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Research Note

Sequences Related to Transposable Elements and Bacteriophages Flank Avirulence Genes of *Pseudomonas syringae*

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Examination of the reported nucleotide sequences containing avirulence genes of *Pseudomonas syringae* pathovars suggested that *avrA, avrB, avrC, avrPphC, avrRpm1,* and *avrPpiA1* are bordered by sequences similar to those of transposable elements of gram-negative bacteria. Repeat sequences and fragments of at least two different insertion sequence elements were identified at the ends of *avrA* and *avrB,* implying multiple transposition events for these areas. A DNA region homologous to a bacteriophage sequence was found upstream of *avrPto*. The linkage of various virulence/avirulence genes of animal- and plant-pathogenic bacteria with transposable elements and bacteriophage sequences, together with the presence of several of these genes on plasmids, supports the idea of horizontal transfer and frequent exchange of virulence/avirulence genes among bacterial pathogens.

Additional keywords: evolution, hypersensitive reaction, pathogenicity island, transposon, type III protein secretion.

Bacterial avirulence (*avr*) genes from pathovars of *Pseudomonas syringae* and *Xanthomonas campestris* elicit a defense response called the hypersensitive reaction (HR) in plants carrying corresponding resistance (*R*) genes (for reviews see Dangl 1994; Leach and White 1996; Vivian and Gibbon 1997). Expression of the avirulence phenotype depends on *hrp* genes, which encode a devoted regulation system and a specialized protein delivery system named the Hrp (type III) pathway (Alfano and Collmer 1996). Inactivation of *avr* genes in a bacterium can lead to virulence on resistant cultivars of the host plant, as indicated by experimental mutation and by observation of natural alleles with variant sequences or insertion elements in them (Leach and White 1996). Although most *avr* genes have been identified based on the incompatibility (avirulence) of *avr*-recipient strains with formerly susceptible host plants, *avr* genes must confer selective advantages to be maintained in bacteria. Indeed, pathogenicity/virulence functions in compatible interactions, or roles in bacterial fitness, have been demonstrated for a functional *avr* homolog in *Erwinia amylovora* (Bogdanove et al. 1998), and several *avr* genes in *P. syringae* pathovars and *Xanthomonas* spp. (Leach and White 1996).

Genes encoding useful, but not essential, traits such as antibiotic/heavy-metal resistance or virulence often are present in or near mobile genetic elements such as plasmids, transposable elements, and bacteriophages (Coplin 1989; Roberts 1996; Hacker et al. 1997; Osborn et al. 1997). Horizontal transfer of *avr* genes between related bacteria has been suggested based on the presence of some *avr* genes on plasmids (Leach and White 1996). Furthermore, inverted repeats similar to the terminal sequences of the Tn*3* family of transposons have been found at both ends of *avr* genes of *Xanthomonas* spp. in the *avrBs3/pthA* family (Leach and White 1996), and a recently described insertion sequence (IS) element, IS*1240*, is located at the 5′ region of *P. syringae* pv. *tomato avrD* separated by plasmid stability genes (Hanekamp et al. 1997).

To investigate the possibility that the linkage of *avr* genes with mobile elements is a general phenomenon, we analyzed the sequences flanking published *avr* genes of *P. syringae* (Table 1), to determine whether they contain transposable elements or other mobile DNA sequences. Deposited sequences that include *avr* genes of *P. syringae* pathovars were retrieved in fall 1997 from GenBank (Benson et al. 1998; available on-line from the National Institutes of Health) with Entrez (Schuler et al. 1996). The regions flanking the *avr* open reading frames (ORFs) were subsequently examined for similar sequences with BLASTN and BLASTX (Altschul et al. 1990, 1997). Also, direct comparisons of similar sequences were made with the BESTFIT program in the GCG software package, version 7.3 (Genetics Computer Group, Madison, WI) with default parameters. From the analysis, DNA sequences similar to those of various transposable elements and bacteriophages of Proteobacteria were found in the flanking regions of *avrA, avrB,* and *avrC* of *P. syringae* pv. *glycinea, avrPphC* of *P. syringae* pv. *phaseolicola, avrRpm1* (*avrPmaA1*) of *P. syringae* pv. *maculicola, avrPpiA1* of *P. syringae* pv. *pisi*, and *avrPto* of *P. syringae* pv. *tomato* (Fig. 1), as detailed below.

avrA and IS*51*.

avrA was the first bacterial *avr* gene cloned from *P. syringae* pv. *glycinea* (Staskawicz et al. 1984). Southern hybridiza-

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tion suggested that, while the gene is present only in race 6 as a single copy, the flanking DNA exists as multiple copies in races 1, 4, 5, and 6, implying the presence of IS(s) in the *avrA*-flanking region (Staskawicz et al. 1984). Indeed, sequence analysis of *avrA* indicated that it is bordered by multiple IS-like sequences (Fig. 1). The upstream region of the *avrA* gene contains a sequence almost identical to the right end of the IS element IS*51* (Fig. 2A). IS*51* was first identified within the inactivated *iaaM* gene of *P. syringae* pv. *savastanoi* (Comai and Kosuge 1983), and related sequences have been found in T-DNA of *Agrobacterium tumefaciens* and in the *ipaH*-flanking region of the invasion plasmid of *Shigella flexneri* (Yamada et al. 1986; Venkatesan et al. 1996).

IS*1240*-related sequences and *avrA*, *avrB*, and *avrC*.

IS*1240* was recently described from a plasmid in *P. syringae* pv. *tomato* PT23, and is located in the 5′ region of *avrD* (Hanekamp et al. 1997). Immediately downstream of *avrA* of *P. syringae* pv. *glycinea* is a sequence matching DNA positioned at the 3′ region of IS*1240* (Fig. 2B). Analysis indicated that similar sequences are present in the vicinity of virulence genes on plasmids of *Salmonella* spp. and downstream of the *yopJ* gene of *Yersinia pseudotuberculosis*. As Kholodii et al. (1997) noted recently, a sequence located at the 3′ region of *avrB* (Fig. 2C) is similar to the inverted repeats of the mobile DNA remnant κγ that is situated in Tn*5041*, the mercury resistance transposon. Related sequences also are positioned at the 5′ region of *avrC* and the flanking sequences of IS*1240*.

IS801 and *avrA*, *avrB*, and *avrPphC*.

IS*801* was isolated from *P. syringae* pv. *phaseolicola* by plasmid entrapment (Romantschuk et al. 1991), and a closely related sequence has been found in the *avrD*-carrying native plasmid of *P. syringae* pv. *tomato* PT23 (Murillo and Keen 1994). Sequences analogous to IS*801* exist in the downstream regions of the indoleacetic acid and cytokinin biosynthetic genes of *P. syringae* pv. *savastanoi*, and the gene of *P. syringae* pv. *phaseolicola* encoding an ethylene-forming enzyme. From a BLASTN analysis, IS*801*-like sequences were identified in the 3′ region of *avrA*, the 5′ region of *avrB*, and the 3′ region of *avrPphC* (Figs. 1; 2D and E). *avrB* was isolated from *P. syringae* pv. *glycinea* race 0, and is homologous to *avrC* from the same race (Staskawicz et al. 1987). Consistent with the presence of a sequence homologous to IS*801*, multiple hybridizing bands were detected in the 5′ region of *avrB* by Southern analysis (Staskawicz et al. 1987). *avrPphC* of *P. syringae* pv. *phaseolicola* is an *avrD*-linked allele of *P. syringae* pv. *glycinea avrC*, and its sequence is highly similar (99% identity at the nucleotide level) to that of *avrC* (Yucel et al. 1994b).

***avrRpm1* and *avrPpiA1*.**

avrRpm1 of *P. syringae* pv. *maculicola* and *avrPpiA1* of *P. syringae* pv. *pisi* are allelic and almost identical in sequence (Dangl et al. 1992). Like Tn*5041*, Tn*501* is a "Tn*3* family" transposon, which confers mercury resistance, and was originally isolated from *P. aeruginosa* (Brown et al. 1985). The 5′ regions of these *avr* genes contain sequences similar to terminal repeats of Tn*501* (Fig. 2F). However, since the published sequences of *avrRpm1* and *avrPpiA1* appear to contain only a part of the right inverted repeat of Tn*501* and the similarities are accordingly marginal (from a gapped BLASTN search, $P = 0.17$), further sequence information on the bordering regions of *avrRpm1* and *avrPpiA1* is necessary to determine whether they are parts of transposons.

***avrPto* and bacteriophage HP1.**

avrPto of *P. syringae* pv. *tomato* is an *avr* gene corresponding to the *Pto* resistance gene of tomato (Ronald et al.

Table 1. Avirulence genes of *Pseudomonas syringae* pathovars examined

Name	Source pathovar	GenBank accession no.	Sequenced nucleotides flanking *avr* gene (no.)		Association with mobile elements[b]
			5′ region[a]	3′ region	
avrA	*glycinea*	M15194	462 (99)	223	Yes; T
avrB	*glycinea*	M21965	476 (344)	829	Yes; T
avrC	*glycinea*	M22219	591 (340)	928	Yes; P/T
avrD	*glycinea*	J03682	140 (28)	2048	Yes; P
avrD	*lachrymans*	L11334	156 (34)	0	Yes; P
avrD	*lachrymans*	L11335	139 (18)	0	Yes; P
avrD	*phaseolicola*	L11336	175 (53)	0	Yes; P
avrPphB	*phaseolicola*	M86401	205 (107)	392	No
avrPphC	*phaseolicola*	U10377	166 (0)	63	Yes; P/T
avrPphE	*phaseolicola*	U16817	–[c]	102	No
avrPpiA	*pisi*	X67807	191 (130)	111	Yes; P/T
avrPpiB	*pisi*	X84843	315 (250)	271	Yes; P
avrRps4	*pisi*	L43559[d]	498 (385)	78	Yes; P
avrRpm1	*maculicola*	X67808	190 (130)	111	Yes; P/T
avrD	*tomato*	J03681	150 (28)	4,500	Yes; P
avrE	*tomato*	U16118	404 (307)	0	No
		U16119	584	0	No
avrPto	*tomato*	L20425	438 (371)	181	Yes; B
avrRpt2	*tomato*	L11355	171 (110)	552	No
hrmA	*syringae*	U96179	705 (204)	–[c]	No

[a] Numbers in parentheses are numbers of nucleotides counted from the first conserved G in the "*hrp* box" (Xiao and Hutcheson 1994).
[b] P, plasmid; T, transposable element; B, bacteriophage.
[c] The *hrpY* gene was found in the 5′ region of *avrPphE* (Mansfield et al. 1994), and the *hrpK* gene in the 3′ region of *hrmA* (Heu and Hutcheson 1993). The homologous *hrpY* and *hrpK* genes mark one end of the *P. syringae hrp* gene cluster.
[d] The sequence described in Hinsch and Staskawicz (1996) was used since the sequence in the data base contains only a part of the coding region.

1992). In the 5′ region of *avrPto*, we detected an incomplete ORF that encodes a potential protein with significant similarity (50% similar and 30% identical residues over 104 amino acids without gaps from a comparison by BESTFIT) to ORF35, the product of the last ORF of bacteriophage HP1 of *Haemophilus influenzae* (Esposito et al. 1996). This suggests the presence of phage DNA flanking *avrPto* (Fig. 3). In animal-pathogenic bacteria, bacteriophages have been postulated to be involved in the transfer of pathogenicity islands (Hacker et al. 1997) and genes encoding proteins secreted by the type III protein secretion system (for example, see Hardt et al. 1998).

avrD.

avrD is a plasmid-borne *avr* gene originally isolated from the PT23 strain of *P. syringae* pv. *tomato* (Kobayashi et al. 1989). Four alleles of *avrD* have been isolated from *P. syringae* pvs. *glycinea*, *lachrymans*, and *phaseolicola* (Yucel et al. 1994a). No IS-element-like sequences were identified at the immediate 3′ regions of *avrD* ORFs from *P. syringae* pvs. *tomato* and *glycinea* in the data base, and the regions were occupied by homologs of phosphoglycerate mutases. However, considerable variation in restriction patterns was observed at the regions surrounding highly conserved *avrD* alleles and immediately flanking regions (Keith et al. 1997), and *avrD* of *P. syringae* pv. *tomato* is indirectly linked to IS*1240* through plasmid stability genes present in between (Hanekamp et al. 1997). This suggests that *avrD* may have experienced multiple relocation.

Other *P. syringae avr* genes.

There was no indication that IS elements or phage DNA are directly linked to *avrPphB* (*avrPph3*), *avrPpiB*, *avrRpt2*, or *avrRps4*. For *avrRps4*, a BLASTX analysis of the published 5′

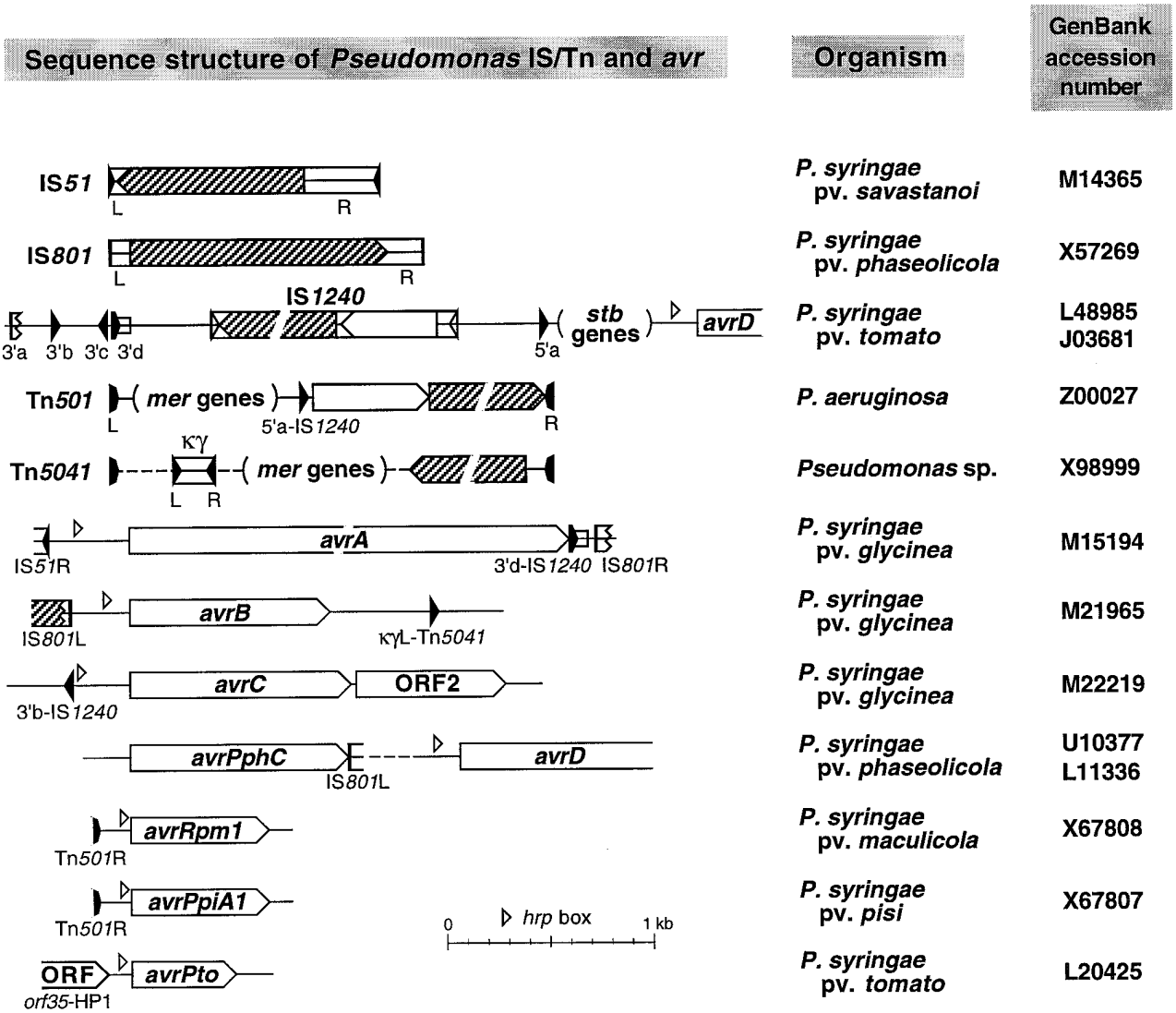

Fig. 1. Diagrammatic overview of published sequences of *Pseudomonas syringae avr* genes that contain DNA similar to transposable elements and bacteriophage DNA. Transposable elements of *P. syringae* and related bacteria shown at top. Open reading frames (ORFs) indicated by arrow boxes; open vertical triangles in front of *avr* ORFs represent "*hrp* box" sequences (Xiao and Hutcheson 1994). Names of closest related sequence from data base search of the *avr*-flanking regions are indicated below each symbol in *avr*-containing sequences. Locations of *avrD* in relation to *avrPphC* and IS*1240* are based on Yucel et al. (1994b) and Hanekamp et al. (1997), respectively.

region of *avrRps4* (Hinsch and Staskawicz 1996) indicated the presence of a sequence that has a weak similarity to a protein encoded by IS*136* of *A. tumefaciens*, but the region of similarity was too short (35% identities over 34 amino acids) and not statistically significant enough ($P = 0.9997$) to be suggestive of homology. In addition, no evidence for mobile elements was observed for *hrp*-linked *avr* genes *avrE*, *avrPphE*, and *hrmA*. However, a recent analysis of the DNA flanking one end of the *hrp* gene clusters of *P. syringae* pvs. *syringae* and *tomato* identified several different mobile DNA elements and homologs of *avrB*, *avrPphE*, and *avrRxv* within a hypervariable region flanking the *hrp* cluster (J. R. Alfano, A. O. Charkowski, and A. Collmer, *in preparation*).

avrRpt2 is an *avr* gene of *P. syringae* pv. *tomato* that corresponds to a resistance gene of *Arabidopsis thaliana, RPS2* (Dong et al. 1991; Whalen et al. 1991). Our analysis of the 3′ region of *avrRpt2* (Innes et al. 1993) predicted an incomplete ORF encoding a protein that shares significant similarities with proteins of the widely conserved SNF2 family (Eisen et al. 1995). Similarly, a partial ORF that could encode a homolog of DEAD-box proteins can be identified from the 5′ region of *avrRxv* of *X. campestris* pv. *vesicatoria* (Whalen et al. 1993). The implication of the linkage of *avrRpt2* and *avrRxv* to genes encoding proteins that are involved in various cellular processes involving protein-DNA interaction is currently unclear.

Overall, our analyses indicated that six out of 19 *avr*-containing sequences of *P. syringae* contain DNA related to transposable elements and one contains DNA related to a bacteriophage. Therefore, among the 19 *P. syringae avr* genes whose sequences are known, 14 (more than 70%) are associated with plasmids, transposable elements, or bacteriophages (Table 1). For the five *avr* genes that apparently are not directly associated with mobile elements, additional mobile elements might be revealed when more flanking sequence becomes available and more mobile elements of *P. syringae* are identified. Interestingly, most of the *P. syringae avr* genes we examined, except for the *hrp*-linked *avr* genes, have significantly lower G+C contents (40 to 52%) than those of the *P. syringae* genome (59 to 61%; Krieg and Holt, 1984) or *P. syringae hrp* genes (61% on average for conserved *hrp* secretion genes *hrcC*, *hrcJ*, *hrcV*, *hrcN*, *hrcQ*$_A$, *hrcQ*$_B$, and *hrcR-U*), raising the possibility that those *avr* genes were introduced into *P. syringae* relatively recently from low G+C bacteria or other organisms.

Several *avr* genes, including *avrC*, *avrPphC*, and *avrRpm1*, are plasmid-borne; others, such as *avrB* and *avrPto*, are chromosomal (Vivian and Gibbon 1997). Furthermore, *avrD* of *P. syringae* pv. *glycinea* and *avrPpiA1* of *P. syringae* pv. *pisi* may be located either on a plasmid or on the chromosome, depending on the strain (Keith et al. 1997; Gibbon et al. 1997). This discrepancy in location of *avrPpiA1* can be explained easily if we assume that *avr* genes "hop" from place to place along with transposable elements or phage DNA. It seems likely that, along with indigenous conjugative plasmids, several active IS elements, including IS*51*, IS*801*, IS*1240*, and their derivatives, have specifically contributed to the distribu-

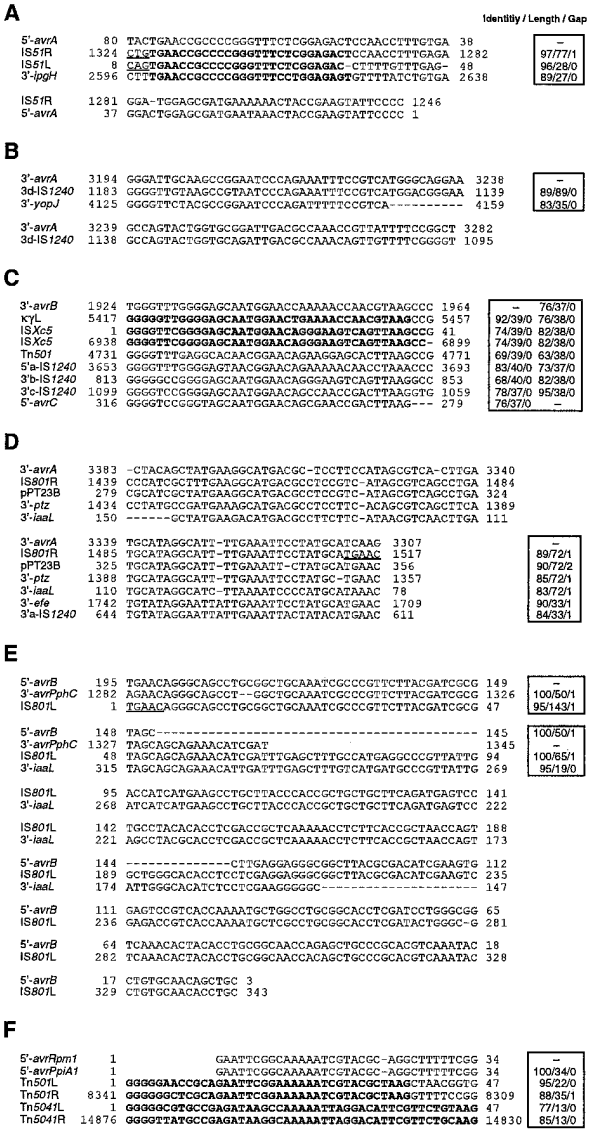

Fig. 2. Alignments of the nucleotide sequences at the 5′- or 3′-flanking regions of *Pseudomonas syringae* **(A,B,D)** *avrA;* **(C)** *avrB* and *avrC;* **(E)** *avrB* and *avrPphC;* and **(F)** *avrRpm1* and *avrPpiA1* with sequences of various transposable elements and virulence genes. Known target sequences of insertions sequences (ISs) are underlined; repeat sequences are bold-face. Dashes in alignments indicate gaps. Base numbers of sequences in the alignments are the same as those assigned in GenBank. Numbers inside the box are percent identity, length of similar region, and number of gaps, compared with reference *avr* sequence indicated by dash. Sequences were compared with the BESTFIT program with default parameters (gap weight = 5.0; gap length weight = 0.3). See Figure 1 for GenBank accession numbers and other information for *avr* sequences and transposable elements. Accession numbers of other sequences are as follows: *ipgH*, U28354; *yopJ*, L33833; *iaaL*, X63466; *ptz*, X03679; *efe*, D13182; and IS*Xc5*, Z73593. pPT23B sequence is from Murillo and Keen (1994).

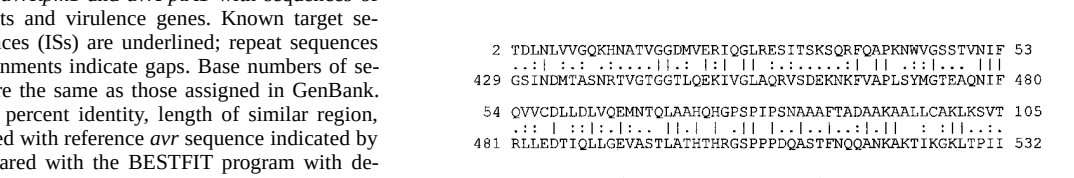

Fig. 3. Alignment of translation product of an incomplete open reading frame (ORF) at the 5′ region of *avrPto* (top) with the *orf35* product of the phage HP1 (bottom; accession number P51739). BESTFIT with default parameters (gap penalty = 3.0; gap-length penalty = 0.1) was used to align the sequences. Vertical lines indicate identical residues; single and double dots indicate conserved residues.

tion of *avr* genes among strains of *P. syringae*. The presence of sequences corresponding to fragments of several different IS elements at the flanking regions of *avrA* and *avrB* of *P. syringae* pv. *glycinea* implies that these genes may have undergone multiple transposition events. It is interesting to note that three out of four *avr* genes of *P. syringae* that show virulence functions, *avrA*, *avrB*, *avrE*, and *avrRpm1* (Lorang et al. 1994; Ashfield et al. 1995; Ritter and Dangl 1995), are flanked by sequences related to transposable elements.

In addition to their ability to carry virulence-associated genes, transposable elements may contribute to the evolution of new virulence genes by gene duplication and to inactivation of virulence genes that have become a liability for a given bacterial strain due to *R*-gene recognition. Two alleles of *avrD*, which exist in *P. syringae* pv. *lachrymans*, may be the result of the action of IS*1240* or other transposable elements (Yucel et al. 1994a). Also, an IS element, IS*476*, has been identified inside the inactivated *avrBs1* gene of spontaneous mutants of *X. campestris* pv. *vesicatoria* that had lost avirulence toward *Bs1*-carrying pepper cultivars (Kearney et al. 1988).

Plants and plant-pathogenic bacteria have coevolved during a long period of coexistence and interaction. A recent model (Alfano and Collmer 1996) states that the proliferation and variable distribution of bacterial virulence/avirulence genes among strains of a given taxon might have resulted from two driving forces. First, changes in plant targets (receptors) of virulence genes would reduce the parasitic ability of the pathogen. Second, the plant *R* gene that recognizes a virulence gene would abolish the value of the corresponding virulence gene. Thus, a successful pathogen would have evolved multiple virulence genes. The finding of the association of bacterial *avr* genes with DNA endowed with mobility is consistent with the model, and provides a basis for the rapid emergence of new strains by acquisition of virulence genes that would contribute to parasitism or by elimination of genes that have become liabilities.

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