* are plasmid-borne, except for an allele in *P. syringae* pv. *pisi* race 2, which is chromosomal, but shows evidence of a previous plasmid location (Gibbon *et al.*, 1999; Arnold *et al.*, 2000).

It has often been suggested that plasmids may serve to further the spread of *avr* genes between strains of the bacteria; however, a study that compared plasmid and host bacterial phylogenies concluded that there was little evidence for horizontal gene transfer among strains of *P. syringae* pv. *syringae* in a pear orchard (Sundin and Bender, 1996). However, one cannot rule

out the likelihood of transient plasmid transfer and delivery of mobile elements associated with effector genes. Certainly the type of replicon which is commonly found in *P. syringae* appears to be adapted to permit compatible co-existence within one strain of closely related plasmids (Sesma *et al.*, 1998; Gibbon *et al.*, 1999).

#### FITNESS ASPECTS

It has always been puzzling to understand why bacteria should find it advantageous to carry *avr* genes, which enable the plant to prevent their access to its nutritional resources. However, for a relatively small number of *avr* genes it has been possible to show that they confer a growth advantage to their bacterial host *in planta* (Table 5). The *avrBs2* gene in *X. campestris* pv. *vesicatoria*, when mutated, has been shown to result in both loss of the HR-induction with pepper carrying the resistance gene *Bs2* and also reduced growth in compatible pepper lines. Functional homologues of this *avr* gene were also found in many *X. campestris* pathovars, representing a wide range of plant hosts, suggesting that it plays a highly conserved role in the fitness of these pathogens in the field (Kearney and Staskawicz, 1990). The *AvrBs2* protein shows homology to genes for agrocinopine synthase and glycerophosphoryl diester phosphodiesterase and may function to modify glucans, implicated elsewhere in plant signalling (Swords *et al.*, 1996). Recently, the *Bs2* resistance gene has been cloned from pepper and shown to confer resistance to *avrBs2*-carrying strains of *X. campestris* pv. *vesicatoria* on tomato (Tai *et al.*, 1999).

Mutation of *avrE* in strain PT23 of *P. syringae* pv. *tomato*, resulted in substantial reductions in virulence and growth of the bacteria in tomato; however mutation of a homologue in the related strain DC3000 had no effect on virulence toward tomato, indicating the importance of the genetic background of the bacterial strain in the phenotype conferred by an *avr* gene (Lorang *et al.*, 1994). A similar situation may explain the conflicting observations for the gene *avrRpm1*, which is virtually identical to *avrPpiA* (Dangl *et al.*, 1992): disruption of *avrRpm1* in *P. syringae* pv. *maculicola* resulted in reduced growth of the bacteria in compatible *Arabidopsis* accessions at low inoculum levels (Ritter and Dangl, 1995), while disruption of the homologous *avrPpiA* gene in *P. syringae* pv. *pisi* had no effect on bacterial growth or pathogenicity toward compatible cultivars of pea (Gibbon *et al.*, 1997).

**Table 4.** Effector genes other than *avrBs3*-like.

Bacterium	Gene	Host plant	R gene	Peptide (kDa)	G + C %	Accession number	Reference
<i>R. solanacearum</i>	<i>avrA</i>	tobacco	nd	nd	nd	–	Carney and Denny, 1990
	<i>popA</i>	petunia	nd	33	67.4	AJ245811	Arlat <i>et al.</i> , 1994
<i>P. syringae</i>	<i>avrA</i>	soybean	<i>RPG2</i>	100	45.0	M15194	Napoli and Staskawicz, 1987
pv. <i>glycinea</i>	<i>avrB</i>	soybean	<i>RPG1</i>	36	46.3	M21965	Tamaki <i>et al.</i> , 1988
	<i>avrC*</i>	soybean	<i>RPG3</i>	39	47.5	M22219	Tamaki <i>et al.</i> , 1988
	<i>avrD2</i>	soybean	<i>RPG4</i>	nd	nd	–	Yucel <i>et al.</i> , 1994a Kobayashi <i>et al.</i> , 1990b
	<i>virPpbA*</i>	soybean	nd	60	54.0	AF141883	Jackson <i>et al.</i> , 1999
pv. <i>maculicola</i>	<i>avrRpm1*</i>	arabidopsis	<i>RPM1</i>	24	43.6	X67808	Dangl <i>et al.</i> , 1992
pv. <i>phaseolicola</i>	<i>avrPpbA</i>	bean	nd	nd	nd	–	Shintaku <i>et al.</i> , 1989
	<i>avrPpbB.R3</i>	bean	<i>R3</i>	38	48.1	M86401	Jenner <i>et al.</i> , 1991
	<i>avrPpbC*</i>	soybean	<i>RPG3</i>	39	47.6	U10377	Yucel <i>et al.</i> , 1994c
	<i>avrPpbD*</i>	pea	nd	75	55.0	AJ277494	Wood <i>et al.</i> , 1994 D.L. Arnold and A. Vivian, unpublished
	<i>avrPpbE.R2</i>	bean	<i>R2</i>	41	57.6	U16817	Mansfield <i>et al.</i> , 1994 Stevens <i>et al.</i> , 1998
	<i>avrPpbF.R1*</i>	bean	<i>R1</i>	15/22	40.0/52.5	AF231452	Tsiamis <i>et al.</i> , 2000
pv. <i>pisi</i>	<i>avrPpiA.R2**</i>	pea	<i>R2</i>	24	44.3	X67807	Dangl <i>et al.</i> , 1992
	<i>avrPpiB.R3*</i>	pea	<i>R3</i>	31	39.7	X84843	Cournoyer <i>et al.</i> , 1995
	<i>avrPpiC</i>	bean	nd	29	46.9	AJ277496	D.L. Arnold and A. Vivian, unpublished
	<i>avrPpiD.R5*</i>	pea	<i>R5</i>	nd	nd	–	C. Gunn and A. Vivian, unpublished
	<i>avrRps4*</i>	arabidopsis	<i>RPS4</i>	24	52.3	L43559	Hinsch and Staskawicz, 1996
	<i>avrPpiG*</i>	bean	nd	41	48.0	AJ277495	D.L. Arnold and A. Vivian, unpublished
pv. <i>syringae</i>	<i>hopPsyA</i> ( $\equiv$ <i>brmA</i> )	tobacco	nd	nd	nd	–	Heu and Hutcheson, 1993 van Dijk <i>et al.</i> , 1999
pv. <i>tomato</i>	<i>avrA</i>	soybean	<i>RPG2</i>	nd	nd	–	Kobayashi <i>et al.</i> , 1989
	<i>avrD*</i>	soybean	<i>RPG4</i>	34	41.0	J03681	Kobayashi <i>et al.</i> , 1990a
	<i>avrE</i>	soybean	nd	195	57.6	U16119	Lorang and Keen, 1995 Bogdanove <i>et al.</i> , 1998
	<i>avrF</i>	soybean	nd	14	56.7	U16119	Bogdanove <i>et al.</i> , 1998
	<i>avrPto</i>	tomato	<i>PTO</i>	18	50.5	S35220	Salmeron and Staskawicz, 1993
	<i>avrRpt2</i>	arabidopsis	<i>RPS2</i>	28	51.5	A40613	Innes <i>et al.</i> , 1993
<i>E. amylovora</i>	<i>dspE</i> ( <i>dspA</i> )	pear	nd	198	54.7	U97504	Bogdanove <i>et al.</i> , 1998
<i>X. campestris</i>	<i>avrBs1*</i>	pepper	<i>Bs1</i>	50	42.2	M32142	Ronald and Staskawicz, 1988
pv. <i>vesicatoria</i>	<i>avrBs2</i>	pepper	<i>Bs2</i>	80	63.3	AF114720	Swords <i>et al.</i> , 1996
	<i>avrBsT*</i>	pepper	nd	39	43.0	AF156163	Ciesiolka <i>et al.</i> , 1999
	<i>avrRxv</i>	bean	<i>Rxv</i>	42	52.3	L20423	Whalen <i>et al.</i> , 1993
	<i>hpaA</i>	pepper	na	30.4	65.0	AF05646	Huguet <i>et al.</i> , 1998
pv. <i>raphani</i>	<i>avrXca</i>	arabidopsis	nd	67	68.2	M99059	Parker <i>et al.</i> , 1993

\* known to be plasmid-borne; \*\* known to be chromosomal and plasmid-borne in different strains.

–: not available; na: not applicable; nd: not determined.

**Table 5.** Genes conferring fitness in planta.

Gene	Fitness	Reference
<i>avrB6</i>	enhanced watersoaking in cotton	Swarup <i>et al.</i> , 1991 Yang <i>et al.</i> , 1994
<i>avrBs2</i>	essential for growth in pepper and for homologues in alfalfa	Kearney and Staskawicz, 1990
<i>avrA</i>	contributes to virulence in tomato	Lorang <i>et al.</i> , 1994
<i>avrE</i>	enhanced virulence and bacterial growth in tomato	Lorang <i>et al.</i> , 1994
<i>avrRpm1</i>	essential for virulence in compatible arabidopsis accessions	Ritter and Dangl, 1995
<i>avrPphF</i>	restores virulence of <i>Pph</i> strain RW60 in soybean	Tsiamis <i>et al.</i> , 2000
<i>pthA</i>	functions as <i>avr</i> gene with bean and cotton: pathogenicity with citrus	Swarup <i>et al.</i> , 1991, 1992 Yang <i>et al.</i> , 1994
<i>virPphA</i>	restores virulence of <i>Pph</i> strain RW60 in bean cultivars	Jackson <i>et al.</i> , 1999
<i>avrXa7</i>	functions in aggressiveness/fitness in rice	Leach and White, 1996

## VIRULENCE

An important factor in the discovery of the first PAI in *P. syringae* was the successful curing (removal) of a large native plasmid from races of *P. syringae* pv. *phaseolicola* by the introduction of a cloned replication region from the race 4 plasmid pAV505 (Gibbon *et al.*, 1999; Jackson *et al.*, 1999). This created in the race 7 strain 1449B, a variant designated RW60, which lacks the plasmid pAV511 and has lost the ability to cause disease in bean. The plasmid was shown to contain a 30 kb region, which included a number of previously known *avr* genes, together with a gene, designated *virP-phA*, required for the partial restoration of virulence in bean; this gene also acts as an *avr* gene in soybean (Jackson *et al.*, 1999).

Recently, a gene *avrPphF.R1*, which confers cultivar specificity in bean and is located on the same PAI, has been shown to be required for partial restoration of virulence toward the compatible bean cv. 'Tendergreen' in strain RW60 and intriguingly, confers an HR toward bean cv. 'Canadian Wonder' – a cultivar that is universally susceptible to all races of *P. syringae* pv. *phaseolicola*. This novel *avr* function, however, was blocked when *avrPphC* – an *avr* gene controlling recognition in soybean – was also present in RW60. Thus, *avrPphF* is involved in both cultivar-specific avirulence and virulence in bean. The same gene product was also shown to be a virulence factor in soybean, confirming its multifunctional role in both recognition and disease (Tsiamis *et al.*, 2000).

There are similarities in the properties of *virPphA*, mentioned above and those of the disease-specific gene, *dspE* from *E. amylovora*. The product of this gene, which is a homologue of AvrE, described above, is absolutely required for disease, but not for elicitation of the HR. Mutation of the *dsp* locus resulted in *E. amylovora* behaving as a *hrp* mutant in pear, while reducing the degree of the HR in tobacco. A second gene *dspF* is required for secretion of DspE and appears to act as a chaperone (Gaudriault *et al.*, 1997; Bogdanove *et al.*, 1998).

In *X. campestris* pv. *vesicatoria*, an interesting gene designated *hpaA*, for *hrp*-associated, is located in the *hrpD* operon, but is required for disease development. The gene contains two functional NLS and mutations in it cause loss of virulence, while retaining the ability to induce an avirulence gene-mediated, host-specific HR. HpaA appears to be a novel kind of effector protein, which shares sequence similarity with HrpV of *R. solanacearum* (Huguet *et al.*, 1998).

## RECOGNITION OCCURS INSIDE PLANT CELLS

Evidence that the products of *avr* genes function inside plant cells, has come from studies where these genes have been introduced either by projectile bombardment or transformation into plant cells carrying matching *R* genes. Expression of the matching genes has been shown to elicit an HR-like cell death in a range of systems (Gopalan *et al.*, 1996; Scofield *et al.*,

1996; Tang *et al.*, 1996; Van den Ackerveken *et al.*, 1996; Alfano *et al.*, 1997; De Feyter *et al.*, 1998; Stevens *et al.*, 1998; Duan *et al.*, 1999; Mudgett and Staskawicz, 1999). Direct interaction of the matching gene products has been demonstrated only between AvrPto and Pto using a yeast two hybrid technology, which was specific for Pto and did not function with the product of the closely related gene for insecticide sensitivity, *Fen* (Frederick *et al.*, 1998).

Processing of effector proteins has been reported for just two avirulence gene products: AvrPphB was found to be N-terminally cleaved in the bacteria (Puri *et al.*, 1997), while AvrRpt2 was N-terminally cleaved in *A. thaliana* (irrespective of whether a compatible or incompatible combination of the pathogen and host was involved), but not in strain DC3000 (Mudgett and Staskawicz, 1999). The significance of these, apparently conflicting, observations remains unclear.

The implication of *in planta* recognition between matching gene products, is that the effectors traverse the Hrp secretion pathway into the plant cell. One system to investigate secretion is that developed by Ham *et al.* (1998). *E. chrysanthemi* is a necrotrophic pathogen with a wide host range, causing soft rots and secreting an arsenal of plant tissue-degrading enzymes using the bacterial general secretory pathway or type II protein secretion system, which involves a two-step process to traverse the bacterial cell membrane and periplasmic space, distinct from type III (Salmond, 1994). Although *avr* genes have not been found in this pathogen, it possesses in addition to the type II pathway, a type III secretion system and mutants lacking the pectic enzymes can elicit an HR in tobacco, confirming its functionality (Bauer *et al.*, 1994). The functional gene cluster was cloned and introduced into *E. coli*: in contrast to similar clusters of *hrp* genes from *P. syringae*, the *E. chrysanthemi* system is not repressed in rich media and Avr proteins are secreted into the surrounding media, suggesting a different or more relaxed gating mechanism (Ham *et al.*, 1998). In the related type III system found in *Yersinia* spp., some Yop proteins encode a signal sequence found in the first 15 codons of the corresponding mRNA (Anderson and Schneewind, 1997). Making use of this knowledge, *E. coli* containing the *E. chrysanthemi hrp* cluster was used to show that the animal pathogen-derived proteins, YopE and YopQ, could be secreted by the plant-pathogen system, while conversely the *Yersinia* system could secrete AvrB and AvrPto. Both of the latter were also shown to possess secretion signals encoded in their first 15 codons of mRNA (Anderson *et al.*, 1999).

Development of another experimental system for the detection of type III protein secretion was described by

Rossier *et al.* (1999) for the pepper pathogen, *X. campestris* pv. *vesicatoria*. Using a mutation in the regulatory gene, *brpG*, to obtain a strain showing constitutive expression of the type III pathway, together with conditions of growth in minimal medium at pH 5.4 in the presence of bovine serum albumin, they were able to detect the secretion of several effector proteins: the specificity of the secretion process was demonstrated by the inability to detect a cytoplasmic (control) protein GUS. Secreted products included the homologous proteins AvrBs3 and AvrRxv, PopA from *R. solanacearum* and AvrB from *P. syringae* pv. *glycinea*. Interestingly, the YopE cytotoxin from *Y. tuberculosis* was also secreted, implying recognition of the secretion signals of both plant and animal effector proteins (Rossier *et al.*, 1999).

However, one should always bear in mind that secretion of effectors into the culture medium may not always reflect the situation of translocation into plant cells: it remains unclear to what extent both the ability to secrete an effector corresponds with its ability to deliver a signal *in planta* and also the effects of genetic background (*i.e.* the presence of other effectors in the bacterial cell) on the hierarchy of delivery.

## CONCLUSIONS

A number of questions remain concerning effector genes and their role in the bacterium-plant interaction. For example it is not clear how many effectors are delivered to the plant during disease establishment – is there a ‘minimal set’ that cross some critical threshold? We still do not understand the precise functions of effector proteins inside the plant cell. However, recent evidence suggests that some, at least, are targeted to the host plasma membrane and that this is important for their function (Nimchuk *et al.*, 2000).

Of growing interest is the science of genomics and the insights it can provide into the likely evolution of pathogenic bacteria. The studies on effector genes, which have tended to be regarded as ‘stamp-collecting’ in recent years, may yet come to be extremely useful as we piece together the intricate assembly of armories designed to facilitate colonisation of plants and animals and the resulting diseases.

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