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Distribution and Sequence Analysis of a Family of Type III-Dependent Effectors Correlate with the Phylogeny of *Ralstonia solanacearum* Strains

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In *Ralstonia solanacearum,* we previously have reported on the characterization of *popP1* and *popP2* genes. These genes encode type III-dependent pathogenicity effectors related to the large family of AvrRxv/YopJ cysteine proteases that are shared among pathogens of plants and animals. In this study, we identify a third gene, named *popP3*, that is inactivated in the genome sequence of strain GMI1000 by insertion of a copy of the insertion sequence ISRso13. The three *popP* genes are localized on two large chromosomal pathogenicity islands, with *popP1* and *popP2* being present on the same island. Phylogenic analysis demonstrated that the PopP2 and PopP3 proteins are clearly distinct from other effectors of this family previously characterized in plant and animal pathogens. Analysis of the distribution and allelic variations of the three genes in 30 strains representative of the biodiversity of *R. solanacearum* established that *popP* genes are distributed widely among strains from two of the three phyla previously defined on the basis of the structure of the core genome. Sequencing of the *popP* genes from the different strains revealed limited allelic variations at the three loci but did not show evidence of recombination between the *popP* genes. Limited allelic variation together with occurrence of insertion sequences within or in the close vicinity of *popP* genes and the presence of gene duplications in these pathogenicity islands suggest that genomic rearrangements might be a major evolutionary driving force controlling evolution of the genes encoded in these regions. The implications of these observations in terms of bacterial evolution, gene acquisition, and horizontal gene transfers are discussed.

Sequencing and the subsequent comparison of complete bacterial genomes has dramatically changed our vision concerning the notion of bacterial species and of the processes that govern evolution of these organisms. A new light has been thrown on the major role played by horizontal gene transfers (Gogarten et al. 2002; Hacker et al. 2003; Jain et al. 2002; Ochman et al. 2000). For bacteria pathogenic to humans, comparison of their genomes to closely related nonpathogenic strains revealed that these organisms share a large number of common genes, referred to as the "core genome", that includes genes encoding basic housekeeping functions. Apart from the core genome, which can be considered as the skeleton for each particular species, different strains from the same species often differ from each other by a large number of genes that are present in one strain but absent in another (Buchrieser et al. 2003; Perna et al. 2001). These genes often are organized in clusters and often have a biased base composition compared with the core genome. In addition, they often encode functions that improve the fitness of the bacteria under particular environmental conditions, including the ability to invade the tissues of a living organism in the case of pathogenic bacteria (Jain et al. 2003). Moreover, these frequently are associated with mobile genetic elements such as prophages and insertion sequences (IS), suggesting that they were acquired from various sources through horizontal gene transfers. These observations led Hacker and associates (2003) to reinforce the concept of a pathogenicity island and to stress the crucial role that these elements may play in bacterial evolution (Hacker and Kaper 2000).

Very limited data is available concerning the role such gene islands could play in the evolution of pathogenicity and host specificity toward plants. The most documented set of data comes from the pathogenicity island of *Pseudomonas syringae* and is centered on *hrp* genes that encode a type-III protein secretion system (Alfano et al. 2000; Charity et al. 2003; Hutcheson 1999).

Sequencing of the genomes of the plant-pathogenic bacteria *Ralstonia solanacearum* (Salanoubat et al. 2002), *Xanthomonas campestris, X. axonopodis* (da Silva et al. 2002), and *P. syringae* (Buell et al. 2003) has established the presence of genomic islands with a G+C biased composition in the genome of each of these organisms. Candidate or established pathogenicity genes are present in several of these genomic islands. In *R. solanacearum,* these regions, named alternative codon usage regions (ACURs), encompass 7% of the genome and are distributed over the two large replicons that constitute the bacterial genome. In contrast to the situation reported for *P. syringae* (Alfano et al. 2000; Hutcheson, 1999), *R. solanacearum hrp* gene cluster cannot be distinguished from the core genome on the basis of DNA composition or on the basis of associated mobile genetic elements. A recent study has established that, in this bacterium, *hrp* genes have co-evolved with ribosomal genes (Gophna et al. 2003) and, therefore, are part of the core genome. On the other hand, a significant proportion of candidate effector genes identified in *R. solanacearum* are located within ACURs (Cunnac et al. in press; Salanoubat et al. 2002). This, together with the abil-

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ity of this bacterium to be naturally competent for DNA transformation (Bertolla et al. 1997), strongly suggests that several type-III effector genes might have been acquired relatively recently through horizontal gene transfers and that this could play a significant role in adaptation of this versatile plant pathogen to a wide range of potential hosts.

R. solanacearum is a complex species, grouping strains with differences in virulence, host range, mode of transmission, and metabolic properties. Based on these differences, several classification systems have been proposed. One is based on the host range of strains and originally distinguished three "races" (Buddenhagen et al. 1962). It later was extended to five races to include strains pathogenic on ginger (Pegg and Moffett 1971) and mulberry (He et al. 1983). An independent classification into six "biovars" is based on the ability of strains to metabolize disaccharides and hexose-alcohols (Hayward et al. 1964, 1991, 1994). A new classification scheme, based on analysis of restriction fragment length polymorphisms (RFLP) at the *hrp* locus and additional loci from the core genome (Cook et al. 1989; Cook and Sequeira 1994), revealed the existence of two divisions corresponding to two evolutionary phyla. Division I, also named "Asiaticum", comprises strains mainly isolated from Asia and Australia, whereas strains from division II, also known as "Americanum", mainly originate from South and Central America.

In addition, within each main division, this classification allows the identification of a subset of strains having a common RFLP patterns at different loci which, therefore, are referred to multilocus genotype or MLG groups. This grouping actually associates strains that share a common core genome structure (Cook and Sequeira 1994; M. Fegan and P. Prior, *personal communication*). The genetic classification thus defined correlates nicely with the biovar classification because strains from division Asiaticum match biovars 3, 4, and 5 and strains from division Americanum match biovars 1, 2, and N2. The diversification of *R. solanacearum* into two major phyla later was confirmed using additional molecular criteria addressing various elements of the core genome including i) polymerase chain reaction (PCR)-RFLP analysis of *pglA* polygalacturonase and *hrp* genes (Gillings et al. 1993; Poussier et al. 1999), ii) comparisons of rRNA sequences (Li et al. 1993; Poussier et al. 2002b; Seal et al. 1993; Taghavi et al. 1996), iii) amplified fragment length polymorphism (AFLP) analysis on genomic DNA (Poussier et al. 2000b), and iv) comparison of a partial nucleotide sequence of the *hrpB* and *eglA* endoglucanase genes (Poussier et al. 2000a). Some of these studies allowed identification of a third phylum corresponding to strains originating from Africa; however, the position of this phylum relative to the two other phyla is still unclear (Poussier et al. 1999, 2000a, 2000b).

Although race 3 classifies as biovar 2, no other correlation can be drawn between host range (races) and the other classifications based on analysis of genes from the core genome. This, together with the fact that a large proportion of effector genes are present in ACURs, suggests that, in *R. solanacearum,* host range might be a biological trait under fast evolutionary adaptation depending on high-frequency horizontal gene acquisitions and deletions. In order to test this hypothesis, we investigated the distribution and allelic variations of a family of three effector proteins among a collection of *R. solanacearum* strains representative of the biodiversity of this species. The three genes chosen for this study encode members of a family of potential cysteine proteases referred to as AvrRxv/YopJ effectors. Members of this family are widely conserved among a type-III secretion system harboring gram-negative pathogens of plants and animals (Orth 2002).

RESULTS

R. solanacearum encodes at least three evolutionarily distant genes for AvrRxv/YopJ-related effectors.

Three structural genes for members of the AvrRxv/YopJ protein family are present in *R. solanacearum* strain GMI1000. Two of them have been identified previously as *popP1* and *popP2* and encode avirulence proteins recognized by petunia (Lavie et al. 2002) and *Arabidopsis thaliana* (Deslandes et al. 2003), respectively. In addition, it was shown that the PopP1 protein modulates aggressiveness of the bacterium toward tomato (Lavie et al. 2002). The third gene, named *popP3*, is inactivated in strain GMI1000 by a copy of the insertion sequence ISRso13, and initially was described as RS02458 and RS02460 in the original annotation of the genome sequence. Analysis of the seven ISRso13 sequences that are present in the genome of strain GMI1000 shows that insertion of this IS generates an 11-bp duplication at the insertion site. Therefore, it was possible to reconstitute the DNA sequence of the native *popP3* gene before insertion of the IS occurred. The corresponding functional gene codes for a protein of 386 amino acid (aa) residues, whereas *popP1* and *popP2* encode 368 and 483 aa proteins, respectively. For convenience, these three genes will be further referred to collectively as "*popP* genes" and the corresponding proteins as "PopP proteins". When the three proteins are aligned with the pairwise alignment program of the Bioedit software package using the PAM250 similarity matrix, global identity of 17% was found between PopP2 and PopP3, 18% between PopP1 and PopP2, and 20% between PopP1 and PopP3. PopP1, PopP2, and PopP3 share 42% global similarity. The highest homology was found in the central region of the proteins which contain the catalytic site of C55 cysteine proteases that is conserved among all members of the AvrRxv/YopJ effector family. In contrast, the three proteins are highly divergent at their N-terminal ends. PopP2 harbors a long N-terminal extension that includes a nuclear localization signal at position 33-53 that promotes addressing of the protein into plant cell nucleus after translocation into the plant cell (Deslandes et al. 2003). On the other hand, PopP3 harbors an N-terminal potential myristoylation site as defined by Johnson and associates (1994) located at position 2-7. Neither of these two signals are predicted in PopP1.

When a multiple alignment of the PopP proteins with other members of the AvrRxv/YopJ family of effectors was generated, the resulting neighbor-joining tree clearly established that PopP1 clusters with one of the two groups of the other members of the family originating from plant-pathogenic and symbiotic bacteria. PopP2 and Pop3 are clearly distinct from these two groups and from the cluster of proteins identified in animal pathogens as well as from the HopPmaD protein from *P. syringae* pv. *maculicola* (Fig. 1).

PopP genes are encoded within two distinct pathogenicity islands.

With a respective G+C base composition of 55, 60, and 62% that is markedly different from the average value of 67% characteristic for the entire genome of strain GMI1000, *popP1, popP2,* and *popP3* are located within ACURs. In addition, *popP1* and *popP2* are part of a 79-kb chromosomal pathogenicity island extending between the RSc0821 gene and the tmRNA gene *ssrA*. In addition to harboring several ACURs, this region is characterized by the presence of several genes originating from phages, two insertion sequences (ISRso9 and ISRso14), and one partial copy of ISRso1. It also carries several potential pathogenicity genes, such as the genes for the hemaglutinin-related protein RSc0887, and the RSc0824 and RSc0895 proteins that share a high homology in their N-ter-

minus with the N-terminus of PthG, a protein controlling both aggressiveness and host specificity in the plant pathogen *Erwinia herbicola* pv. *gypsophylae* (Ezra et al. 2000). This pathogenicity island also harbors several stretches of DNA that are duplicated elsewhere in the genome.

Similarly, *popP3* is part of an 83-kb genomic island located near the origin of replication of the chromosome. This island is bordered on one side by the *cysC1* gene that codes for an adenylylsulfate kinase and by an Ala-tRNA (RS02400) on the other side. This region is characterized by the presence of several long ACURs and two copies of both ISRso1 and ISRso13. Furthermore, several phage genes and numerous structural genes for proteins with unknown functions are present in this island. This region also is particularly rich in DNA stretches that are duplicated elsewhere in the genome. In particular, two duplications are shared between the *popP1–popP2* and the *popP3* pathogenicity islands, with rs16 occurring in direct orientation and rs22 in reverse orientation.

popP genes are present in most Asiaticum and African isolates but are generally absent from Americanum isolates.

As a first step toward the analysis of the potential contribution of horizontal gene transfer to strain variability in *R. solanacearum*, we used a collection of 30 strains representative of the different races, biovars, and genetic groups previously described to monitor the distribution of *popP* genes in this species. The positioning of each of these strains according to the Americanum, Asiaticum, or African divisions is shown in Table 1 and was confirmed using multiplex PCR as defined by M. Fegan and P. Prior (*personal communication*). In the case of race 2 strains originally described as pathogenic on *Musa* spp., further genomic characterization was performed to differentiate them according to the MLG classification (Cook and Sequeira 1994; M. Fegan and P. Prior, *personal communication*).

The distribution of *popP* genes among this strain collection was first analyzed by Southern hybridization of total genomic DNA with various probes corresponding to PCR amplicons of *popP1* and *popP2* genes obtained from strain GMI1000 and to the *popP3* gene amplified from strain GMI8084. The nucleotide sequences of these three probes are highly divergent so that no cross-hybridization occurred under the experimental conditions used (data not shown). Clear hybridization signals were detected with at least one of the probes in all but one (Rd15) of the strains from the Asiaticum and African divisions (Table 1). The majority of these strains gave a positive signal with two of the three probes. Three other strains only hybridized with *popP1* and two strains (GMI8160 and GMI1000) gave a positive signal for the three genes. Another strain (JT685) gave a positive signal only with *popP1*. All three genes appear to be almost equally represented among the Asiaticum strains tested. Positive hybridization signals with the *popP1* probe also were obtained with the three African strains included in this study. These strains did not hybridize with the *popP2* probe and only one of them hybridized with the *popP3* probe.

Conversely, with the exception of strains from MLG25, all of the Americanum strains tested were consistently negative when probed with the three *popP* genes.

A gene family with limited allelic variability at each locus.

To confirm the positive signals observed in Southern hybridization and to establish the nucleotide sequences at each *popP* locus, PCR experiments were conducted to amplify the genes, or parts thereof, from strains that were positive in Southern hybridizations. This allowed us to determine the sequence for the full-length PopP1 and PopP2 coding genes for strains from the Asiaticum and the African divisions. For *popP3* genes, sequences covering 1,137 bp of the 3′ end of the 1,161-bp *popP3* gene were obtained. In the case of strains

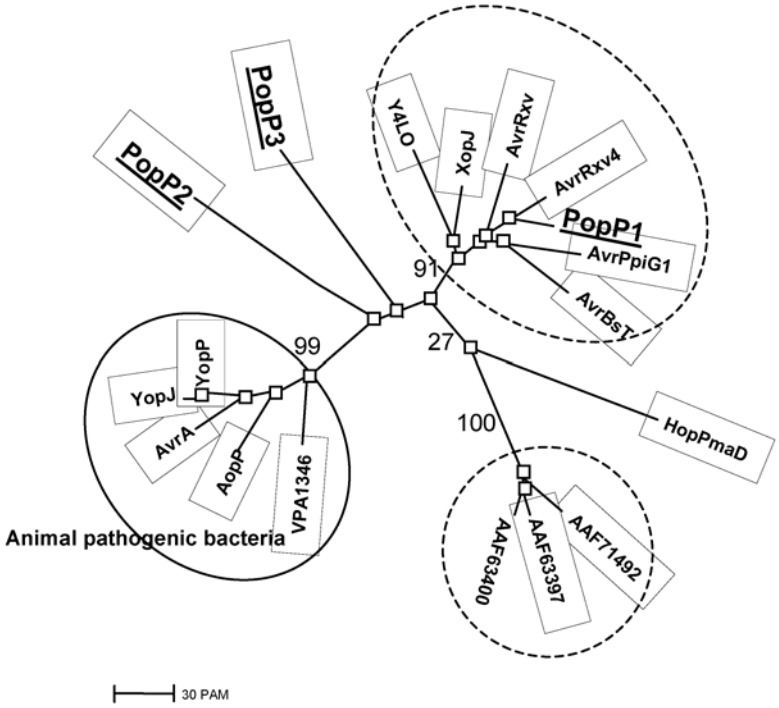

Fig. 1. Phylogenic tree of members showing the relationship between members of AvrRxv/YopJ family of type III-dependent pathogenicity effectors. Accession numbers for the proteins are: *Yersinia pseudotuberculosis* YopJ, P31498; *Y. enterocolitica* YopP, NP-052382; *Salmonella enterica* AvrA, AAB83970; *Xanthomonas axonopodis (campestris)* AvrRxv, Q08678; AvrBsT, AAD39255; XopJ, AAK72486; AvrXv4, AAG39033; *Pseudomonas syringae* AvrPpiG1, CAC16700; HopPmaD, AAL84243; AAF71492, AAF71492; *Erwinia amylovora* AAF63400, AAF63400; AAF63397, AAF63397; *Aeromonas salmonicida* sp. *salmonicida* AopP, NP_710166; *Vibrio parahaemolyticus* VPA1346, NP_800856; *Rhizobium* sp. Y4LO, NP-443964. Relevant bootstrap values are shown on the graph.

from the subgroup MLG25, only partial *popP1* gene fragments of 771 bp covering the 3′ end of the 1,107-bp PopP1 coding sequence could be amplified and sequenced. The results from PCR amplifications confirmed the presence of *popP* genes in all the strains that gave a positive hybridization signal in Southern hybridization, with the exception of *popP2* amplicons from the MLG25 strains (discussed below). All these sequences are available in the GenBank database under accession numbers AJ628876 to AJ628895.

In several cases, the sequences thus generated revealed the presence of an insertion sequence within or flanking the corresponding gene (Table 1). Where this occurred, the sequence of the native gene was established by removal of the IS sequence and of its flanking base pair duplication generated by the insertion process. The sequences obtained for each locus were aligned using Clustal X software and the corresponding neighbor-joining trees were constructed.

The neighbor-joining tree corresponding to the alignment of 771 bp covering the last codons of the *popP1* loci is shown in Figure 2A. It establishes that, with a bootstrap value of over 70, the *popP1* genes can be classified in three major allelic groups. Group 1A includes three Asiaticum isolates, group 1B includes both African and Asiaticum isolates, and group 1C is characteristic of the MLG25 subgroup of Americanum isolates. The six *popP1* partial sequences from group 1C were completely identical even though the corresponding strains originated from different geographical areas, including French West Indies and South and Central America. Comparison of the full-length *popP1* genes from group 1A were found to encode identical proteins, although one single silent base change was detected in one strain. Genes of the 1B group, however, are more variable and can be distinguished from the group 1A alleles on the basis of 54 conserved base changes resulting in 10 conserved amino acid changes at the protein level (Table 2). The PopP1 proteins from group 1A share 95% identity with the PopP1 proteins from group 1B. The partial C-terminal sequence of proteins from group 1C shares 97 to 98% amino acid identity with the corresponding regions of proteins from both group 1A and 1B. Three of the characterized *popP1* genes, one from group 1A and two from group 1B, are disrupted by an IS. The IS differs from strain to strain and is inserted at different locations within the gene, indicating that these insertions correspond to recent evolutionary events (Fig. 3).

Table 1. Characteristics of the *Ralstonia solanacearum* strains used in this study and summary of the distribution of the allelic forms of *popP* genes present in these strains

							Presence of popP alleles[a]		
Strains[b]	**Other designation[b]**	**Geographical origin**	**Isolated from**	**Ref[c]**	**Bv[d]**	**Div, MLG[e]**	***popP1***	***popP2***	***popP3***
UW20	…	Venezuela	Banana	1	1	Am/MLG28	–	–	–
UW9	GMI8132, JT644	Costa Rica	*Heliconia* sp.	2	1	Am/MLG24	–	–	–
UW135	…	Honduras	Banana	1	1	Am/MLG24	–	–	–
UW167	…	Costa Rica	Banana	1	1	Am/MLG24	–	–	–
UW11	…	Costa Rica	*Heliconia* sp.	3	1	Am/MLG24	–	–	–
MOLK2	GMI8238	Indonesia	Banana	3	1	Am/MLG24	–	–	–
UW30	GMI8124	Trinidad	Tomato	2	1	Am	–	–	–
UW26	GMI8122	United States	Tomato	2	1	Am	–	–	–
UW25	K60	United States	Tomato	1	1	Am	–	–	–
NCPPB3987	GMI8227, JT677	Brazil	Potato	2	N2	Am	–	–	–
1609	…	Nederlands	Potato	6	2	Am	–	–	–
UW23	GMI8139	Israel	Potato	1	2	Am	–	–	–
UW127	GMI8135	Perou	Plantain	1	1	Am/MLG25	1C	+	3C*
UW129	…	Perou	Plantain	3	1	Am/MLG25	1C	+	3C*
UW163	GMI8235	Perou	Plantain	3	1	Am/MLG25	1C	+	3C*
CFBP1419	JS847, UW156	Costa Rica	*Musa* sp.	3	1	Am/MLG25	1C	+	3C
Ant307	…	French West Indies	Anthurium	3	1	Am/MLG25	1C	+	–
Ant1121	…	French West Indies	Anthurium	3	1	Am/MLG25	1C	+	–
GMI1000	…	Guyana	Tomato	4	3	As	1A	2A	3A::ISRso13
GMI8160	…	French West Indies	Eggplant	7	4	As	1A	2A	3A*
JT685	GMI8228	China	Mulberry	2	5	As	1A::IS1420	–	–
GMI8081	…	Australia	Tomato	7	3	As	–	2A	3A*
GMI8085	…	Australia	*Xanthium pungens*	7	3	As	–	2A	3A*
GMI8084	…	Australia	Tomato	7	3	As	–	2A	3A*
UW74	JS841, GMI8230	Sri Lanka	Potato	2	4	As	1B	2B*	–
GMI8093	…	Australia	Ginger	7	4	As	1B::IS1021	2B	–
NCPPB332	JS949	Zimbabwe	Potato	2	1	Af	1B	–	3B
CFBP3059	JS904	Burkina Faso	Eggplant	2	1	Af	1B	–	–
JT525	GMI8237	Reunion Island	*Pelargonium asperum*	2	1	Af	1B::IS	–	–
Rd15	…	Taiwan	Radish	5	3	As	–	–	–

[a] *popP*: presence of the genes *popP1, popP2,* or *popP3* in the strains; – indicates that no signal was obtained in Southern hybridization with the corresponding *popP* probe; + indicates that a signal was detected in Southern hybridization with the corresponding *popP* probe but no amplicon of the gene or part of it could be produced. 1A, 1B, 1C, 2A, 2B, 3A, 3B, and 3C correspond respectively to the different allelic groups of *popP1, popP2,* and *popP3* genes defined on the basis of phylogenetic analysis that were found in the strains. The presence of an insertion sequence in certain gene is indicated by ::IS; * indicates strains where multiple copies of the corresponding gene are detected based on Southern hybridization.

[b] Strain designation: CFBP, Collection Française de Bactéries Phytopathogènes, INRA Angers, France; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, U.K.; UW, D. Cook and L. Sequeira, Department of Plant Pathology, University of Wisconsin–Madison, U.S.A.; GMI, CNRS-INRA, Auzeville, Castanet Tolosan Cedex, France; JS and JT, laboratoire de phytopathologie, CIRAD-FLHOR, Saint-Pierre, La Réunion, France.

[c] References: 1, Cook et al. 1989 ; 2, Poussier et al. 2000b; 3, P. Prior and M. Fegan, *personal communication*; 4, Salanoubat et al. 2002; 5, Deslandes et al. 1998; 6, Van Elsas et al. 2000 ; 7, this study.

[d] Biovars.

[e] Division (Div): As, Asiaticum; Am, Americanum; and Af, African as defined by Cook and associates (1989) and Poussier and associates (1999). Multilocus genotype (MLGs) as defined by Cook and Sequeira (1994).

A similar analysis was performed on the full-length *popP2* genes (Fig. 2B) allowing the definition of two clearly distinct groups of alleles referred to as 2A and 2B.

These two groups can be distinguished on the basis of 13 specific base substitutions resulting in six amino acid changes in the proteins (Table 2). Proteins from group 2A are identical and share 98% of identity with group 2B proteins. The two proteins from group 2B differ from each other by one single amino acid change, Q81H.

No amplicon could be obtained for *popP2* in strains from MLG25. However, Southern hybridizations performed under high-stringency conditions reproducibly gave a clear hybridization pattern on these strains. The pattern observed for the six MLG25 strains is not enlightened by a *popP1* or a *popP3* probe (data not shown) and clearly is distinct from the patterns obtained with the *popP2* probe on the other strains (Fig. 4). Altogether, this is a good indication that a *popP2* gene might be present in MLG25 strains and that these genes might form a distinct allelic group referred to as 2C.

Concerning PopP3, the neighbor-joining tree obtained after alignment of the 1,137 bp located at the 3′ end of the *popP3* open reading frame identifies three groups of alleles (3A, 3B, and 3C) with a bootstrap value of above 99% (Fig. 2C). Strains from group 3A harbor *popP3* genes whose sequences are identical to the sequence of the allele found in strain GMI1000, except that these genes are not interrupted by insertion of ISRso13. Group 3B is composed of a single gene originating from an African isolate. The corresponding protein sequences (379 aa) share 88 and 92% identity with the PopP3 sequences from groups 3A and 3C, respectively.

Group 3C corresponds to four identical *popP3* genes present in strains from the MLG25 subdivision of the Americanum phylum. The corresponding protein sequences share 88% identity with the protein sequences from group 3A.

Table 2: Positions and identity of discriminative amino acids between alleles 1A and 1B of the PopP1 proteins and between alleles 2A and 2B of the PopP2 proteins

	Amino acids at allelic forms			
	PopP1		PopP2	
Positions	**1A**	**1B**	**2A**	**2B**
71	M	A	…	…
90	S	A	…	…
97	H	S	…	…
132	Q	E	…	…
156	G	A	…	…
239	A	V	…	…
280	H	R	…	…
282	Y	Q	…	…
285	H	Y	…	…
326	R	S	…	…
13	…	…	S	N
115	…	…	L	P
146	…	…	R	H
156	…	…	G	D
288	…	…	S	N
396	…	…	G	E

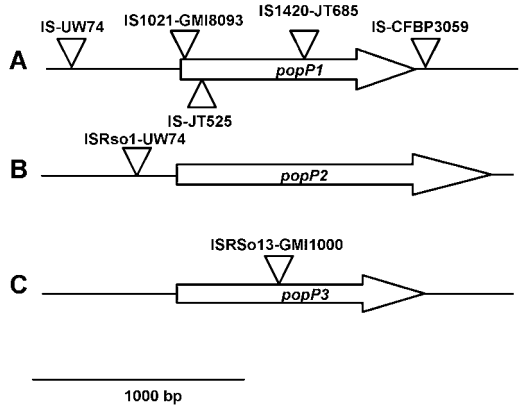

Fig. 3. Schematic representation of the locations of the insertions sequences present within or in the vicinity of **A,** *popP1,* **B,** *popP2,* and **C,** *popP3* genes in the *Ralstonia solanacearum* strains studied. The positions of insertion sequence (IS) insertions are indicated by an arrow; the name of the corresponding IS and the strain in which the insertion was present are indicated next to the arrow.

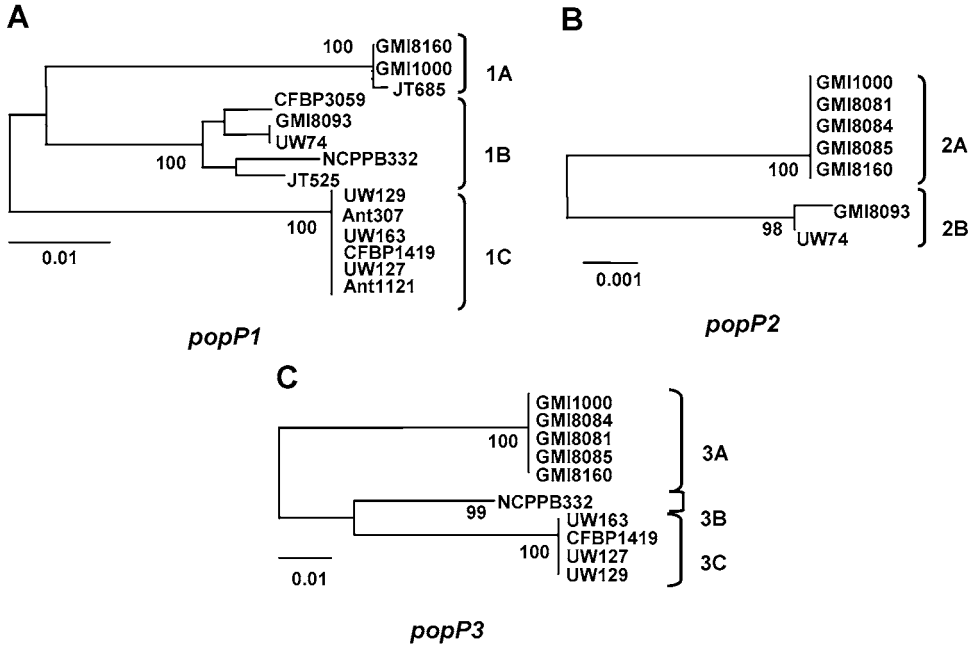

Fig. 2. Neighbor-joining tree established upon alignments of the nucleotide sequences of **A,** partial *popP1,* **B,** full-length *popP2,* and **C,** partial-length *popP3* genes showing the grouping in allelic forms. The numbers at the branch points correspond to the percentages of bootstrap replicates in which the clusters are found.

Taken all together, these results confirm the presence of at least one *popP* gene in all but one of the strains from the Asiaticum division (Table 1). They also confirm that MLG25 strains form a specific group among Americanum strains because they are the only strains from this phylum to harbor *popP* genes. However, the *popP* alleles found in these strains are clearly distinct from the alleles identified in Asiaticum strains. In addition, our data show that, when more than one *popP* gene is present in a strain, no recombination is observed between the three loci because the same combination of alleles are always found (i.e., a *popP1* allele from group 1A exclusively is found associated with a group 2A allele of *popP2* and with a group 3A allele of *popP3*). The same is true for *popP* genes from allelic groups B and C.

Occurrence of *popP* gene duplications.

In several cases, the banding patterns obtained in Southern blots of *Eco*RI-digested genomic DNA suggested that more than one copy of the gene was present in a single strain. For example, strain UW74, which carries a 2B allele of *popP2,* harbors four fragments that hybridize with the *popP2* (Fig. 5A). Two of these fragments are shared with strain GMI8093, which carries a 2B allele of *popP2.* This allele harbors two internal *Eco*RI restriction site generating three restriction fragments with only two that are large enough to be visualized after hybridization. Therefore, the two additional hybridizing restriction fragments that are detected in strain UW74 correspond to at least one additional copy of a *popP2* gene. As far as the 3C alleles of *popP3* are concerned, even though a single hybridizing band was found for the strain CFB1419, additional bands with variable hybridizing intensity were detected in strains UW127, UW163 (Fig. 5B), and UW129 (data not shown). No *Eco*RI site was present in the sequence of the corresponding 3C allele of *popP3* amplicons; this demonstrates the presence of multiple copies of *popP3*-related genes in strains UW127, UW163, and UW129. The same reasoning was applied for the *popP3* genes that are present in two copies in the strains GMI8160, GMI8081, and GMI8085 (Fig. 5B) and GMI8084 (data not shown). This data is summarized in Table 1.

When thermal asymmetric interlaced (TAIL) PCR amplification was conducted on the upstream and downstream regions of *popP* in the corresponding strains, no difference was detected in the sequence generated at each locus. This established that the duplication covered a region of at least 170 bp on each side of PopP coding sequence. However, the difference in the size of the restriction fragments of the hybridizing fragments strongly suggests that the different copies of the duplicated genes are located within different genetic environments. Alternatively, differences in hybridization intensities of these additional bands may be an indication that the different copies of *popP* gene within a strain might be too different to be amplified with a common set of primers.

Allelic variations of *popP1* and *popP2* do not affect their avirulence function.

In strain GMI1000, the *popP1* and *popP2* genes behave as avirulence determinants on Petunia St40 and *A. thaliana* ecotype Nd-1, respectively (Deslandes et al. 2003; Lavie et al. 2002). To test whether the allelic variations observed within these genes actually affected their avirulence function, the *popP1* 1B allele from strain UW74, the *popP2* 2B allele from strain GMI8093, and the *popP2* 2B allele from UW74 were tested individually for avirulence activity following introduction of pML1, pML2, and pML3 plasmids into strain Rd15 that is virulent on petunia St40 and *A. thaliana* Nd-1. The 1B allele used in these experiments was chosen as being the most divergent from the *popP1* gene present in strain GMI1000. The 2B PopP2 proteins from strains UW74 and GMI8093 differ by six and seven amino acid changes, respectively, from

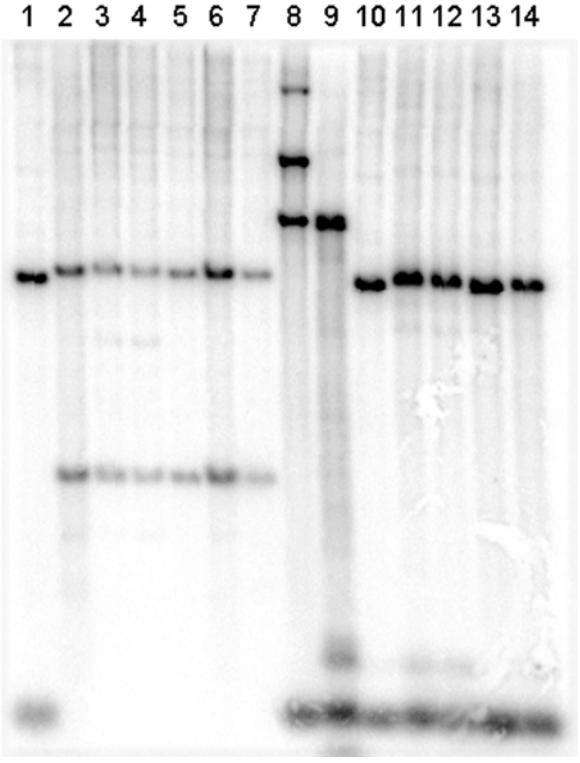

Fig. 4. Hybridization patterns of a *popP2* probe against *Eco*RI digests of genomic DNA from selected positive strains of *Ralstonia solanacearum* belonging to the phyla Americanum and Asiaticum. Individual strains are: Lanes 1 and 10, GMI1000; lane 2, UW163; lane 3, Ant1121; lane 4, Ant307; lane 5, CFBP1419; lane 6, UW129; lane 7, UW127; lane 8, UW74; lane 9, GMI8093; lane 11, GMI8160; lane 12, GMI8085; lane 13, GMI8084; and lane 14, GMI8081.

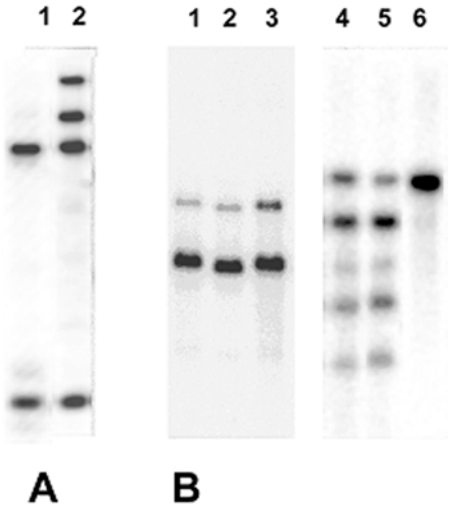

Fig. 5. Southern blot hybridization profiles of *Eco*RI-digested genomic DNA obtained with **A,** a *popP2* probe and **B,** a *popP3* probe establishing the existence of *popP* gene duplication in several strains of *Ralstonia solanacearum.* Hybridization probes used correspond to **A,** *popP2* and **B,** *popP3*. Genomic DNA originate from stains UW74 (A, lane 2), GMI8160 (B, lane 1), GMI8085 (B, lane 2), GMI8081 (B, lane 3), UW127 (B, lane 4), UW163 (B, lane 5), and CFBP1419 (B, lane 6). The hybridization profiles for strains GMI8093 (A, lane 1) and CFBP1419 (B, lane 6), which carry a single copy of the *popP2* and *popP3* genes, respectively, also are presented for comparison.

GMI1000 PopP2. These tests were conducted as previously described using *popP1* and *popP2* genes from strain GMI1000 as controls (Deslandes et al. 2003; Lavie et al. 2002). In each case, no difference in avirulence activity was observed between the new allelic forms tested and the controls.

DISCUSSION

We previously characterized two genes in strain GMI1000 of *R. solanacearum* that encode proteins belonging to the AvrRxv/YopJ family of cysteine proteases that are secreted by type-III protein secretion machinery from bacterial pathogens (Deslandes et al. 2003; Lavie et al. 2002). In this study, we identified a third gene, called *popP3,* encoding a new member of this gene family. In addition, we show that occurrence of multiple members of this gene family is a common feature among *R. solanacearum* strains. This is a rather unusual situation among plant pathogens because the occurrence of several members of this gene family in a single strain has been reported only in *X. campestris pv. vesicatoria* (XcvT, race 1 75-3), in which the two closely related genes *AvrRxv* and *AvrBsT* are present (Ciesiolka et al. 1999).

In strain GMI1000, the structural genes for these PopP effectors are located at two distant regions of the chromosome harboring most of the features characteristic of pathogenicity islands. The *popP1* gene encodes a protein closely related to other members from this family that have been characterized in plant pathogens. The two others genes, *popP2* and *popP3*, encode proteins that do not cluster with the other members of this family identified in either plant or mammal pathogens.

We also have established that the three *popP* genes are not evenly distributed among the three phyla identified in this species. They are found almost exclusively in strains that belong to the divisions Asiaticum and African and usually are absent from strains classified as Americanum. Considering the wide conservation of this gene family among bacteria pathogenic to plants and mammals (Orth 2002), it is surprising that such genes were absent from most Americanum strains. Therefore, this raises the question of whether other members of this gene family that remain to be identified are present in these strains. We know, however, that these genes are not essential for pathogenicity because a mutant of strain GMI1000 in which both *popP1* and *popP2* have been inactivated (in addition to *popP3* that already is nonfunctional in this strain) retained the ability to cause disease on tomato and *A. thaliana* (data not shown).

Several features indicate that the three PopP proteins might not be functionally equivalent. It has been shown that PopP2 is addressed to the plant nucleus (Deslandes et al. 2003), whereas PopP1 and PopP3 are devoid of a predicted nuclear localization signal. A potential myristoylation site is present at the N-terminus of PopP3, suggesting that this protein could be addressed to the plant cell membrane as established for several Avr proteins from *P. syringae* (Nimchuk et al. 2000; Shan et al. 2000). In addition, PopP1 is predicted to remain in the cytoplasm after translocation into plant cell. This is a strong indication that the three effectors have different targets in the plant and, therefore, act differently in controlling plant–bacteria interactions.

We have shown that allelic variations of *popP* genes in *R. solanacearum* are rather limited and that they might have little effect on the functions of the corresponding proteins because the *popP1* and *popP2* variant alleles tested retained the avirulence function of the corresponding alleles in strain GMI1000. In contrast, great variability exists concerning the presence or absence of each individual gene and the number of copies of each *popP* gene present in a particular strain. Moreover, we found that, when present, expression of these genes frequently might be affected by the presence of IS elements within or in close vicinity to the coding sequence (Fig. 3). In addition to the *popP3* gene that is disrupted in strain GMI1000, 3 of the 14 *popP1* genes sequenced were disrupted by an insertion sequence. Three IS also were found in *popP* flanking regions (one located 34 bp after the *popP1* stop codon of strain CFBP3059, one at 500 bp before the *popP1* start codon of strain UW74, and one 180 bp upstream of the *popP2* start codon of strain UW74). In addition, several DNA duplications have been identified within the *popP1–popP2* and *popP3* pathogenicity islands and several strains were identified in which individual *popP* genes have been duplicated. Taken together, these results indicate that genomic rearrangements may play a major role in the control of expression of the pathogenicity functions encoded at these loci.

In this study, a strong correlation between the distribution of *popP* genes and the phylogenic position of strains was established. Taking into account the large background of evidence that suggests that these genes have been acquired relatively recently through horizontal gene transfer, this result is rather unexpected. Contrary to what would be expected in case of independent horizontal acquisition of the *popP1–popP2* and *popP3* pathogenicity islands, recombination between *popP1–popP2* and *popP3* was never observed. Therefore, these observations favor a model in which a common ancestor to the African and Asiaticum phyla acquired *popP* genes through horizontal gene transfers. In addition, these genes would have been acquired more recently by a common ancestor of the subset of MLG25 strains from the phylum Americanum. According to such a model, one would predict that the long co-evolution of the *popP* genes with the core genome of Asiaticum and African strains should have resulted in adjustment of *popP* genes base composition to the average base composition of the genome. This clearly is different from what we observe.

An alternative scenario to explain the correlation between *popP* gene distribution and strain phylogeny would be that, in order to acquire or stably maintain the *popP* genes, the host strains require particular functions encoded only in the core genome of the Asiaticum and African strains. Such functions could include, for example, phage receptors, restriction or modification enzymes, other functions required for heterologous recombination of the incoming DNA, or alternatively, pathogenicity functions such as specific type-III effectors such as PopA, PopB, or PopW (Arlat et al. 1994; Guéneron et al. 2000; Salanoubat et al. 2002*)* that are encoded by genes indistinguishable from the core genome. However, again, the absence of recombination between the *popP* genes is in contradiction with this hypothesis.

In conclusion, the present study reveals that the mechanisms governing ACUR acquisition by *R. solanacearum* might be much more complex than we originally thought. Further work, based on the analysis of the distribution of a large number of effectors from ACUR, will be needed in order to understand the mechanisms which govern distribution and evolution of these genes in *R. solanacearum* and how these genes might be involved in adaptation of this versatile pathogen to its wide range of potential hosts.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Strain TG1 of *Escherichia coli* grown in Luria-Bertani medium (Sambrook et al. 1989) at 37°C was used for cloning experiments. *R. solanacearum* strains used in this study are listed in Table 1. All *R. solanacearum* strains were grown at 28°C in B medium (Boucher et al. 1985). When required, anti-

biotics were used at the following concentrations: tetracycline at 10 mg liter^{-1} and ampicillin at 50 mg liter^{-1}.

R. solanacearum electroporation.

Plasmids were introduced into *R. solanacearum* strains by electroporation (2.5 kV, 200 ohms, 25 μFd, 0.2-cm cuvette gap).

Molecular biology techniques.

Unless stated otherwise, standard methods were used (Ausubel et al. 1990). Genomic DNA was extracted from *R. solanacearum* strains as described by Arlat and associates (1992). PCR amplifications were done using the Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN, U.S.A.).

For Southern analysis, approximately 3 μg of *Eco*RI-digested genomic DNA was loaded per lane on an agarose gel. DNA was transferred to Hybond N+ membranes (Amersham Biosciences, Piscataway, NJ, U.S.A.) and hybridizations were performed in 0.5 M sodium phosphate, pH 7.2; 1 mM EDTA, pH 8; 7% sodium dodecyl sulfate (SDS); and salmon sperm DNA at 20 μg/ml at 65°C. Membrane were washed at 65°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS buffer. The DNA probes were labeled with [α-P^{32}]dCTP using the kit Ready to Go (Amersham Biosciences). *popP1* and *popP2* probes were obtained by PCR amplifications of the *popP1* and *popP2* full gene sequences from strain GMI1000 using primers Fw4-P1/Rev1-P1 and Fw3-P2/Rev3-P2, respectively. The *popP3* probe was obtained by PCR amplification of the coding sequence of *popP3* from strain GMI8084 using primers Fw1-P3 and Rev1-P3.

DNA amplifications and sequencing.

popP gene flanking sequences were isolated using TAIL-PCR performed according to the method described by Liu and Whittier (1995), except that the Expand Long Template PCR System (Roche) and corresponding elongation conditions were used for amplifications. Two different degenerate primers were tested for each amplification. The largest products obtained were approximately 2.5 kb long.

popP gene amplification.

Several methods were used to amplify *popP* genes prior to sequencing.

In most of the cases, primers located in the flanking regions of *popP* genes in strain GMI1000 genome were used successfully to amplify *popP* genes from other strains. When no product could be obtained in this way, primers internal to the *popP* coding sequences were tested. In such cases, the missing sequences corresponding to the 5′ and 3′ ends of the corresponding genes were determined on TAIL-PCR amplification products.

TAIL-PCR and PCR products were analyzed by gel electrophoresis and purification of amplicons was performed by passage through a S400 column (Amersham Biosciences) when a single amplicon was present in the reaction mixture. When several amplicons were present, individual amplicon products were purified by extraction of excised gel fragments containing each individual replicons. This extraction was performed using the Wizard SV gel and PCR clean-up system (Promega Bioscience, San Luis Obispo, CA, U.S.A.). The products thus obtained were used directly for sequencing.

Plasmid constructions.

pML2 and pML3 were constructed by cloning the *popP2* genes from strains GMI8093 and UW74, respectively, between the restriction sites *Bam*HI and *Hin*dIII of the pLAFR3 vector (Lindgren et al. 1986). *popP2* genes were obtained by PCR amplification of a 1,489-bp DNA fragment, extending from the ATG of the *popP2* gene down to 23 bp downstream of the stop codon, using the primers Fw3-P2 and Rev3-P2 that introduced the *Bam*HI and *Hin*dIII restriction sites at the end of the amplicon (Table 2). In the resulting constructs, gene expression was under the control of the p*lac* promoter of pLAFR3.

To construct pML4, a fragment *Eco*RI-*Hin*dIII of 1.4 kb, containing the *popP1* gene from strain GMI1000 together with 307 bp of its 5′ flanking region and 35 bp of its downstream sequence, was PCR amplified with the primers Fw2-P1/Rev2-P1 that carry *Eco*RI and *Hin*dIII restriction sites, respectively, at their 5′ extremities. This fragment then was cloned between the *Eco*RI and *Hin*dIII restriction sites of pLAFR3.

pML1 is a pLAFR3 derivative in which a 1.4-kb *Eco*RI-*Eco*RI fragment containing the *popP1* gene from strain UW74 was cloned into the *Eco*RI restriction site of the pLAFR3. In strain GMI1000, the 5′ extremities of primers Fw3-P1 and Rev4-P1 are located 48 bp upstream of the *popP1* start codon and at 17 bp downstream of the *popP1* stop codon, respectively. These primers allow the amplification of a 1.2-kb DNA fragment. Using strain UW74 genomic DNA as template, the PCR amplification with Fw3-P1 and Rev4-P1 produced a DNA fragment of 2.5 kb. The partial sequencing of this fragment showed that the entire *popP1* gene was contained between the Fw3-P1 primer and an *Eco*RI site present within the amplified DNA fragment. Hence, *Eco*RI digestion of the 2.5-kb amplicon allowed isolation of a 1.4-kb DNA fragment that contained a full-length *popP1* gene. This fragment was cloned under the control of the p*lac* promoter in pLAFR3.

Plant tests.

Pathogenicity assays on *Arabidopsis* (ecotypes Col-5 and Nd-1) and petunia plants were performed as previously described by Marenda and associates (1998) and Deslandes and associates (1998).

Phylogenetic analysis.

popP gene sequences were analyzed using the PHYLOWIN software package (Galtier et al. 1996). Sequences were aligned with the Clustal X 1.8 software (Thompson et al. 1997). Phylogenetic trees were constructed from the alignments by using the neighbor-joining method (Saitou and Nei 1987) with a correction of the evolutionary distances for multiple mutations with the Kimura formula. Bootstrapping was performed with 1,000 replicates. The resulting phylogenetic trees were visualized using the Treeview 1.6.6 software.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

North Carolina State UniversityBioedit biological sequence alignment editor website: www.mbio.ncsu.edu/BioEdit/bioedit.html

INRA web server *Ralstonia solanacearum* page: sequence.toulouse.inra.fr/R.solanacearum.html