Novel Exchangeable Effector Loci Associated with the *Pseudomonas syringae* *hrp* Pathogenicity Island: Evidence for Integron-Like Assembly from Transposed Gene Cassettes

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Pseudomonas syringae strains use a type III secretion system (TTSS) to translocate effector proteins that assist in the parasitism of host plant cells. Some genes for effector proteins are clustered in the exchangeable effector locus (EEL) associated with the *hrp* pathogenicity island. A polymerase chain reaction-based screen was developed to amplify the EEL from *P. syringae* strains. Of the 86 strains screened, the EEL was successfully amplified from 30 predominantly North American *P. syringae* pv. *syringae* strains using *hrpK* and *queA*-derived primers and from an additional three strains using *hrpL* and *queA*-derived primers. Among the amplified EEL, ten distinct types of EEL were identified that could be classified into six families distinguishable by genetic composition, but other types of EEL may be present in strains isolated in other geographical regions. No linkage with the host range of the source strain was apparent. Gene cassettes carrying conserved flanking, coding, and intergenic sequences, present in different combinations, were identified in the characterized EEL. Six new alleles of known effectors were identified that differed from the homolog in sequence, size, or both of the gene. One of these apparently novel effector proteins, HopPsyB, retained a strongly conserved amino terminus similar to that of HopPsyA, but other regions of the two polypeptides were only weakly similar. *hopPsyB* was expressed from an apparent operon that included *hrpK* and a *shcA* homolog, *shcB*. *Escherichia coli* MC4100 expressing the *hrp* TTSS, *ShcB*, and HopPsyB elicited the hypersensitive response (HR) in tobacco, consistent with effector production. Indicative of translocation as an effector, *P. syringae* pv. tomato DC3000 expressing a HopPsyB':AvrRpt2 fusion elicited the HR in RPS2⁺ *Arabidopsis thaliana*. *P. syringae* pv. tomato DC3000 carrying HopPsyB exhibited slightly enhanced virulence in several *Brassica* spp. These results are consistent with the hypotheses that the EEL is a source of disparate effectors functioning in pathogenicity of *P. syringae* strains and that it evolved independently of the *hrp* pathogenicity island central conserved region, most likely through integron-like assembly of transposed gene cassettes.

The pathogenicity of *Pseudomonas syringae* strains has been linked to activities encoded by the *hrp* pathogenicity island (PAI) (Alfano et al. 2000; Hutcheson 1999). This tripartite PAI is composed of an exchangeable effector locus (EEL), a conserved effector locus (CEL), and a central conserved region (CCR) (Alfano et al. 2000) that expresses the *hrp*-encoded type III secretion system (TTSS). The *hrp*-encoded TTSS is required for the translocation of effector proteins, known as Avr, Vir, or Hop proteins, into the cytoplasm of host plant cells (Gopalan et al. 1996; Leister et al. 1996; Mudgett and Staskawicz 1999; Pirhonen et al. 1996). In susceptible plants, the translocated effectors suppress or modify the cellular defense responses of the host (Chen et al. 2000) or alter the physiology of the host cell to favor growth of the pathogen (Dangl and Jones 2001; Hutcheson 2001). In resistant plants, one or more of the effectors elicit the programmed cell death associated with hypersensitive response (HR) (Dangl and Jones 2001; Hutcheson 2001). Translocated effectors are, therefore, essential for the virulence of the strains and control host range by initiating cellular defense responses in resistant plants. Since strains of *P. syringae* frequently have distinct host ranges, these strains likely differ in the effectors they translocate into host cells. Conversely, strains with similar host ranges are thought to retain a subset of translocated effectors that are necessary for virulence in a specific host. Therefore, to understand the pathogenicity and host range of a given *P. syringae* strain, it is important to identify the effectors that are expressed and translocated by that strain.

Several methods have been used to survey *P. syringae* strains for translocated effectors. Most *avr* genes have been identified by screening a genomic library of one strain for genes that affect the host range of another strain in a 'gene-for-gene' manner (Leach and White 1996; Staskawicz et al. 1984; Vivian and Gibbon 1997). The analysis of the *P. syringae* pv. tomato DC3000 genome has revealed at least 51 candidate or known effector proteins (Collmer et al. 2002; Fouts et al. 2002; Petnicki-Ocweija et al. 2002), many of which share sequence similarity with effectors originally identified in other strains through phenotypic screens. In each case, these effectors appear either to be expressed from HrpL-dependent promoters (Fouts et al. 2002), to carry apparent TTSS secretion signals (Guttman and Greenberg 2001; Guttman et al. 2002; Petnicki-Ocweija et al. 2002), or both. Another approach has been to screen the genome of a strain for translocated proteins by using randomly generated fusions to an '*avrRpt2*' cassette that requires TTSS-dependent translocation for activity in RPS2 lines of *Arabidopsis thaliana* (Guttman et al. 2002). In some cases,

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culture filtrates have been used to identify secreted proteins (Mudgett and Staskawicz 1999; van Dijk et al. 1999; Yuan and He 1996). Unfortunately, most *P. syringae* strains secrete TTSS effectors into liquid media at levels below the threshold of detection (Alfano and Collmer 1997; Hutcheson 1999; Li et al. 1992). The results of these screens indicate that effector genes can be dispersed throughout the genome (Kim et al. 1998), clustered in plasmid-borne 'pathogenicity islands' (Jackson et al. 1999), or associated with the *hrp* PAI in either the EEL or CEL (Alfano et al. 2000; Hutcheson 1999).

Although the effectors encoded by the EEL are unlikely to be solely responsible for a strain's host range, the EEL appears to be a highly variable source for effectors (Alfano et al. 2000; Hutcheson 1999). Each strain characterized thus far contains a distinct EEL that encodes at least one demonstrated effector (Alfano et al. 2000; Heu and Hutcheson 1993; Mansfield et al. 1994; Petnicki-Ocweija et al. 2002). In *P. syringae* pv. *syringae* 61, the EEL consists of *hopPsyA* (also known as *hrmA*), which encodes a known TTSS-dependent effector (Alfano et al. 1997; Heu and Hutcheson 1993), and *shcA*, producing a chaperone for HopPsyA (van Dijk et al. 2002). The EEL of *P. syringae* pv. *syringae* B728A includes six genes, previously named *orf1* through *orf6*, three of which encode potential translocated effectors (Alfano et al. 2000). *P. syringae* pv. *syringae* B728A HopPsyC product shares weak sequence similarity with AvrPphC; *hopPsyE* expresses a homolog of AvrPphE; and the HopPsyV product exhibits sequence similarity to AvrXct (Alfano et al. 2000). The *P. syringae* pv. tomato DC3000 EEL consists of five genes previously designated *orf1* through *orf5* that share little sequence similarity with the genes of the *P. syringae* pv. *syringae* 61 or *P. syringae* pv. *syringae* B728A EEL (Alfano et al. 2000). *orf1* of the *P. syringae* pv. tomato DC3000 EEL encodes the effector HopPtoB (Fouts et al. 2002; Petnicki-Ocweija et al. 2002). The functions of the other genes in the *P. syringae* pv. tomato DC3000 EEL have not been reported.

During a promoter trap screen used to isolate HrpL-dependent promoters from *P. syringae* strains (Hutcheson et al. 2003),

a 2-kb fragment was identified in a genomic library of *P. syringae* pv. *syringae* 61 that carries the HrpL-dependent promoter for the *shcA-hopPsyA* operon. Sequence analysis of this region revealed a homolog of *queA* and a tRNA_{leu} gene, as reported at the time for *P. syringae* pv. *syringae* B728A and *P. syringae* pv. tomato DC3000 (Alfano et al. 2000; A. Collmer, *personal communication*), and subsequently for *P. syringae* pv. *syringae* 61 (Alfano et al. 2000). *queA* is a housekeeping gene involved in the modification of tRNA nucleoside 34 (Reuter et al. 1991) and is not functionally linked to the TTSS, whereas *hrpK* is thought to be a component of the *hrp* PAI CCR (Alfano et al. 2000). The presence of these conserved flanking genes adjacent to the EEL introduced the possibility of using polymerase chain reaction (PCR) to amplify the EEL from various *P. syringae* strains for identification of the resident genes. This report describes the properties of the EEL and effectors amplified from 30 strains isolated mostly from North American sources. (A preliminary report of this work has been presented [Hutcheson et al. 2003]).

RESULTS

Development of a PCR screen for the *P. syringae* EEL.

In order to amplify the EEL from *P. syringae* strains, several primers were constructed, using regions within *queA* and *hrpK* that were conserved among alleles found in the previously characterized strains. Different combinations of these primer pairs were then tested for their ability to amplify previously characterized EEL, using commercial long-range PCR amplification kits. A primer pair was identified (K2688 and Q920) that could amplify the EEL from *P. syringae* pv. *syringae* 61, *P. syringae* pv. *syringae* B728A and *P. syringae* pv. tomato DC3000. The expected fragments were detected (Fig. 1) and were identified as EEL by sequence analysis of flanking regions (data not shown). Consistent with the findings of Mansfield and associates (1994), a sequencing error was detected in the originally reported coding sequence for *P. syringae* pv. *syringae* 61 *hrpK* (Xiao et al. 1994). The corrected deduced product of *hrpK* is a 767-aa polypeptide with a predicted mass of 80,070 kDa.

Characterization of *hrpK*-associated EEL amplified from *P. syringae* strains.

Using the primer pair described above, we attempted to amplify the EEL from 86 *P. syringae* strains, using commercial long range PCR amplification kits, and were successful for 30 predominantly North American *P. syringae* pv. *syringae* strains isolated from a variety of host plants (Table 1). These amplified EEL were initially classified based on size of the amplified fragment, the nucleotide sequence adjacent to *hrpK* and *queA*, and in some cases, the amplification of diagnostic internal fragments. The complete nucleotide sequence was then obtained for at least one representative of each novel type of EEL (Table 1).

Apparent homologs to the EEL of *P. syringae* pv. *syringae* 61, *P. syringae* pv. *syringae* B728A, and *P. syringae* pv. tomato DC3000 were detected among the amplified EEL, along with several variants of these EEL. Several previously unreported types of EEL differing from the previously characterized EEL in genetic composition and nucleotide sequence (Fig. 2) were identified among the characterized EEL. These EEL could be further classified into six apparent families that could be distinguished by constituent genes. Strains with the same type of EEL (Table 1) retained >98% identity across sequenced portions of the region. Overall, 10 distinct types of EEL were detected among the amplified EEL. Representative strains for each type of EEL are indicated in Table 1.

For several of the strains that were recalcitrant to amplification of the EEL using the *hrpK*- and *queA*- derived primers, the

Fig. 1. The exchangeable effector locus (EEL) amplified from *Pseudomonas syringae* strains. Polymerase chain reaction amplification of the EEL from **A**, *P. syringae* pv. *syringae* 61 **B**, *P. syringae* pv. tomato DC3000, and **C**, *P. syringae* pv. *syringae* B728A employed primers Q920 and K2648 and ProofPro polymerase. Whole cells were used as the template. The manufacturer's recommended conditions for long-range amplification and 10-min extension reaction were used after a 60°C annealing step. After 35 cycles, amplified fragments were fractionated by agarose gel electrophoresis and stained with ethidium bromide. The amplified fragments were gel-purified, and the nucleotide sequence obtained. The nucleotide sequences obtained were identical to the corresponding regions of the respective EEL reported in GenBank.

attempt was made to amplify the EEL using compatible *hrpL*- and *queA*-derived primers designed using conserved regions of each gene. The EEL of three additional strains were amplified, which, upon characterization as described above, were homologous to one of the EEL amplified in the previous survey. The reason for the inability to amplify the EEL from the other strains has not been determined, but it may be that other types of EEL are carried by these strains, that sequence divergence at the primer sites prevented amplification, or that *queA* and *hrpK/hrpL* are not closely linked in these strains.

Family I EEL. Sixteen strains were found to carry an EEL related to that of *P. syringae* pv. *syringae* 61, henceforth designated Family I EEL (Fig. 2). In eight strains (Table 1), this region was highly conserved (Fig. 2IA). The fragments amplified from these strains were the same size as the fragment amplified from *P. syringae* pv. *syringae* 61 (Table 1) and the nucleotide sequence of the entire region exhibited >98% identity with the *P. syringae* pv. *syringae* 61 EEL. Strongly conserved homologs of the *P. syringae* pv. *syringae* 61 ShcA and HopPsyA were detected (>98% aa identity), and the genetic organization of the locus was conserved relative to the EEL of *P. syringae* pv. *syringae* 61.

The EEL of eight additional strains (Table 1) only carried partial homologs to *P. syringae* pv. *syringae* 61 ShcA and

HopPsyA (Fig. 2IB). The orientation of the two genes was reversed relative to the EEL of *P. syringae* pv. *syringae* 61, and they appeared to be expressed as part of the *hrpK* operon. Notably, the apparent translational start of the first open reading frame (ORF), a *shcA* homolog designated *shcB1*, overlapped the stop codon of *hrpK*, and the stop codon of *shcB1* was immediately followed by the translational start of a second ORF, a weak homolog of *hopPsyA* designated *hopPsyB1*. Differences were also detected in gene products. For example, the deduced *P. syringae* pv. *syringae* B5 ShcB1 product was 65 aa longer at the amino terminus than *P. syringae* pv. *syringae* 61 ShcA and had a predicted mass of 19.5 kDa (Fig. 3A). Three minor sequence variants were detected. Overall, the deduced ShcB1 product shared 56% identity (I) and 69% similarity (S) with *P. syringae* pv. *syringae* 61 ShcA. The *P. syringae* pv. *syringae* B5 HopPsyB1 encoded a 382-aa polypeptide with a predicted mass of 41.9 kDa (Fig. 3B). The amino terminal 44-aa domain retained 68% I and 82% S with the amino terminus of HopPsyA. This domain, which likely carries the TTSS secretion signal (Mudgett and Staskawicz 1999), was serine rich, as observed in other *P. syringae* TTSS-dependent effectors (Guttman et al. 2002). It did not, however, fit many of the criteria proposed by Petnicki-Ocwieja and associates (2002) for TTSS translocated effectors. For example, the deduced

Table 1. Properties of the *Pseudomonas syringae* strains used in these experiments and the amplified exchangeable effector locus (EEL)

Strain ^{a,b}	Plant source ^c	Geographical location ^d	Amplified PCR product ^e	EEL type ^f	Accession no.
Psy-B76	Tomato	GA	4.0	IA	
Psy-B458	Orange	CA	4.0	IA	
Psy-PDDCC3523	Tomato	AU	4.0	IA	
Psy-PS-1	Peach	NC	4.0	IA	
Psy-PS-14	Apricot	NC	4.0	IA	
Psy-Ps6-SB	Soybean	NE	4.0	IA	
Psy-84-15	Tomato	GA	4.0	IA	
Psy-85-274	Tomato	GA	4.0	IA	
Psy-B5	Soybean	MC	3.5	IB	AY147022
Psy-B6	Soybean	MC	3.5	IB	AY147023
Psy-464	Corn	SD	3.5	IB	AY147017
Psy-B301D	Pear	UK	3.5	IB	AY147020
Psy-BK034	Wheat	MN	3.5	IB	AY147024
Psy-PDDCC3907	Pear	GR	3.5	IB	AY147027
Psm-567	Cherry	UK	3.5	IB	
Psy-5D4198	Plum	CA	8.0	IC	AY147019
Pph-BK378	Bean	MN	4.0	II	AY147025
Pph-B130	Bean	GA	4.0	II	
Psy-B362	Bean	CA	8.5	IIIA	
Psy-Ps1Bean	Bean	WA	8.5	IIIA	
Psy-B452	Orange	CA	5.5	IIIB	AY147021
Psy-B460	Lemon	CA	5.5	IIIB	
Psy-BK035	Wild Rice	MN	5.5	IIIB	
Psy-W4N15	Apple	WA	5.5	IV	AY147028
Psy-W4N27	Pear	OR	5.5	IV	
Psy-NCPPB1053	Millet	ET	7.5	VA	
Pto-2844	Tomato	UK	7.5	VA	
Ppe-5846	Peach	FR	6.0	VB	AY147018
Psy-DH015	Bean	WI	5.0	VI	AY147026

^a See Denny et al. 1988 for references for the listed strains. Abbreviations: Pmo, *P. syringae* pv. morsprunorum; Ppe, *P. syringae* pv. persicae; Pph, *P. syringae* pv. phaseolicola; Psy, *P. syringae* pv. *syringae*; Pto, *P. syringae* pv. *tomato*. Representative strains used for each type of EEL are shown in bold. Psy-61, Psy-B728A and Pto-DC3000 were used as the representative strains for type IA, IIIA, and VA EEL.

^b The following strains were negative for PCR amplification of the EEL using Q920 and K2468: Psy-480, *P. syringae* pv. delphinii-529, Pto-2424, *P. syringae* pv. berberidis-4116, *P. syringae* pv. antirrhini-4303, *P. syringae* pv. savastanoi-4352, Pmo-5795, *P. syringae* pv. maculicola (Pma)-#1, Pma-#5, Pma-#10, Pma-1083-3, Psy-5D417, Pto-832F, Pto-B117, Pto-B118, Pto-B120, Pto-B121, Pto-B122, Pto-B125, Psy-B15, Pto-B19, Psy-B359, Psy-B368, Psy-B382, Psy-B3A, Psy-B407, Psy-B427, Psy-B455, Pto-B67, Pto-B88, Psy-BK036, Pto-CNBPI323, Pto-DAR26742, Pto-30555, Pto-DAR31861, Psy-HS191, Pto-JL1053, Pto-JL1060, Pto-JL1075, Pto-JL1105, Pto-JL1120, Pto-NCPPB880, Pph-NK343, Pto-PDDCC 3357, Pto-PDDCC 4355, Pto-PT14, Pto-PT21, Pto-PT30, Pto-RG-4, Psy-S-4B-1, Psy-SD19, Pto-T1, Pto-T4B1, Psy-W4N108, Psy-W4N43.

^c Host plant from which the strain was isolated from or reported to cause symptoms.

^d Geographical region where the strain was isolated. Abbreviations: AU, Australia; CA, California, U.S.A.; ET, Ethiopia; FR, France; GA, Georgia, U.S.A.; GR, Greece; MC, Manitoba, Canada; MN, Minnesota, U.S.A.; NC, North Carolina, U.S.A.; NE, Nebraska, U.S.A.; OR, Oregon, U.S.A.; SD, South Dakota, U.S.A.; UK, United Kingdom; WA, Washington, U.S.A.; WI, Wisconsin, U.S.A.

^e The indicated strain was used to initiate PCR amplification of the EEL using primers Q920 and K2468 as described in the Materials and Methods. The size indicated was determined by agarose gel electrophoresis and represents the amplified EEL plus 1.5 kb of sequence internal to *queA* and *hrpK*.

^f Genetic organization of EEL families is shown in Figure 2. Placement into the indicated families was based upon sequence similarities.

HopPsyB1 product had hydrophobic amino acids at positions 3 and 4, carried a glutamic acid at position 12, and had multiple cysteines in the polypeptide. However, other translocated effectors, such as HopPsyA, also displayed these characteristics (data not shown). Overall, the HopPsyB1 polypeptide exhibited 31% I and 45% S with HopPsyA and required several gaps to align with HopPsyA. This suggests that the effector activity of the two proteins may be different. Since the EEL of these strains appeared to be an inversion of a *P. syringae* pv. *syringae* 61-like EEL, they were retained as a Family I EEL.

A third variant of the Family I EEL was identified in strain *P. syringae* pv. *syringae* 5D4198. This strain retained the *P. syringae* pv. *syringae* B5-like ShcB and HopPsyB alleles (>97% aa identity) but carried three additional genes, *eelA*, *eelB*, and *eelC*, immediately downstream of the *hopPsyB2* allele, that have unknown functions (Fig. 2IC). *EelA* encoded a 256-aa polypeptide with a predicted mass of 30 kDa. *EelB* is predicted to be a 243-aa product with a mass of 27.2 kDa, whereas *EelC* is a 545-aa polypeptide with a predicted mass of 62 kDa. Neither homologs nor conserved functional domains were detected in the databases for any of the three proteins. No obvious secretion signals were apparent. A partial duplication of the tRNA_{leu} gene was detected, suggesting that *eelA* and *eelBC* are part of a 4-kb insertion into the tRNA gene. Consistent with this interpretation, the nucleotide sequence of the region between the truncated tRNA_{leu} gene and *P. syringae* pv. *syringae* 5D4198 *hopPsyB2* retained 95% identity with the corresponding region of the *P. syringae* pv. *syringae* B5 EEL. No obvious HrpL-dependent promoters were detected in the apparent promoter region between the divergently expressed *P. syringae* pv. *syringae* 5D4198 *eelA* and *eelBC*, suggesting that neither are part of the HrpL-dependent regulon. *eelBC* likely form an operon, since they are expressed collinearly, and no obvious transcriptional terminators were detected in the intergenic region between them.

Family II EEL. The EEL of *P. syringae* pv. *phaseolicola* BK378 and *P. syringae* pv. *phaseolicola* B130 consisted of two genes, one of which was a strong homolog to the AvrPphE allele originally described for *P. syringae* pv. *phaseolicola* 1032A (race 4) (Mansfield et al. 1994)(Fig. 2). This EEL amplified as a 4-kb fragment (Table 1), and the nucleotide sequence of the *P. syringae* pv. *phaseolicola* BK378 *avrPphE* homolog retained >98% identity with the reported sequence for *avrPphE* of *P. syringae* pv. *phaseolicola* 1032A. Residues G191, W235, and E310, which have been reported to be essential for R2-specific 'avirulence' activity (Stevens et al. 1998), were conserved in this allele, but the *P. syringae* pv. *phaseolicola* BK378 AvrPphE homolog was 60 aa longer than its 1032A homolog. This carboxy terminal extension was nearly identical to the carboxy terminal extension present in *avrPphE8* allele (Stevens et al. 1998), and this designation was retained. The 95-bp *P. syringae* pv. *phaseolicola* BK378 *hrpK-avrPphE8* intergenic region exhibited little sequence similarity with the corresponding regions of Family III and IV EEL, which also carried alleles of AvrPphE (less than 41% identity with either the *hopPsyC1-avrPphE* or *hrpK- hopPsyC1* intergenic regions [data not shown]). This indicates that the genetic arrangement of Family II EEL was unlikely to be the result of a deletion event that occurred in a Family III or Family IV EEL and, conversely, that Family III and Family IV EEL did not arise due to an insertion event.

A second ORF, designated *eelF4*, was identified 154 bp downstream of the *P. syringae* pv. *phaseolicola* BK378 and *P. syringae* pv. *phaseolicola* B130 *avrPphE* homologs that exhibited 83% I and 87% S with ORF 3 (*eelF1*) of the Family IV EEL of *P. syringae* pv. tomato DC3000 described below. *EelF4* lacked homologs of known function and was unusual because,

whereas *EelF1* consisted of six 17-aa repeats and exhibited properties suggestive of an outer membrane localization, the *EelF4* was missing the fifth repeat. Possible functions of this gene product have not yet been explored.

Homologs to *queA* and the gene for tRNA_{leu} were also detected flanking Family II EEL (Fig. 2). Although nearly identical *avrPphE* alleles had been identified in the EEL of *P. syringae* pv. *phaseolicola* 1032A (Mansfield et al. 1994; Stevens et al. 1998), the chromosomal location of the *hrp* PAI in this strain had not been established. The ability to amplify a closely related EEL from several strains carrying Family II EEL using *queA*- and *hrpK*-specific primers and the detection of *queA* and tRNA_{leu} homologs adjacent to this EEL indicated that the chromosomal location of the *hrp* PAI in strains carrying Family II EEL is identical to those strains with characterized EEL.

Family III EEL. Two strains were identified that carried a *P. syringae* pv. *syringae* B728A-like Family III EEL. The fragments amplified in the initial PCR were of similar size to the EEL amplified from *P. syringae* pv. *syringae* B728A (Fig. 1; Table 1), and the 600-nt sequence at each end of the amplified fragments exhibited strong sequence similarity (>98% identity) to the EEL of *P. syringae* pv. *syringae* B728A (data not shown). To determine if the strains carrying the *P. syringae* pv. *syringae* B728A-like EEL retained all six ORFs identified in the *P. syringae* pv. *syringae* B728A EEL, a PCR screen was used to amplify diagnostic fragments extending between adjacent genes, using gene-specific primers as described below. The expected fragments could be amplified from the EEL for each of these strains (data not shown), indicating that the genetic organization was conserved, but no attempt was made to further identify strain-specific sequence polymorphisms that could be present in these EEL. Proposed genetic designations for each of the detected genes are shown in Figure 2.

Three additional strains were identified that carried truncated versions of the Family III EEL (Fig. 2IIIB). Although the EEL of these strains retained a partial homolog of *P. syringae* pv. *syringae* B728A HopPsyC1 and the *queA*-associated sequences exhibited at least 95% identity at the nucleotide level to the corresponding region of *P. syringae* pv. *syringae* B728A, the amplified EEL was 3 kb smaller than the *P. syringae* pv. *syringae* B728A EEL (Table 1). A PCR screen similar to the one used to screen full size *P. syringae* pv. *syringae* B728A-like EEL indicated that these strains lack the *avrPphE* allele as well as the transposition-associated *eelDE* operon. Sequence analysis of the *P. syringae* pv. *syringae* B452 EEL confirmed the deletion in these genes and revealed a nearly complete deletion of the *hopPsyC* allele. Only the N-terminal 3 to 71 aa were conserved, and the truncated ORF lacked both the probable ribosome binding site and the start codon. The remaining *hrpK- hopPsyC* intergenic region, however, was nearly identical to the corresponding region of the *P. syringae* pv. *syringae* B728A EEL. Homologs of *shcV* encoding an apparent chaperone (van Dijk et al. 2002) and the *avrXct*-like *hopPsyV* were present. In *P. syringae* pv. *syringae* B452, the *hopPsyV2* allele included a frameshift mutation in codon 208 that likely inactivates effector activity. The intergenic sequences were conserved, indicating that the EEL of these strains were likely deletion derivatives of a *P. syringae* pv. *syringae* B728A-like EEL and, hence, their classification as part of this EEL family.

Family IV EEL. A fourth family of EEL was identified in *P. syringae* pv. *syringae* W4N15 (type strain) and *P. syringae* pv. *syringae* W4N27 (Table 1), which consisted of four genes (Fig. 2). The EEL of these strains contained the *hrpK-hopPsyC1-hopPsyE1* operon found in the Family III EEL and homologs to *eelF1* and *eelG2* of the Family V EEL found in *P. syringae* pv. tomato DC3000. The W4N15 HopPsyC2 retained 74% I and 80% S to the *P. syringae* pv. *syringae* B728A HopPsyC1 (ORF

1) product, both of which also exhibited weak similarity to AvrPphC. The *P. syringae* pv. *syringae* W4N15 HopPsyE2 homolog was 97% identical to the *P. syringae* pv. *syringae* B728A HopPsyE1. *P. syringae* pv. *syringae* W4N15 EelF3 and EelG3 exhibited 85% and 89% identity with *P. syringae* pv. tomato DC3000 EelF1 and EelG1.

Family V EEL. Three strains were identified whose EEL was nearly identical to the EEL described for *P. syringae* pv. tomato DC3000 (Table 1). The amplified regions from each strain were the same size as the EEL amplified from *P. syringae* pv. tomato DC3000 (type strain) and the initial sequences were nearly identical to that of the *P. syringae* pv. tomato DC3000 EEL. Similar to the experiments described for Family III EEL, diagnostic fragments linking adjacent genes could be amplified that were of similar size to those amplified from *P. syringae* pv. tomato DC3000 (data not shown), indicating that the genetic organization of these Family V EEL was likely conserved in these strains (Fig. 2). As before, no attempt was made to further identify sequence polymorphisms in these strains.

A variant of the *P. syringae* pv. tomato DC3000 EEL was identified in *P. syringae* pv. *persicae* 5846. The EEL of *P. syringae* pv. *persicae* 5846 was 2.5 kb smaller than the *P. syringae* pv. tomato DC3000 EEL (Table 1), but sequence analysis

of the region flanking *hrpK* showed high sequence identity to *P. syringae* pv. tomato DC3000 *hopPtoB*. The 5846 HopPtoB homolog, HopPtoB5, retained 99% aa sequence identity to its *P. syringae* pv. tomato DC3000 homolog over the majority of its length but contained a 138-aa C terminal deletion. Since the activity of HopPtoB has not been established, it is unclear whether *P. syringae* pv. *persicae* 5846 HopPtoB5 retains similar function to the *P. syringae* pv. tomato DC3000 HopPtoB. Nonetheless, the *P. syringae* pv. *persicae* 5846 HopPtoB5 is likely secreted by the *hrp* TTSS, since the amino termini of the two proteins are identical. Sequence analysis revealed that the deletion detected within the *P. syringae* pv. *persicae* 5846 EEL extended into the *eelG* allele. The *P. syringae* pv. *persicae* 5846 *eelG2* lacked the coding sequence for the C-terminal 53 aa of other *eelG* alleles. The remaining polypeptide retained 99% aa identity to *P. syringae* pv. tomato DC3000 EelG1. In the *P. syringae* pv. *persicae* 5846 EEL, IS53-like sequences were also detected between the 5846 *hopPtoB5* and *eelG2* (Fig. 2).

Family VI EEL. A distinct EEL was identified in *P. syringae* pv. *syringae* DH015 (Table 1). Although superficially similar to the EEL of *P. syringae* pv. tomato DC3000, the EEL of this strain was 2.5 kb smaller. Only partial sequence similarity was

Fig. 2. The exchangeable effector loci (EEL) amplified from *Pseudomonas syringae* strains. The EEL was amplified, and the complete nucleotide sequence was obtained. The EEL for strains shown in bold in Table 1 were used as representatives of each type of EEL. The *P. syringae* pv. *syringae* 61 EEL served as the representative for the type IA EEL, and the *P. syringae* pv. *syringae* B728A and *P. syringae* pv. tomato DC3000 EEL were used for type IIIA and type VA EEL, respectively. Known or deduced genes are indicated by the filled and labeled boxes. Boxes with dashed line borders indicate probable pseudogenes that lacked translational initiation signals or appeared to be gene fragments. Apparent transcription units are shown by the arrows. If no arrow is shown the organization is conserved relative to the EEL shown above. Strains sharing similar or related genetic compositions are grouped into families labeled with Roman numerals, and variants of each family are indicated by upper case letters on the left. Strains carrying the indicated variants of the EEL are shown in Table 1. Boxes filled with the same color indicate apparent gene cassettes. Apparent transposable elements are shown in purple. Deletions are indicated by the labeled bridges. The numbers above the boxes represent the percent GC for the adjacent coding sequence.

detected between the first ORF the *P. syringae* pv. *syringae* DH015 EEL, designated as *hopPsyG*, and the *P. syringae* pv. tomato DC3000 *hopPtoB* locus. In addition, the *hrpK-hopPsyG* intergenic region was larger and lacked sequence similarity to its *P. syringae* pv. tomato DC3000 counterpart. A similar result was obtained for the *hopPsyG-eelF5* intergenic region, indicating that this EEL was unlikely to be a deletion derivative of a Family V EEL. The *P. syringae* pv. *syringae* DH015 *HopPsyG* product was of similar size to the *P. syringae* pv. tomato DC3000 *HopPtoB* product but retained only 50% identity at the amino acid level. Most predicted properties of known TTSS-secreted effectors (Petnicki-Ocwiejka et al. 2002) were retained in *HopPsyG*. *EelF5* and *EelG5* showed stronger similarity to their counterparts of the *P. syringae* pv. tomato DC3000 EEL. The *P. syringae* pv. *syringae* DH015 *EelF5* shared 78% identity with *EelF1* of *P. syringae* pv. tomato DC3000, whereas the *P. syringae* pv. *syringae* DH015 *EelG5* retained 88% identity with the *P. syringae* pv. tomato DC3000 *EelG1*. The apparent Family VI EEL can be distinguished from Family V EEL by the divergent *HopPsyG* allele and its flanking intergenic regions.

Identification of gene cassettes conserved among several EEL.

Several sets of genes appeared to be conserved between EEL families. For example, alleles of *hopPsyC-hopPsyE* present in Family III EEL were also present in Family IV EEL. To determine if these genes were part of conserved gene cassettes, the nucleotide sequences of noncoding intergenic and flanking regions were compared. Within the apparent W4N15 (Family IV) *hopPsyC2-hopPsyE2* cassette, intergenic regions were highly conserved with corresponding regions of Family III EEL (Fig. 4, regions D, E, and F). For example, the 190-bp *hrpK-hopPsyC2* intergenic region (Fig. 4, region D) retained 98% identity, and the 148-bp *hopPsyC2-hopPsyE2* intergenic region (Fig. 4, region E) exhibited 92% identity with corresponding regions of the *P. syringae* pv. *syringae* B728A *hopPsyC1-hopPsyE1* cassette. This conservation extended 85 bp past the 3' end of the *hopPsyE* coding sequence (Fig. 4, region F). Thus, flanking, coding, and intergenic regions of this cassette are conserved in both types of EEL.

Similarly, convergently expressed *eelF1* and *eelG1* of Family V EEL were detected in Family II, IV, and VI EEL as part of an apparent gene cassette. Like above, the 154-bp intergenic region between the *P. syringae* pv. tomato DC3000 *eelF1* and *eelG1* (Fig. 4, region H) retained 91% identity with the corresponding regions of *P. syringae* pv. *syringae* W4N15 or *P. syringae* pv. *syringae* DH015. High identity (93%) was also observed in the 300-bp region 5' to *eelF* in strains *P. syringae* pv. *syringae* DH015 and *P. syringae* pv. *syringae* W4N15 (labeled G in Figure 4), but only 50% identity was detected to this region of *P. syringae* pv. tomato DC3000, while in other strains higher conservation was observed. The 100-bp region 5' to *eelG* (Fig. 4, region I) also exhibited >75% sequence identity in *P. syringae* pv. tomato DC3000, *P. syringae* pv. *persicae* 5846, *P. syringae* pv. *syringae* W4N15, and *P. syringae* pv. *syringae* DH015. Two modified forms of the *eelF-eelG* cassette were also detected. In the type VB EEL of strain *P. syringae* pv. *persicae* 5846, the region 5' to *eelG2* (Fig. 4, region I) retained at least 80% identity with corresponding regions of the *P. syringae* pv. tomato DC3000, *P. syringae* pv. *syringae* W4N15, and *P. syringae* pv. *syringae* DH015 EEL, but other portions of the cassette were deleted as described above.

The *eelF4* of Family II EEL also appears to have been derived from the *eelF-eelG* gene cassette. The 154-bp region 5' to *eelF4* (Fig. 4, region G) retained 85% identity with the corresponding region of the *P. syringae* pv. *syringae* DH015 and *P.*

syringae pv. *syringae* W4N15 EEL. In contrast, the 129-bp region next to the tRNA gene appeared to be a chimera between the tRNA-*eelG* intergenic region (Fig. 4, region I) and the *eelG-eelF* intergenic region (Fig. 4, region H). Approximately 36 bp adjacent to the 3' end of *eelF4* were nearly identical to the *eelF-eelG* intergenic region of *P. syringae* pv. tomato DC3000, W4N15, and DH015. The remaining 31 nt after subtraction of the generally conserved 62-bp region next to the tRNA gene exhibited strong sequence similarity to the corresponding portion of the tRNA-*eelG* region of *P. syringae* pv. tomato DC3000, *P. syringae* pv. *syringae* W4N15, and *P. syringae* pv. *syringae* DH015.

Consistent with the presence of apparent gene cassettes, the percent GC content of the EEL exhibited mosaicism. While the percent GC of other portions of the *hrp* PAI were around 58.7%, many of the genes identified in the characterized EEL were closer to 50% GC (Fig. 2). In contrast, the sequenced portions of *hrpK* were $58.8 \pm 1.2\%$ GC, and those of *queA* were $60.9 \pm 1.1\%$ GC. Thus, the percent GC of EEL genes is significantly different from other chromosomal or PAI genes. This suggests these apparent cassettes were acquired by horizontal transfer.

Analysis of *queA* and *hrpK* sequence variants.

The amplified EEL from the aforementioned strains also carried portions of *queA* and *hrpK*. To determine if there was evolutionary relationship of either gene with the adjacent gene cassettes, the partial *queA* and *hrpK* sequences were subjected to phylogenetic analyses. Insufficient sequence polymorphisms were detected in the approximately 100-bp portion of *queA* that was sequenced to use in phylogenetic analyses. The *hrpK* locus, however, was highly polymorphic, and numerous base substitutions were detected that were parsimony informative. Although the sampled sequence was relatively small (231 bp), 69 bases were variable. Using this information, two distinct clusters of *hrpK* variants were identified after minimum evolution and maximum likelihood analysis using PAUP*4.0. The *hrpK* of strains carrying a Family V EEL formed a separate cluster with strong bootstrap support (100%) from the *hrpK* of strains with other types of EEL (Fig. 5). Although some correlations were detected between resident EEL of other families and the adjacent *hrpK* allele, there was insufficient bootstrap support of the phylogenetic tree to defend the apparent correlations.

Linkage

between EEL, host range, and geographical source.

Few correlations were detected between the resident EEL and the apparent host range of strains, as expected. Strains carrying type IIIA EEL tended to be bean pathogens isolated in North America, but bean pathogens were found among the strains carrying other types of EEL (Table 1). Strains carrying Family IV EEL were apparent pathogens of Roseaceae plants; nonetheless, strains with apparently similar host ranges were found to carry other types of EEL. Some groups of EEL showed no correlation with host range. For example, included among the nine strains carrying a type IA EEL were tomato, soybean, citrus, and *Prunus* sp. pathogens (Table 1). Tomato and *Prunus* pathogens were also identified among the strains carrying type VA EEL, and citrus pathogens were also found among the strains carrying a type IIIB EEL. The inability to link the apparent host range of a strain with the resident EEL is consistent with current models that predict that multiple effectors control host range of a strain (Fouts et al. 2002; Hutcheson 1998). A possible geographical correlation was noted, however. Strains carrying Family V EEL were either isolated or derived from strains isolated in the U.K., France, or North Africa. Strains carrying other types of

EEL were predominately North American isolates. As most strains surveyed in this study were of an apparent North American origin, additional studies are required to confirm this possible geographical relationship.

The Family I Orf 2 encodes a translocated effector.

In EEL Families II to V, sequence variants of known effectors were identified that retained at least 75% sequence identity and carried strong conserved amino termini, suggesting that

A

B5	1	MQWSRAATLSSVLFRECVVLQTSMTNTPPGGEVIYRPRSFVQCSGHRGVLMTDRLYKTVLA
46	1	MQWSRAA-LSSILFRECVVLQTSMTNTPPGGEVIYRPRPFVQCSGHEGVLMTDRLYKTVLA
5D4198	1	MQWSRAAALSSILFRECVVLQTSMTNTPPGGEVIYRPRPFVQCSGHRGVLMTDRLYKTVLA
ShcA	1	-----
B5	61	DLSTSLTMQPLMFDDTGACDVVVDEEIALKVVVDP-VFORLLLIIGLMDISPDLPQRLLS
464	60	DLSTSLTMQPLMFDDTGACDVVVDEEIALKVVVDP-VFORLLLIIGLMDISPDLPQRLLS
5D4198	61	DLSTSLTMQPLMFDDTGACDVVVDEEIALKVVVDP-VFORLLLIIGLMDISPDLPQRLLS
ShcA	1	-----MEMPALAFDDKAGACNMIDKAFALTLLRDDTHQORLLLIIGLLEPHEDLPQRLLA
B5	120	GALNPLFNDGPGGLGWHAGSELYIGFKAIPRENVSVVTLKQAI AELVEWIKRWDAH-
464	119	GALNPLFNDGPGGLGWHAGSELYIGFKAIPRENVSVVTLKQAI AELVEWIKRWDAH-
5D4198	120	GALNPLFNDGPGGLGWHAGSELYIGFKAIPRENVSVVTLKQAI AELVEWIKRWDAH-
ShcA	56	GALNPLVNA GPGTGWDEQSGLYHAYQSIPREKVSVEMLKLEIAGLVEWMK CWREART

B

B5	1	MNPIQTRFSNVEALRHSEVDVQELKAHQIEVGGKCYDIRAAANNDLTVQRSDKQAMAMSK
464	1	MNPIQTRFSNVEALRHSEVDVQELKAHQIEVGGKCYDIRAAANNDLTVQRSDKQAMAMSK
5D4198	1	MNPIQTRFSNVEALRHSEVDVQELKAHQIEVGGKCYDIRAAANNDLTVQRSDKQAMAMSK
HopPsyA	1	MNPIHARFSSVEALRHSNVDIQAIKSEGOLEVNGRYFIRAAADGSTAVLRPDQOSKADK
B5	61	FFKKAGLSGSSGSQSDQIAQVLNDRKRGSSVSRLIRQGQTHLGRMQFNIEEGQGSSAATS
464	61	FFKKAGLSGSSGSQSDQIAQVLNDRKRGSSVPRLMRQGQTHLGRMQFNIEEGQGSSAATS
5D4198	61	FFKKAGLSGSSGSQSDQIAQVLNDRKRGSSVPRLMRQGQTHLGRMQFNIEEGQGSSAATS
HopPsyA	61	FFKGAAHLIGGQSQRACIAQVLN-EKAAAVPRLDLDR---MLGRRFDLEKGCSSAVCAAIK
B5	121	QNSRLPNGRLVNSSILQWAEKAKANGSTSSSALYQIYAKELPRVELLPRTTEHRACLAHMY
464	121	QNSRLPNGRLVNSSILQWAEKAKANGSTSSSALYQIYAKELPRVELLPRTTEHRACLAHMY
5D4198	121	QNSRLPNGRLVNSSILQWAEKAKANGSTSSSALYQIYAKELPRVELLPRTTEHRACLAHMY
HopPsyA	116	AADSRLTSKQTFASFQOWAEKAEALGRYRNRYLHDLQEGHARINAYECGRVKNITWKRYR
B5	181	KLNGKDGISIWPQFLDGVRGLQLKHDTKVFMMNPNKAADEFYKIERSGTQFPDEAVKARL
464	181	KLNGKDGISIWPQFLDGVRGLQLKHDTKVFMMNPNKAADEFYKIERSGTQFPDEAVKARL
5D4198	181	KLNGKDGISIWPQFLDGVRGLQLKHDTKVFMMNPNKAADEFYKIERSGTQFPDEAVKARL
HopPsyA	176	LSITRKTL SYAPQTHDRE--EELDLGRYIAEDRNARTGFERMVPK-DQRAPE TNSGRL
B5	241	TINVKPQFQKAMVDAAVRLTAERHDIITAKVAGPAKIGTITDAAVFYVSGDFSAAQTLAK
464	241	TINVKPQFQKAMVDAAVRLTAERHDIITAKVAGPAKIGTITDAAVFYVSGDFSAAQTLAK
5D4198	241	TINVKPQFQKAMVDAAVRLTAERHDIITAKVAGPAKIGTITDAAVFYVSGDFSAAQTLAK
HopPsyA	233	TIGVEPKYGAQIALAMATIMDKHKSVTQCKVVGPAKYGQQTDSATLYINGDLAKAVKLGE
B5	301	ELQAL--LPDDAFINHTPAGMQSMGKGLCYAERTPQDRTSHGMSRASIIESALADTSRSS
464	301	ELQAL--LPDDAFINHTPAGMQSMGKGLCYAERTPQDRTSHGMSRASIIESALADTSRSS
5D4198	301	ELQAL--LPDDAFINHTPAGMQSMGKGLCYAERTPQDRTSHGMSRASIIESALADTSRSS
HopPsyA	293	KLKKLSGTPPEGFVEHTFLSMQSTGLGLSYAESVEGQPS SHGQARTHVIMDALKGQG--P
B5	359	LEKKLRNAFKSAGYNPDNPAFRLE-
464	359	LEKKLRNAFKSAGYNPDNPAFRLE-
5D4198	359	LEKKLRNAFKSAGYNPDNPAFRLE-
HopPsyA	351	MENRLKMA LAERGYDPENPALLRARN

Fig. 3. Sequence alignments between **A**, ShcA orthologs and **B**, HopPsyA orthologs. The deduced amino acid sequences for representative ShcB and HopPsyB products are shown. The sequences were aligned to the *P. syringae* pv. *syringae* 61 ShcA or *P. syringae* pv. *syringae* 61 HopPsyA using the ClustalW algorithm and displayed using the Boxshade output. Identical amino acids are highlighted with black, whereas similar amino acid residues are shown in gray.

they are alleles of known effectors. Thus, the EEL of these families, with the possible exception of the *P. syringae* pv. *persicae* 5846 type VB EEL, appear to express at least one functional effector. Family IB and IC HopPsyB, in contrast, only retained 31% identity with the known effector, HopPsyA. The apparent retention of an amino terminal TTSS secretion signal and association with a ShcA-like apparent chaperone suggested that this locus might also encode a translocated effector. The *P. syringae* pv. *syringae* B5 EEL was cloned into pDSK600, such that *shcB1-hopPsyB1* operon was expressed from the vector's P_{lacUV5} promoters to create pSHB5EEL1-600. When this construct was transformed into *Escherichia coli* MC4100 (pHIR11-2070)(pYXL2B), a strain that had been used previously to test for *hrp* TTSS-dependent effector activity (Pirhonen et al. 1996), transformants exhibited an HR⁺ phenotype in tobacco leaves, consistent with the presence of effector activity (Table 2).

To confirm that the HopPsyB product is translocated into host cells by the *hrp* TTSS, a fusion was constructed between the amino terminal 78 aa of HopPsyB1 and 'AvrRpt2 in pJCB5EEL2-AR2, and the resulting fusion was screened for activity in *RPS2* and *rps2* lines of *A. thaliana* (Guttman et al. 2002). *P. syringae* pv. tomato DC3000 (pJCB5EEL2-AR2) elicited the HR in *A. thaliana* ecotype Columbia (*RPS2*) but not in the *rps2* derivative (Table 2). These observations are consistent with translocation of HopPsyB into host cells and indicate that HopPsyB, like HopPsyA, is a TTSS-dependent effector.

When tested for an effect on the virulence of *P. syringae* pv. tomato DC3000 in *A. thaliana* (Whalen et al. 1991) and several *Brassica oleracea* cultivars (C. Bender, personal communication), *P. syringae* pv. tomato DC3000 transformants carrying pSHB5EEL1-600 exhibited a slight reduction in virulence on *A. thaliana* but showed slightly enhanced virulence in some *B. oleracea* lines (Table 2). Populations of *P. syringae* pv. tomato DC3000 (pSHB5EEL1-600) were consistently higher than *P.*

syringae pv. tomato DC3000 alone or the transformant expressing the *P. syringae* pv. *syringae* 61 EEL. In contrast, growth of *P. syringae* pv. tomato DC3000 (pYXL1L) expressing HopPsyA was reduced in these same cultivars. Symptom formation roughly paralleled the population levels. The responses in the *B. oleracea* varieties did not seem to be classical *R* gene-mediated responses. *E. coli* expressing the *hrp* TTSS and HopPsyA and HopPsyB did not elicit the HR in these plants (Table 2) as observed with other *avr* genes (Pirhonen et al. 1996).

DISCUSSION

The EEL of the *hrp* PAI is one source for effectors, and unique EEL expressing distinct effectors had been identified in each of the previously characterized PAI of *P. syringae* strains (Alfano et al. 2000; Hutcheson 1999). To identify novel effectors that could function in *P. syringae* pathogenicity, the attempt was made to amplify and characterize the EEL from 86 *P. syringae* strains. The EEL was successfully amplified from 33 of these *P. syringae* strains that had been isolated primarily in North America from a diversity of hosts. The results presented here indicate that each strain does not carry a unique EEL. Additional strains of distinct host range were identified that carried an EEL related to one of the four previously characterized EEL. Several variants of previously characterized EEL as well as apparently novel types of EEL were identified among the amplified EEL. Some of these EEL retained sufficient similarity to be grouped into six apparent families that differed in genetic composition. As expected, no apparent correlation was detected between resident EEL and host range of a strain.

Candidate effectors were found in each of the newly characterized EEL that differed in sequence or length from previously characterized homologs. For example, HopPsyB of the type IB and IC EEL exhibited partial sequence similarity to HopPsyA. Effector activity of HopPsyB was indicated by the HR elicited in tobacco leaves by *E. coli* strains expressing this locus and the *P. syringae* pv. *syringae* 61 TTSS. Translocation of HopPsyB into host cells was demonstrated using 'AvrRpt2 fusions. Consistent with the observed sequence polymorphisms, the effector activity of HopPsyB was distinct from HopPsyA, as different indicator plants responded to strains expressing these effectors. Most significantly, expression of HopPsyB affected the virulence of *P. syringae* pv. tomato DC3000 in several hosts. Other candidate effectors likely to have activities distinct from their homolog due to divergent sequence include *P. syringae* pv. *syringae* W4N15 HopPsyC2, *P. syringae* pv. *syringae* DH015 HopPsyG, and *P. syringae* pv. *syringae* B452 HopPsyV2. The *P. syringae* pv. *persicae* 5846 HopPtoB5 allele lacked the carboxy terminal 138 aa. Some effectors have been shown previously to be tolerant of carboxy terminal alterations without apparent alteration of function (Gopalan et al. 1996; Mudgett and Staskawicz 1999) but others, such as *avrPphE*, exhibit a change in activity upon single point mutation or C terminal extension (Stevens et al. 1998). Since indicator plants for the aforementioned effectors have not been established, it was not possible to determine at this time the phenotype of the candidate effectors.

The EEL of the characterized *P. syringae* strains appear to have assembled from transposed gene cassettes. Several gene cassettes were identified in the EEL of these strains that were conserved within or between EEL families, or both. A *hopPsyC1-hopPsyE* cassette with conserved flanking, coding, and intergenic sequences was detected in Family III and IV EEL. Likewise, an *eelF-eelG* cassette was detected in Family IV, V, and VI EEL, and a vestige was present in Family II EEL. A third cassette was apparent in the strains carrying Family I EEL. The

Region	Location	Size(bp)	% Identity
A	<i>hrpK-hopPsyC</i>	190	98
B	<i>hopPsyC-hopPsyE</i>	148	92
C	<i>hopPsyE-eelE</i>	85	98
D	<i>eelF-hopPsyE</i>	150	50
E	<i>eelF-eelG</i>	154	91
F	<i>eelG-tRNA</i>	170	86

Fig. 4. Conserved gene cassettes identified in the *Pseudomonas syringae* *hrp* pathogenicity island exchangeable effector loci. Proposed gene cassettes are indicated by the bars. The conservation of intergenic region indicated by the upper case letter (Region) is shown. The size of the indicated intergenic region is reported in base pairs (bp), and the calculated percent identity for this region (I) is reported after alignment using ClustalW. The indicated sequences from *P. syringae* pv. *syringae* B728A and *P. syringae* pv. *syringae* W4N15 were used to compare regions A, B, and C. The sequences from *P. syringae* pv. *syringae* W4N15 and *P. syringae* pv. tomato DC3000 were used for regions D, E, and F. Similar results were obtained during comparison of regions D, E, and F from *P. syringae* pv. tomato DC3000 with those from DH015, except that region D retained 94% identity.

shcA-hopPsyA cassette found in the type IA EEL appears to be an inverted and truncated derivative of the *shcB-hopPsyB* cassette found in the type IB and IC EEL (or vice versa). It is then likely that the loci for type II EEL AvrPphE8 allele, the Family V HopPtoB alleles, and the type VI HopPsyG gene could also represent gene cassettes. Thus, as indicated by the divergent

percent GC of identified genes, each EEL appears to have a mosaic structure formed by horizontally acquired gene cassettes assembled in different combinations.

In nine out of the ten types of EEL identified in this study, a gene cassette encoding one or more effectors was expressed as part of the *hrpK* operon. Since *HrpK* also appears to be se-

Fig. 5. Relationship between *hrpK* alleles. The partial *hrpK* coding sequences obtained during characterization of the exchangeable effector loci (EEL) were subjected to minimum evolution and maximum likelihood analysis using PAUP*4.0 after alignment by ClustalW. Sixty-nine of the 231 nt utilized were parsimony informative. Substitutions per site are indicated by the bar. Bootstrap analyses are shown in parentheses. The type of EEL carried by each strain and the geographical source for the strain is indicated on the right. NA = North America; Eur = Europe; Aust = Australia; and AF = Africa.

creted by the *hrp* TTSS (J. Alfano, *personal communication*), it was possible that *hrpK* could be a previously unrecognized component of the EEL. Four arguments, however, can be made against this conclusion. First, HrpK is essential to the activity of the *hrp* TTSS. Insertional inactivation or deletion of *hrpK* results in an Hrp⁻ phenotype (Huang et al. 1991). Second, consistent with an essential function for HrpK and inclusion in the *hrp* CCR, the genetic location of *hrpK* is conserved in all *hrp* PAI characterized to date, whereas the location of gene cassettes carrying effectors, such as alleles of *avrPphE* (Jackson et al. 1999; Stevens et al. 1998), appears to be variable. Third, the percent GC of *hrpK* is very similar to that of the *hrp* PAI and is substantially higher than most EEL genes. Finally, sequence polymorphisms in *hrpK* only weakly correlated with the adjacent gene cluster, and the correlations observed, in most cases, lacked strong bootstrap support. The only exceptions were the *hrpK* alleles associated with the Family V EEL. Since these strains had been isolated or derived from European or North African sites, this clustering could be due to the geographical isolation of the strains. Although the limitations of the small sample size can not be fully excluded, the inability to link sequence polymorphisms in *hrpK* with the adjacent gene cassette is consistent with an independent evolution for the EEL and *hrpK*.

The mosaic structure of characterized EEL is suggestive of an integron-like assembly of the EEL from transposed gene cassettes. Integrons were first identified during studies of the localized and sequential accumulation of promoterless antibiotic resistance genes in enteric bacteria but have since been found in many taxonomic groups of bacteria (Hall and Collis 1995). Integrons assemble through the activity of a transacting, site-specific integrase that mediates the insertion of transposed gene cassettes through homologous recombination of *att* sites (Grainge and Jayaram 1999). An imperfect inverted repeat is a key feature of the *att* site (Grainge and Jayaram 1999; Hall and Collis 1995) and is retained after the transposition event. Many transposed pathogenicity islands of bacteria are associated with a tRNA locus that carries inverted repeats (Hacker and Kaper 1999). Transposition of the LEE pathogenicity island in REPEC strains of *E. coli* has been linked to the activities of a tRNA gene-linked integrase (Tauschek et al. 2002), and tRNA-linked excision of virulence factors has been detected in other *E. coli* strains (Blum et al. 1994).

Adjacent to the EEL of *P. syringae* strains is a tRNA_{leu} locus (Alfano et al. 2000) that could function as the predicted *att* site, but analysis of the region flanking the EEL revealed that, with the exception of Family III and IC EEL, a 62-bp region 3' to the tRNA gene was conserved between most types of EEL. If recombination occurred within the tRNA gene, little sequence conservation would be expected in this region. Within this conserved 3' region, however, is an inverted repeat. A survey of the *P. syringae* pv. tomato DC3000 genome revealed at least two apparently functional homologs of a tRNA-linked integrase (S. W. Hutcheson, *unpublished data*) that could mediate excision and transposition of gene cassettes. tRNA-dependent excision of *avr* genes, an activity indicative of integrase activity (Hall and Collis 1995), has been observed in *P. syringae* strains (Jackson et al. 2000). When taken together, the mosaic structure of the EEL, the association of the EEL with a candidate *att* site, the presence of tRNA gene-associated integrases in the genome of at least one *P. syringae* strain, and the apparent integrase-dependent excision of an *avr* gene cassette argue in favor of an integron-like assembly of the EEL.

All of the EEL characterized in this study exhibited at least some similarity to a previously characterized EEL, and the majority of EEL were related to the EEL of *P. syringae* pv. *syringae* 61. The similarity of these newly characterized EEL to previously characterized EEL could be due to an experimental bias resulting from the PCR amplification strategy and the strains surveyed. Although sequences obtained from *P. syringae* pv. *syringae*, *P. syringae* pv. *phaseolicola*, and *P. syringae* pv. *tomato* strains of North American and European origin were used in the initial primer design, some strains may simply be too divergent to be able to amplify the EEL using these primers. This could explain why the strategy predominately worked on *P. syringae* pv. *syringae* strains. A modified screen employing primers specific to conserved regions of *queA* and *hrpL*, however, did not reveal any additional types of EEL in some of the *P. syringae* strains with previously unamplifiable EEL. Due to the design of the primers, only the EEL linked with *hrpK* were amplified in the initial screen. It may be that the PAI of the other strains are not inserted near *queA*. Another bias in the screen was the choice of strains surveyed. The majority of the *P. syringae* strains tested were *P. syringae* pv. *syringae* strains that had been iso-

Table 2. Ectopic expression of HopPsyB alters the plant response phenotypes of *E. coli* and *Pseudomonas syringae* transformants

Bacterium	Expressed effector	Tobacco ^a	Response observed in					
			<i>A. thaliana</i> ^a		<i>B. oleracea</i> ^b			
			<i>R</i>	<i>r</i>	Brus.	Cab	Kale	Kohl.
<i>E. coli</i> ^c	None	N	N					
	HopPsyA	HR	N	N	N	N	N	
	HopPsyB1	HR	N	N	N	N	N	
<i>P. syringae</i> ^d	None	HR	D	D	2.7 ± 0.4	4.2 ± 0.2	3.5 ± 0.1	4.4 ± 0.3
	HopPsyA		D		1.8 ± 0.3	2.2 ± 1.7	2.6 ± 1.5	2.6 ± 0.4
	HopPsyB1		D		4.5 ± 0.9	3.6 ± 0.2	4.9 ± 0.4	4.9 ± 0.4
	'AvrRpt2		D	D				
	HopPsyB1': 'AvrRpt2		HR	D				

^a The plants were infiltrated with the indicated bacterial suspension and scored for plant responses at 16, 48, 72 and 96 h. N, Null response; HR, necrotic reaction observed at 18 h; D, expanding lesion observed by 72 h. Blank cells, Not tested R, *A. thaliana* ecotype Columbia (RPS2'); r, rps2 derivative.

^b Response described as above or log population growth in diseased tissue after 48 h. Populations were estimated by plate counts as described in the Materials and Methods and the mean ± standard error of three replicates are shown. The experiments were repeated three times with similar results. Abbreviations: Brus., Brussel Sprouts cv. Long Island; Cabb., Cabbage cv. Earliana; Kale, Kale cv. Dwarf Blue; Kohl., Kohlrabi cv. Early White Vienna. No substantial differences in growth of the tested strains were detected in Cauliflower cv. Early White Hybrid, Broccoli cv. Green Goliath, or Cabbage cv. Early Jersey Wakefield. Less than one log of growth was detected in Broccoli Raab cv. Spring for any tested strain. P values for the comparison of the mean populations reported for DC3000 expressing HopPsyA and HopPsyB were: Brus., 0.05; Cabb., 0.24; Kale, 0.14; Kohl., 0.05.

^c *E. coli* MC4100 (pHIR11)(pYXL2B)(pDSK600) transformant (expresses HopPsyA) or *E. coli* MC4100 (pHIR11-2070)(pYXL2B) transformant carrying pSHB5EEL1-600 (expresses HopPsyB1).

^d *P. syringae* Pto-DC3000 transformant carrying pYX1L (expresses HopPsyA), pSHB5EEL1-600 (expresses HopPsyB), pJC'avrRpt2-600 (expresses 'AvrRpt2) or pJCB5EEL2-AR2 (HopPsyB': 'AvrRpt2).

lated from North American sites. Since a possible geographical linkage was detected among strains with similar EEL, it may be that *P. syringae* populations indigenous to geographical regions not included in this study will have novel types of EEL. Thus, examination of the EEL from other geographical regions is a high priority. The size of the EEL also influences the ability to amplify the region. Although several long-range PCR protocols were employed to circumvent the difficulties of amplifying longer fragments, smaller fragments such as Family I EEL are simply easier to amplify and, thus, more likely to have been detected in these studies. Alternatively, the mechanism by which the EEL assembles could limit what genes transpose into this locus. Only genes associated with an *att* site would be able to insert into this site if the integron model is correct. Fusion of these gene cassettes to the *hrpK* operon would place expression of these genes under control of HrpL, as observed for most other effector genes (Fouts et al. 2002; Xiao and Hutcheson 1994), and would provide a direct means to coordinate expression of the effector with expression of the TTSS.

In summary, this partial survey of the EEL of *P. syringae* strains suggests that the EEL is a semiconserved site for TTSS-dependent effectors and provides a starting point for identification of the effectors produced by previously uncharacterized strains. Alterations in genetic composition of the EEL coupled with the sequence divergence detected among homologs of known effectors likely contribute to the genetic

diversity of strains. Many of the effectors identified exhibited sequence divergence at the amino acid level from previously characterized homologs that likely affected their activity, as described above. The apparent insertion of transposed gene cassettes into the *hrp* PAI would add new effectors to the strain's genome if acquired by horizontal transfer and could explain, in part, the putative geographical correlations observed. Several strains had large deletions within the EEL that abolished expression of one or more effectors. The inversion of a cassette, like what apparently occurred in Family I EEL, could affect the level of expression by altering which promoter controls expression. Overall, these results suggest that the genetic diversity detected in the EEL can be attributed in part to i) acquisition of effector gene cassettes via horizontal transfer and integron-like assembly into the PAI, ii) accumulation of point mutations in the effector genes, and iii) genetic rearrangements such as gene duplications, deletions, insertion of IS elements, and inversion.

MATERIALS AND METHODS

Bacterial strains.

P. syringae and *E. coli* strains were grown in King's medium B media at 25°C and 37°C, respectively. Rifampin (200 µg per ml) and spectinomycin (100 µg per ml) were added to the media, as indicated. Previously described strains, plasmids, and primers used in amplifications are described in Table 3.

Table 3. Other strains used in these studies, plasmids, and primers

Strain, plasmid, or primer	Relevant properties	Source or reference
<i>Pseudomonas syringae</i> strains		
DC3000	Tomato/ <i>Arabidopsis</i> pathogen; Rif ^r derivative of NCBPP1106 isolated in the U. K.; carries a Type VA EEL	Cuppels 1986
Psy-61	Weak bean pathogen; carries a Type IA EEL	Baker et al. 1987
B728a	Bean pathogen; carries a Type IIIA EEL	Hirano et al. 1999
1032A	Bean pathogen; carries a Type II EEL	Mansfield et al. 1994
<i>Escherichia coli</i> strains		
DH5α	(<i>r</i> _k ⁻ <i>m</i> _k ⁻) <i>recA1 relA1 Δ(argF-lacZYA)</i> U169 φ80 <i>dlacZ</i> DM15	Invitrogen
MC4100	F ⁺ <i>Δ(argF-lacZYA)</i> U169	Casadaban 1976
Plasmids		
pDSK519	<i>incQ</i> , Kn ^r	Keen et al. 1988
pDSK600	<i>incQ</i> , Sp ^r , triple <i>lacUV5</i> promoter, <i>mcs</i>	Murillo et al. 1994
pHIR11	<i>hrp</i> PAI from Psy-61 cloned into pLAFR3	Huang et al. 1988
PHIR11-2070	pHIR11 derivative carrying a <i>hopPsyA::TnpA</i> insertion	Huang et al. 1991
pJC' <i>avrRpt2</i> -600	617-bp <i>EcoR1-HindIII</i> fragment carrying C terminal effector domain of <i>avrRpt2</i> ligated into pDSK600	This report
pJCB5EEL2-AR2	259-bp <i>XbaI-HindIII</i> fragment carrying <i>hopPsyB</i> RBS and N terminal 86 codons ligated into pDSK519	This report
pLAFR3	<i>incP-1</i> Tc ^r cosmid vector	Staskawicz et al. 1987
pSHB5EEL1-600	The 3.5-kb EEL from <i>P. syringae</i> B5 cloned as an <i>XbaI-EcoR1</i> fragment into pDSK600	This report
pYX1L	The <i>shcB-hopPsyA</i> operon clones as a <i>BamHI-EcoR1</i> fragment in pLAFR3	Pirhonen et al. 1996
pYXL2B	Expresses the <i>hrpL</i> locus from vector promoter	Xiao et al. 1994
Primers		
K2688	CTGGGCGGACAGATGATC	This report
Q920	AACGCCGAAACCAGCATCAA	This report
D1F	GCAGAGTCAGGGTCATCAG	This report
D2R	TTCCAGCACAGCATCCAGTT	This report
D2F/	GGTGCGGTAAGTGTCAGAAA	This report
D3R	CGGTGTGGTGGTCATTGTC	This report
D3F	TCAATTGGCACTTCGCTACCT	This report
B1F	CGATGTGGGTGAGCCTAATG	This report
BER	GGAGCGGGATTTCAGGATGT	This report
BEF	GTGGTCGCTATCGGCAAGAA	This report
B5F	TACTTTCAGCATTAGGCAACG	This report
B5R	GTCTGCCCTCGCCACGCTGTA	This report
B5EEL1950-X	GCTCTAGAGCTGTATATCGGCTTTA	This report
B5EEL1565-RI	CGGAATTCCGCGCGCTTGTCAATTCAGTA	This report
<i>avrRpt2</i> -412-RI	CGGAATTCACGAGACGGGCGGTTCAAG	This report
<i>avrRpt2</i> -1028-H	CCCAAGCTTTAGGGACCAAAAAGCCAGAC	This report
P197-R1	CCGGAATTCGGACGGTCTATATAAGGAGG	This report
PB5ORF1- <i>XbaI</i>	GCTCTAGACAGTTCGGGATTGACAGG	This report

Amplification of the EEL by PCR.

Colony PCR reactions employed a Hybaid PCR Sprint thermocycler using a 10-min extension time and either Proof-sprinter (Hybaid, Teddington, Middlesex, U.K.) or ProofPro (Continental Lab Products, San Diego, CA, U.S.A.) DNA polymerase enzyme mixtures. Primers K2688 and Q920 were chosen based on their ability to amplify previously characterized EEL in *P. syringae* pv. *syringae* 61, *P. syringae* pv. *syringae* B728a, and *P. syringae* pv. tomato DC3000. All other variables were set according to standard protocol or manufacturer's instructions for long-range PCR. PCR products were gel-purified and extracted using the Bio-Rad Prep-A-Gene Master Kit (Hercules, CA, U.S.A.) prior to sequencing. DNA was sequenced at the University of Maryland Biotechnology Institute using an ABI Model 3100 Automated Sequencer.

Diagnostic PCR to determine conservation of *P. syringae* pv. tomato DC3000- and B728s-like EEL.

Published sequences of *P. syringae* pv. tomato DC3000 and *P. syringae* pv. *syringae* B728a were used to create primers internal to selected ORFs. Primers were then paired appropriately and utilized in *Taq* polymerase-based (Invitrogen, San Diego, CA, U.S.A.) PCR amplification, using a 5-min. extension time. For the *P. syringae* pv. *syringae* B728a-like EEL, the following primer sets were used: *hopPsyC1-hopPsyE1* B1F, BER; *hopPsyE1-hopPsyV1* BEF, B5R; and *hopPsyV1-queA* B5F, Q920. For the *P. syringae* pv. tomato DC3000-like EEL, the following primer sets were used: *hopPtoB-eelH* D1F, D2R; *eelH-eelF1* D2F, D3R; and *eelF1-queA* D3F, Q920.

Construction of a *P. syringae* pv. *syringae* B5 EEL clone.

The EEL was amplified from *P. syringae* pv. *syringae* B5, using tailed primers P197-R1 and PB5ORF1-Xba. After PCR, the fragment was digested with *Xba*I and *Eco*RI and was gel-purified using the Bio-Rad Prep-A-Gene Master Kit. The resulting fragment was ligated into *Xba*I-*Eco*RI-digested pDSK600 and was transformed into electrocompetent *E. coli* DH5 α cells. Plasmids were isolated from spectinomycin-resistant colonies and inserts were confirmed by PCR and sequencing.

Construction of 'avrRpt2 fusions to *P. syringae* pv. *syringae* B5 ORF 2.

A 259-nt fragment carrying the 5' portion of *hopPsyB1* (includes the native RBS and first 86 codons) was amplified by PCR from strain *P. syringae* pv. *syringae* B5 using primers B5EEL1950-X and B5EEL1565-R1. A 528-nt fragment carrying the C-terminal 176 codons for *avrRpt2* was amplified using tailed primers *avrRpt2*-412-R1 and *avrRpt2*-1028-H. Both fragments were gel-purified using the Bio-Rad Prep-A-Gene Master Kit, were digested with *Eco*RI, and were ligated. The desired fusion was amplified from the ligation mixture, using primers B5EEL1950-X and *avrRpt2*-1028-H, gel-purified as described above, and ligated into pDSK519 as an *Xba*I, *Hind*III fragment to create pJCB5EEL2-AR2.

Plant assays.

For pathogenicity trials, overnight cultures grown at 25°C were diluted to an optical density at 600 nm of 0.1, diluted 10³-fold into water and infiltrated into leaves of the indicated plants. Plant responses were scored daily, and bacterial populations were monitored using the leaf disk assay of Bertoni and Mills (Bertoni and Mills 1987). For assessment of the ability to elicit the HR, overnight cultures were harvested, were washed in water, and were resuspended to 10⁸ CFU/ml for *P. syringae* pv. tomato DC3000 derivatives and 10⁹ CFU/ml for MC4100 derivatives. Leaf tissue was inoculated using a syringe and was scored for responses at 24 h.

Phylogenetic analyses.

Sequences were aligned using ClustalW and an initial tree generated using minimum evolution and HKY85 distances in PAUP*4.0. Parameters were estimated using maximum likelihood and the GTR + I + Γ model. These estimated parameters were used to perform a minimum evolution search. The parameter estimation was then repeated, and the maximum likelihood-minimum evolution search was completed using the revised parameters. Bootstrapping was performed with 100 replicates.

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NOTE ADDED IN PROOF

Similar findings are being reported by Deng, W. L., Rehm, A., Charkowski, A., Rojas, C. M., and Collmer, A. *Pseudomonas syringae* exchangeable effector loci: Sequence diversity in representative pathovars and virulence function in *P. syringae* pv. *syringae* B728a. *J. Bacteriol.* In press.

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