PopP1, a New Member of the YopJ/AvrRxv Family of Type III Effector Proteins, Acts as a Host-Specificity Factor and Modulates Aggressiveness of *Ralstonia solanacearum*

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A functional analysis of an 11-kb-long region of the genome of the plant-pathogenic bacterium *Ralstonia solanacearum*, previously identified as an alternative codon usage region (ACUR), reveals that it was probably acquired through horizontal gene transfer. This ACUR encodes an insertion sequence and eight potential proteins, one of which is partially homologous with a host-specificity factor from a plant-pathogenic *Erwinia* sp., and another, PopP1, which is homologous to members of the YopJ/AvrRxv family of type III-secreted bacterial effectors controlling interaction between bacteria and their hosts. The analysis of mutants affecting all except one of the genes identified in the ACUR showed that only the *popP1*-deficient strain had an altered phenotype in plant infection tests. This mutant strain became pathogenic to a *Petunia* line that is resistant to the wild-type strain. Therefore, *popP1* behaves as a typical avirulence gene. We demonstrate that PopP1 protein is secreted and that secretion of this protein requires a functional type III-secretion pathway. In contrast to the structural genes for other type III-secreted proteins identified in *R. solanacearum*, transcription of the *popP1* gene is not coregulated with transcription of *hrp* genes but is constitutive.

Additional keywords: *Arabidopsis thaliana*, HR, type III secretion pathway, virulence, YopP

Type III secretions systems (TTSS) have a key role in the control of the interactions between pathogenic bacteria and their hosts (Cornelis and Van Gijsegem 2000; Galan and Collmer 1999; Hueck 1998). In human and animal pathogens, TTSS act to deliver bacterial proteins into the host cell cytoplasm where they express antihost functions, therefore reorienting host cell physiology to favor successful multiplication of the pathogen in host tissues.

In plant-pathogenic bacteria, TTSS are encoded by *hrp* genes which are found in all the major groups of gram-nega-

tive plant-pathogenic bacteria except *Agrobacterium*. Indirect evidence indicates that, similarly to TTSS from mammal pathogens, the Hrp secretion machinery acts to deliver effector proteins into plant cells (Staskawicz et al. 2001). This hypothesis recently was confirmed on *Xanthomonas* and *Pseudomonas* spp. (Lahaye et al. 2002; Casper-Lindley et al. 2002). Among plant pathogens, the majority of known type III secreted proteins are Avr proteins. These proteins are host specificity factors that trigger defense mechanisms in plants harboring a matching resistance gene (Keen 1990). Type III effectors from plant pathogens, such as the DspA/E protein of *Erwinia amylovora*, also act as pathogenicity determinants (Bogdanove et al. 1998; Gaudriault et al. 1997). Although the functions of most of these pathogenicity factors are still mostly unknown, they are hypothesized to act by preventing the initiation of plant defense reactions or by promoting the release of nutrients from the plant cell into the apoplast, therefore allowing active bacterial multiplication.

Avirulence functions and pathogenicity functions are not mutually exclusive and an increasing number of Avr proteins are known to act as effectors that increase the severity of symptoms when the bacterium infects sensitive plants that are devoid of a matching resistance gene (White et al. 2000).

During our analysis of the complete genome sequence of *Ralstonia solanacearum*, we identified several genes that code for proteins homologous to Avr proteins previously identified in other plant-pathogenic bacteria (Salanoubat et al. 2002). This situation was rather unexpected because, contrary to the situation found with most other plant-pathogenic bacteria, no gene-for-gene interaction had ever been reported for *R. solanacearum*. Only one avirulence gene, named *avrA*, had previously been identified in this bacterium but, unlike canonical *avr* genes, *avrA* determines host specificity at the plant species level rather than at the genotype-specific level within a particular plant species (Carney and Denny 1990).

Here we address the question of whether the *R. solanacearum* PopP1 protein, which shares homology to the YopP/YopJ pathogenicity factors from human pathogen *Yersinia* (Orth 2002; Orth et al. 2000) and with members of the AvrBsT family of *avr* genes from plant-pathogenic bacteria (Lahaye and Bonas 2001), behaves as a host-specificity determinant or as a pathogenicity TTSS effector protein. In addition, we investigate the roles of several genes flanking *popP1*, which were probably simultaneously acquired through horizontal gene transfer, in pathogenicity.

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RESULTS

Genetic organization of the alternative codon usage region that encodes PopP1, a new member of the YopJ/AvrRxv family.

The gene locus originally defined as RS05177 in the annotation of the complete genome sequence of *R. solanacearum*

strain GMI1000 is located on the bacterial chromosome and spans 11,028 bp from position 864,361 to 875,388.

With a base composition of 57% G+C, this region differs significantly from the average value of 68% established for the complete genome (Fig. 1A). In addition, genes identified in this region have a codon usage that significantly differs from

Fig. 1. Genetic organization of alternative codon usage region (ACUR) RS05177. **A**, Variation of G+C% (window size: 5,000 bp, window step 250) along the ACUR and flanking regions is shown together with **B**, its genetic organization. Big arrows represent the predicted protein coding genes, arrows with a thick border correspond to proteins that have partial or global homology with other proteins encoded in the genome of strain GMI1000. Open arrows correspond to proteins with no homology to known proteins, arrows filled with dots to proteins having partial homology with other proteins, gray arrows correspond to the two open reading frames coding for the transposase of insertion sequence ISRs014, and a horizontally hatched arrow represents the structural gene for the PopP1 protein. The two 280-bp DNA direct repeats present in the ACUR are shown as headless diagonally hatched arrows below the open reading frame arrows. The scale shows the position on the *R. solanacearum* strain GMI1000 chromosome. **C**, Several constructions used in this work, including deletion mutants GMI1695 and GMI1696 (dashed lines), insertion sites for the Ω interposon (arrow heads), and plasmid inserts (solid lines and open arrows). The narrow white arrows represent the coding sequence for β -galactosidase.

the general codon usage of the organism and were thus classified as an alternative codon usage region (ACUR) (Salanoubat et al. 2002). It harbors one of the four copies of the insertion sequence ISRso14 identified in the complete genome and two 280-bp-long nearly perfect DNA direct repeats at positions 865,112 to 865,391 and 866,636 to 866,916 (Fig. 1B). At least four additional copies of this repeat also are present elsewhere in the genome. Moreover, two stretches of DNA located within ISRso14 and spanning positions 868,630 to 869,036 and 869,239 to 869,661 have 87 and 83% homology, respectively, at the DNA level with two internal fragments of IS407 first identified in *Burkholderia cepacia* (Wood et al. 1991).

In addition to the two open reading frames (ORF) coding for the ISRso14 transposase, eight additional proteins encoding genes have been predicted in this region. Two of these genes, (RS05169 and RS06018) code for proteins which have no homology with any known proteins, whereas two others (RS05171 and RS05157) code for proteins which have partial homology only with other *R. solanacearum* hypothetical proteins.

Accession RS05161 codes for a protein of unknown function shared with *Agrobacterium* spp., various *Rhizobium* spp.,

and *Brucella melitensis*. This protein and its corresponding homologues all are predicted to harbor a potential esterase/lipase/thioesterase active site as defined in Prosite (PS0187).

Accession RS05156 encodes a protein which shares homology with a domain of 220 amino acid (aa) residues present in the secA-related protein from *Vibrio cholerae* (accession TrEMBL: Q9KLW3). In addition, the C-terminus of this protein harbors the Sec-C motif present in the SecA protein (Interpro IPR004027). This domain is predicted to chelate zinc and potentially to bind nucleic acids. RS05156 also is predicted to harbor a P-loop ATP/GTP binding motif (PR00017) at position 86-93.

The two remaining proteins share homology with proteins reported or suspected to play a role in the control of plant-bacterial interactions.

RS05166 has 54% identity and 72% similarity over 33 aa located at the N-terminus of the protein PthG (accession AAC24862) encoded on the pPATH plasmid from *Erwinia herbicola* pv. *gypsophilae* (Ezra et al. 2000). PthG acts to increase aggressiveness of *E. herbicola* pv. *gypsophilae* toward *gypsophilum* and as an avirulence protein in beet. RS05166

Fig. 2. Phylogenetic tree, showing the relationships between proteins from the YopJ/AvrRxv protein family. This tree was generated from a protein alignment generated by Multalin. 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; Orf5, AAF71492; *Rhizobium* sp. Y4LO, NP-443964; *Erwinia amylovora* OrfB Ea246, AAF63400; and OrfB Ea321, AAF63397.

has a homologue (RS04524) in strain GMI1000. The N-terminus of RS05166 also shares homology with the N-terminus of RS05218 from the GMI1000 proteome.

Finally, RS05162 encodes a protein subsequently referred to as PopP1, which is a member of the YopJ/P/AvrRxv family of effector proteins translocated through type III secretion pathways (Lahaye and Bonas 2001; Orth et al. 2000). These effectors are shared among mammalian and plant pathogens and have been proposed to act as cysteine proteases because they all harbor three specific amino acids at certain positions that are characteristic of the active site of such enzymes (Orth et al. 2000). A multiple alignment of the members of this family shows that, although these proteins are relatively well conserved in their central domain, they are significantly divergent both at the N and C termini (data not shown).

The *popP1* gene is predicted to encode a 41-kDa protein with a isoelectric point of 7.87 and harbors the conserved triad of amino acids characteristic for cysteine proteases (Orth et al. 2000). Analysis of the relationship between PopP1 and other related proteins performed using alignments generated by Multalin (Corpet 1988) revealed that the PopP1 protein is closely related to the avirulence proteins AvrRxv, AvrBsT, AvrXv4, and XopJ from *Xanthomonas* spp., to the AvrPpiG1 protein from *Pseudomonas syringae* pv. *Pisi*, and to the Y4LO protein from *Rhizobium* sp. NGR234 (accession NP-443964). On the other hand, although PopP1 clearly shows weak homology to members of the family found among human pathogens (YopP, YopJ, and AvrA) and from a third group consisting of three other avirulence proteins accessions (AAF63400, AAF63397, and AAF71492), identified in the plant pathogens *E. amylovora*, and *P. syringae* pv. *syringae*, respectively, it is nevertheless evolutionarily much more distant to the latter groups (Fig. 2).

PopP1 encodes an Avr protein which modulates aggressiveness of the bacterium toward host plants.

In order to investigate whether genes located within ACUR RS05177 are involved in the control of plant–bacteria interac-

tions, three mutants were constructed. The *popP1*-deficient mutant GMI1658 was generated by insertion of the Ω interposon at the *Bam*HI restriction site of the gene. The two deletion mutants GMI1695 and GMI1696 were constructed by replacing the right and left ends of the *popP1*-flanking region of the ACUR by the Ω interposon. Therefore, mutant GMI1696, in addition to lacking one copy of ISRso14, was deficient for the functions encoded by RS05161, RS05157, and RS05156; and mutant GMI1695 was deficient for RS05166, RS05169, RS05171, and RS05178 but still retained RS06018 (Fig. 1C).

These mutants were compared with the wild-type strain for their ability to induce a hypersensitive response (HR) on tobacco and to cause wilting on tomato and *Arabidopsis thaliana* ecotype Col-5. Following infiltration into tobacco leaves, none of the three mutants were affected in the HR-inducing ability (data not shown). The evolution of the average disease index from three independent pathogenicity assays performed on batches ranging from 10 to 46 *A. thaliana* plants is shown in Figure 3. The distribution of the wilting index in the population is not normal; therefore, standard deviations are inappropriate, and a nonparametric statistical test is necessary to analyze these results. The results of eight independent pathogenicity tests on *A. thaliana* ecotype Col-5 and *Lycopersicon esculentum* cv. Supermarnade using the χ^2 test, where the plants are noted simply as healthy or diseased, is summarized in Table 1. Apparition of the first symptoms and development of the disease reproducibly occurred 1 or 2 days earlier among plants that were inoculated with the *popP1* mutant compared with plants inoculated with the wild-type parent, but the time required for appearance of the first symptoms differed slightly from one experiment to another, probably depending on variation on the plant growth stage at inoculation time between the different batches of plants used for the different assays and the bacterial inoculum density used. In contrast, no clear phenotypic trait was observed following inoculation of the two deletion mutants GMI1695 and GMI1696 on tomato plants or on *A. thaliana* ecotype Col-5 (data not shown).

Fig. 3. Aggressiveness of the *popP1* deficient mutant on *Arabidopsis thaliana* Col-5 evolution of the average disease index over time of plant inoculated with the wild type strain GMI1000 (bars) and with the *popP1* mutant GMI1658 (triangles). Values are averages of three independent assays.

Different lines of both *Arabidopsis* (Deslandes et al. 1998) and *Petunia* (Arlat et al. 1994) show variation in their responses to inoculation with strain GMI1000. The PopP1 protein shares homology with a class of avirulence proteins; therefore, we compared the ability of the wild-type strain and of its *popP1* mutant derivative to cause disease on the resistant line of *Petunia* St40 and on the resistant ecotype Nd-1 of *Arabidopsis*. The *popP1* mutant became virulent on line St40 and avirulence was restored when the mutant was complemented with plasmid pGMI3623, which harbors a functional *popP1* gene (Fig. 4A). Therefore, the *popP1* gene behaves like a classical avirulence gene. This was further confirmed when plasmid pGMI3623 was introduced into strain Rd15 of *R. solanacearum*, a strain virulent for *Petunia* St40 (Fig. 4B). The transconjugants thus obtained had lost their ability to wilt *Petunia* St40. On the other hand, no phenotypic difference was observed between the wild-type strain and its *popP1* mutant derivative when the bacteria were tested for virulence on *Arabidopsis* ecotype Nd-1, which remained resistant to both strains (data not shown).

PopP1 is secreted via the TTSS of *R. solanacearum*.

The data presented above strongly suggest that, like other members of the YopJ/AvrRxv protein family, PopP1 might be translocated through the TTSS encoded by *hrp* genes. In order to test this hypothesis, Western blot analyses were conducted to compare the partitioning of PopP1 between bacterial cells and concentrated culture supernatants in the wild-type strain GMI1000 and in the two *hrp* mutants GMI1402 (*hrpV*) and GMI1410 (*hrpY*) after growth in minimal medium which is known to induce TTSS. This was done using a polyclonal antiserum which we raised against purified PopP1 protein. These experiments established that the PopP1 protein is present in the culture medium from the wild-type strain but not from the *hrp* mutants (Fig. 5). On the other hand, a clear band was detected in cell lysates prepared from the wild-type strain and its *hrp* derivatives, demonstrating that the PopP1 protein actually is secreted through the TTSS. As a control, we found that this band was absent from the culture supernatant and the cell lysate of the *popP1* mutant, although a weak (nonspecific) band with a similar electrophoretic mobility still was observed in the cell lysate of the mutant. The *popP1* mutant was not affected in its ability to secrete other type III-secreted proteins such as PopA (Fig. 5B). In additional experiments using β -galactosidase-producing

strains, we ruled out the possibility that the presence of PopP1 in the culture supernatant could be due to cell lysis, because no β -galactosidase was detected in the supernatants from bacterial cultures (data not shown).

popP1 does not belong to the *hrpB* regulon.

So far, three effector proteins (PopA, PopB, and PopC) which are secreted via *R. solanacearum* TTSS have been identified. All three are encoded within a single operon which belongs to the *hrpB* regulon (Guneron et al. 2000). Genes belonging to this regulon are not expressed when the bacterium is grown in rich culture medium and require the presence of the transcriptional activator HrpB to be transcribed during growth in minimal medium. A consensus motif (TTCGN₁₆TTCG) recently has been identified in the promoter of *hrpB*-regulated genes (S. Cunnac, S. Genin, and C. Boucher, unpublished data). Such a motif is present 203 to 178 bp upstream of the *popP1* start codon, suggesting that this gene might belong to the *hrpB* regulon. In order to test this hypothesis, a transcriptional gene fusion was generated between the *popP1* promoter and a promoterless *lacZ* reporter gene. This construction was cloned in the wide host range vector pLAFR6, thus generating plasmid pGMI3622, which then was introduced into the wild-type strain GMI1000. The resulting transformant was tested for β -galactosidase activity during growth in rich and minimum media. In contrast to a control construction where the reporter gene was driven by the typically *hrpB*-regulated promoter of *hrpQ*, growth in rich medium of the strain carrying the *ppopP1::lacZ* did not affect expression of β -galactosidase activity (Fig. 6A). In addition, expression of the *ppopP1::lacZ* fusion was not affected in minimal medium when plasmid pGMI3622 was introduced into an *hrpB*-deficient mutant (Fig. 6B). This clearly demonstrates that transcription of the *popP1* gene does not require a functional HrpB protein to be present and thus proves that this gene does not belong to the *hrpB* regulon.

popP1 transcription is not dependent on regulatory genes acting upstream of *hrpB* in the regulatory cascade.

We previously identified a set of regulatory genes acting upstream of *hrpB* (Brito et al. 1999, 2002; Marenda et al. 1998). Among these, the *prhA* gene, which acts as a sensor for contact of the bacterium with plant cells (Aldon et al. 2000) and *hrpG*, which acts immediately upstream of *hrpB*, are probably involved in the control of *hrp*-independent pathogenicity functions (Vasse et al. 2000). Therefore, we investigated whether transcription of the *popP1* gene could be placed under the control of one of these two genes. We first introduced plasmid pGMI3622 into the *hrpG* mutant GMI1578 and compared the level of β -galactosidase activity produced during growth in minimal medium with that produced under the same conditions when the plasmid was present in the wild-type strain GMI1000. No significant effect of the *hrpG* mutation was observed in this experiment (Fig. 7).

Similarly, a *prhA* mutation had no effect on the activity of the *popP1* promoter when bacteria were grown in co-culture with *A. thaliana* plant cells (data not shown).

Therefore, the *popP1* gene appears to be expressed constitutively under all of the conditions that we tested and it is very likely that none of the transcriptional activators of the *hrp* regulation cascade as defined by Brito and associates (2002) are required for its expression.

DISCUSSION

Like other plant pathogens, *R. solanacearum* harbors avirulence genes.

We show that the PopP1 protein in the strain GMI1000 of *R. solanacearum* is a TTSS-dependent effector which acts as

Table 1. Comparison of the aggressiveness of a *popP1* mutant strain of *Ralstonia solanacearum* with its wild-type progenitor in different host plants

Test no.	Plant line (inoculum density) ^a	DPI ^b	No. diseased/ no. inoculated plants ^c
1	Col-0 (high)	8	23/35***
2	Col-0 (high)	6	20/46***
3	Sm (high)	3	15/48**
4	Sm (high)	3	12/50*
5	Sm (high)	3	10/40 ^d
6	Sm (medium)	4	27/48***
7	Sm (medium)	4	70/80***
8	Sm (low)	4	39/80 ^d

^a *Arabidopsis* (tests 1–2) ecotypes or tomato cv. Supermarmande (tests 3–8) per milliliter inoculated with differing densities of bacterial cultures of GMI1000/*popP1::* (high = 108/ml., medium = 107/ml., low = 106/ml).

^b DPI = days post inoculation. Notation of symptoms on the day showing the clearest difference between GMI1000/*popP1::* and GMI1000 wild-type inoculations.

^c The number of diseased plants was counted and compared, by the χ^2 test, to a similar number of plants inoculated with the wild-type strain (data for the latter is not shown). The level of significance is indicated by asterisks; * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$.

^d The χ^2 test could not be applied because none of the plants in the wild-type control had wilted at this time point.

an avirulence factor toward *Petunia* St40. In contrast to the structural genes for the PopA, PopB, and PopC TTSS-dependent effectors previously characterized in this bacterium, the *popP1* gene is not located in the vicinity of the *hrp* gene cluster on the megaplasmid but on the bacterial chromosome. This validates our previous prediction made from genome analysis (Salanoubat et al. 2002) that, in *R. solanacearum*, both replicons are involved in the production of TTSS-dependent bacterial effectors.

We show that *popP1* is a canonical avirulence gene because it controls host specificity in a plant genotype-dependent pattern, acting as an avirulence gene toward *Petunia* St40 but not toward sensitive *Petunia* Tr66 (Arlat et al. 1994) or the resistant ecotype Nd-1 of *A. thaliana* (Deslandes et al. 2002).

In contrast, the *avrA* avirulence gene previously identified in *R. solanacearum* strain AW1 showed no differential response among cultivars of *Nicotiana tabacum* (Carney and Denny 1990). We hereby present the first clear evidence that

mechanisms which govern host-specificity in *Pseudomonas* and *Xanthomonas* leaf pathogens may be shared in rhizospheric *Ralstonia*. As a corollary, we might expect that major dominant resistance genes to *R. solanacearum* acting in a “gene-for-gene” manner probably are present in plants, although they had not yet been clearly identified among crop plants. Existence of a novel recessive resistance gene to *R. solanacearum* has also been exemplified recently with the cloning of a resistance gene from *A. thaliana* for which the matching avirulence gene also remains to be identified (Deslandes et al. 2002).

Does PopP1 act as a weak avirulence protein in some cases?

When inoculated on tomato and on the compatible ecotype Col-5 of *A. thaliana*, the *popP1*-deficient mutant was found to be more aggressive than the wild-type strain. This could be explained if *popP1* is recognized as a weak avirulence gene in

Fig. 4. Avirulence function of *popP1*. **A**, Behavior of petunia St40 inoculated with the wild-type strain GMI1000 (1), with the *popP1* mutant GMI1658 (2), and with this mutant complemented with plasmid pGMI3623 which carries a functional *popP1* gene (3). **B**, Virulence of strain Rd15 (1) and of a transconjugant carrying plasmid pGMI3623 (2) on petunia St40. Pictures were taken 15 days post inoculation.

these plants, a notion which is in agreement with the discovery of more complex polygenic resistance to bacterial wilt in tomato (Mangin et al. 1999; Wang et al. 2000).

Could PopP1 be a pathogenicity effector?

The discovery of *popP1* in *R. solanacearum* extends the distribution of proteins of the YopJ/AvrRxv family to the group of β -proteobacteria. Members of this family previously had been identified in the α - (*Rhizobium*) and γ - (*Salmonella*, *Yersinia*, *Erwinia*, *Xanthomonas*) subdivisions of proteobacteria. Among these bacteria, members of YopJ/AvrRxv family are found specifically in bacteria that have a TTSS and that do interact with plant or animal cells. Considering the wide conservation of this gene family among bacterial pathogens (and the bacterial symbiont *Rhizobium*), it seems likely that these proteins may play a crucial role in host colonization. This actually has been demonstrated for YopP in *Yersinia enterocolitica* (Denecker et al. 2001). Our data establish that *popP1* is not essential for pathogenicity of strain GMI1000 on tomato or *Arabidopsis* spp. However, no definitive conclusion can be drawn concerning the contribution of PopP1 to pathogenicity functions because a second ORF (RS04882) potentially encoding a member of this protein family has been identified in the genome of strain GMI1000 (Salanoubat et al. 2002). Further experiments, including the generation of a mutant inactivat-

ing both *popP1* and the gene coding for its homologous protein PopP2, are required before the status of the PopP1 protein in the control of plant-bacteria interactions can be definitively established. Similar double and triple mutants also are needed before a definitive conclusion can be drawn concerning RS05166, for which two homologous proteins are encoded in the genome of strain GMI1000 (Salanoubat et al. 2002).

Two classes of hrp effectors can be defined based on regulation patterns.

We show that the *popP1* gene is expressed in *R. solanacearum* independently of the transcriptional activator HrpB. This is reminiscent of the situation found for *avrRxv* in *Xanthomonas campestris*, where this gene is expressed constitutively, independently of the transcriptional activator HrpX and of integrity of the PIP-box present upstream of the gene (Ciesiolka et al. 1999). The constitutive expression of *popP1* contrasts with the status of the *popABC* operon which belongs to the *hrp* regulon (Guneron et al. 2000). Accordingly, based on the regulatory pattern of the corresponding structural genes, at least two classes of type III effectors can be distinguished in *R. solanacearum*. Whether the constitutive expression of a subset of effectors might have a biological significance, for example in the timing of production or secretion of different effector proteins during the infection process, remains to be established.

Fig. 5. Hrp-dependent secretion of the PopP1 protein. Western blots of concentrated supernatant preparations (CSPs) (lanes 2, 5, 7, 9) and of cell free lysates (lanes 1, 4, 6, 8) from the wild-type strain GMI1000 (wt), the *hrpV* mutant GMI1492 (*hrpV*), the *hrpY* mutant GMI1410 (*hrpY*), and the *popP1* mutant GMI1658 (*popP1*) using antibodies raised against the **A**, PopP1 and **B**, PopA proteins. Lane 3 in **A** is a control for the electrophoretic mobility of purified PopP1 protein.

ACUR RS05177 might be part of a larger pathogenicity island.

ACUR RS05177 displays several features characteristic of a pathogenicity island (PAI) (Hacker et al. 1997): (i) it has a base composition that is shifted 10% from the average G+C% of the genome; ii) in addition to *popP1*, two other genes (RS05166, a homologue of the *pthG* gene of *E. herbicola* [Ezra et al. 2000] and RS05156, a homologue of a *V. cholerae* protein [Heidelberg et al. 2000]) are likely to play a role in plant-bacteria interactions; and (iii) an insertion sequence is present in this element. However, ACUR RS05177 might itself be part of a larger PAI extending 75 kb downstream of *popP1* and bordered by the *ssrA* gene which codes for a bacterial tmRNA (previously known as 10Sa RNA in *Escherichia coli*) (Komine et al. 1994). In *Dichelobacter nodosus* and *V. cholerae*, the *ssrA* gene is thought to be a target site for horizontal transfer of virulence genes, because virulence or virulence-related gene clusters are found physically linked to this locus (Cheetham and Katz 1995; Hacker et al. 1997; Kovach et al. 1996). Extension of the PAI down to the *ssrA* locus also is justified by the presence of two additional ACURs, several bacteriophage remnants, and two additional insertion sequences in this region. It also would explain why the borders of ACUR RS05177 do not correspond to the location of the G+C% shift around the ACUR (Fig. 1A).

MATERIALS AND METHODS

Molecular biology techniques.

Standard methods were used (Ausubel et al. 1990) unless otherwise stated. Genomic DNA was extracted from *R. solanacearum* strains as described by Arlat and associates (1992). Polymerase chain reaction (PCR) amplifications were done using the Expand Long Template PCR System (Roche Molecular Systems, Inc. Branchburg, NJ, U.S.A.).

Bacterial strains, plasmids, and growth conditions.

Bacterial strains and plasmids used in this study are listed in Table 2. *E. coli* strains were grown in Luria-Bertani medium

(Sambrook et al. 1989) at 37°C. *R. solanacearum* strains were grown at 28°C on either B medium or minimal medium (Boucher et al. 1985) supplemented with 20 mM glutamate (MMG). When indicated, MMG was supplemented with Congo red (100 µg ml⁻¹). Antibiotics were used at the following concentrations: tetracycline, 10 mg l⁻¹; kanamycin, 50 mg l⁻¹; ampicillin, 50 mg l⁻¹; spectinomycin (Sp), 50 mg l⁻¹; and streptomycin (Sm), 50 mg l⁻¹.

Bacterial transformation.

Transformation of strains of *R. solanacearum* leading to homologous recombination of the incoming DNA was performed according to the method described by Boucher and associates (1985). Plasmids were introduced into *R. solanacearum* strains by electroporation (2.5 kV, 200 ohms, 25 µF, 0.2-cm cuvette gap).

Sequence analysis.

Homology searches and PROSITE pattern searches were performed using the NCBI Blast server.

Construction of bacterial strains carrying mutations in and around *popP1*.

The *popP1* gene was disrupted by insertion of the Ω interposon. A 1,655-bp DNA fragment containing the *popP1* gene was amplified by PCR using strain GMI1000 genomic DNA as template and primers A1 (5'-AAGCTTAAGAGTTGCGGTGGC-3') and A2 (5'-TCTAGAAATGTAGAAGGAAGC-3'). The underlined sequences correspond to restriction sites: *Hind*III in primer A1 and *Xba*I in primer A2. After amplification, the DNA fragment was digested with *Hind*III and *Xba*I and cloned into *Hind*III-*Xba*I-digested pBluescript, generating plasmid pBluescript(*popP1*). This plasmid was partially digested with *Bam*HI and the digestion product was ligated with the Ω interposon purified after *Bam*HI digestion of plasmid pHP45Ω (Prentki and Krisch 1984). This interposon confers resistance to Sp and Sm. Clones which contained a single Ω insertion in the *Bam*HI site of *popP1* were selected based on restriction profile analysis. This construct then was linearized

Fig. 6. The *popP1* gene does not belong to the *hrpB* regulon. **A**, Effect of culture medium and **B**, dependence of *popP1* transcription on *hrpB*). **A**, Comparative β-galactosidase activity of a *popP1::lacZ* transcriptional fusion carried by plasmid pGMI3622 in strain GMI1000 grown in minimal medium (unfilled columns) and B medium (gray columns) or **B**, in minimal medium in a wild-type strain versus the *hrpB*-deficient mutant GMI1525 (hatched columns). The β-galactosidase activity driven by the *hrpQ* promoter carried on plasmid pGMI1957 under similar conditions is given as a control for a gene belonging to the *hrpB* regulon. Bars correspond to standard error.

by digestion with *Xba*I and used to transform *R. solanacearum* strain GMI1000, selecting for Sm resistance.

In order to construct strain GMI1695, which is deleted for RS05166, RS05169, RS05171, and RS05178, a 973-bp DNA fragment spanning from position 866,236 to 867,209, upstream of the *popP1* start codon, was generated by PCR using GMI1000 genomic DNA as a template and primers P1-*Hind*III (5'-AAGCTTGCAGAGGCAAACTCT-3') and P2-*Xba*I (5'-TCTAGACGTTGCGAGATTCCA-3'). The PCR product was digested by *Hind*III and *Xba*I and cloned into the *Xba*I-*Hind*III digested pBluescript SK(-) vector, generating pBSF1. In addition, the 815-bp DNA fragment spanning from position 862,809 to 863,624 was amplified using primers dela-*Hind*III (5'-AAGCTTTAGCTTATTTT-3') and delb-*Xho*I (5'-CTCGAGAGATGGCTGACGCGTAAT-3'). The PCR product was digested by *Xho*I and *Hind*III and cloned into pBSF1 digested with *Xho*I and *Hind*III, thus generating plasmid pBSF2. A *Hind*III-restricted Ω interposon then was cloned into the

*Hind*III restriction site of pBSF2, generating plasmid pBSF3. This last plasmid was linearized by *Xba*I and used to transform *R. solanacearum* GMI1000, selecting for Sm resistance.

Mutant strain GMI1696 was constructed by deletion of all the ORF located downstream of *popP1* in the ACUR RS05177. The 754- and 789-bp fragments spanning positions 867,802 to 868,556 and 874,886 to 875,675, respectively, were amplified by PCR using the respective pairs of primers D-*Sma*I, *Bam*HI, *Eco*RI (5'-CCCGGGGGATCCGAATTCGTCATCTCTGACGTAGCC-3'), and D-*Xba*I (5'-TCTAGAATACCGAACTGGCGAGTG-3') on one side and U-*Hind*III (5'-AAGCTTATCCCTAAGGGCCATCAG-3'), U-*Eco*RI, *Bam*HI, and *Sma*I (5'-GAATTCGGATCCCCCGGGTGTTCCTCCACTCGCA-3') on the other side. The overlapping complementary sequences of fragment D and U were combined and the resulting 1,543-bp D-*Xba*I/U-*Hind*III fragment was amplified in a third PCR using D-*Xba*I and U-*Hind*III primers as described by Higuchi (1990). The D-*Xba*I/U-*Hind*III fragment

Fig. 7. Transcription of *popP1* is independent of the HrpG transcriptional activator. Comparison of β -galactosidase activity of the *ppopP1*::*lacZ* fusion carried by plasmid pGMI3622 when expressed in the wild-type strain GMI1000 (white columns) or in the *hrpG* mutant GMI1578 (vertical hatched columns) under inducing conditions. Activity driven by the *hrpQ* promoter carried on plasmid pGMI1957 under similar conditions is given as a comparison with a *hrpG* regulated gene. Bars correspond to standard error.

Table 2. Bacterial strains and plasmids

Strain/plasmid	Relevant characteristics ^a	Reference/source
Bacteria		
<i>Escherichia coli</i>		
DH5 α	F– <i>recA lacZ</i> AM15	Bethesda Research Laboratories Stratagene
BL21-CodonPlus(DE3)-RIL	F– <i>ompT hsdS</i> (rB–mB–) <i>dcm</i> + Tc ^r <i>gal</i> λ (DE3) <i>endA</i> Hte [<i>argU ileY leuW</i> Cam ^r]	
<i>Ralstonia solanacearum</i>		
GMI1000	Wild type	Boucher et al. (1985)
GMI1402	Tn5-B20:: <i>hrpV</i> mutant	Arlat et al. (1992)
GMI1410	Tn5-B20:: <i>hrpY</i> mutant	Arlat et al. (1992)
GMI1525	<i>hrpB</i> :: Ω mutant	Genin et al. (1992)
GMI1575	<i>prhA</i> :: Ω mutant	Marenda et al. (1998)
GMI1578	<i>hrpG</i> :: Ω mutant	Brito et al. (1999)
GMI1658	<i>popP1</i> :: Ω mutant	This work
GMI1695	Mutant in upstream <i>popP1</i> ORFs in the ACUR	This work
GMI1696	Mutant in downstream <i>popP1</i> ORFs in the ACUR	This work
Rd15	Wild type	S. T. Hsu, A.V.R.D.C., Tainan, Taiwan
Plasmids		
pHP45 Ω	Sp ^r , Sm ^r , Ω	Prentki and Krisch (1984)
pLAFR6	pLAFR1 with <i>trp</i> terminators, Tc ^r	Huynh et al. (1989)
pCZ205	pUC19 carrying <i>lacZ</i> in <i>Kpn</i> I- <i>Xba</i> I, Amp ^r	This work
pGEM-T	Cloning vector, Amp ^r	Promega
pbluescript II SK(-)	Amp ^r	Stratagene
pGMI1957	pLAFR6 carrying <i>hrpQ</i> promoter/ <i>lacZ</i> fusion	Van Gijsegem et al. (1995)
pGMI3622	pLAFR6 carrying <i>popP1</i> promoter/ <i>lacZ</i> fusion in <i>Hind</i> III- <i>Kpn</i> I	This work
pGMI3623	pLAFR6 carrying a 2.08 kb <i>Hind</i> III- <i>Kpn</i> I fragment containing <i>popP1</i> gene	This work

^a Tc = tetracyclin, ORFs = open reading frames, ACUR = alternative codon usage region, Sp = spectinomycin, Sm = streptomycin, and Amp = ampicillin.

was directly cloned into the pGEM-T vector, giving the pGEM-TDU plasmid. The *EcoRI*-restricted Ω integron then was cloned into the *EcoRI*-digested pGEM-TDU. The resulting plasmid was linearized by *XbaI* digestion and used to transform *R. solanacearum* GMI1000.

In the three mutants thus generated, gene replacement was confirmed by Southern hybridization or PCR (data not shown).

Plasmid constructions.

Plasmid pCZ205 is a pUC19 derivative in which a fragment *XbaI-KpnI* containing the Tn5-*lacZ* transposon (Simon et al. 1989) has been cloned.

Plasmid pGMI3622 is a pLAFR6 derivative (Huynh et al. 1989) carrying a *lacZ* coding sequence placed under the control of the *popP1* promoter. The *popP1* promoter/*lacZ* fusion was generated by cloning the 973-bp DNA fragment located immediately upstream of the *popP1* start codon by PCR amplification with P1-*HindIII* and P2-*XbaI* primers, into the *HindIII-XbaI*-digested pCZ205 plasmid. The fragment *HindIII-KpnI* containing the *popP1* promoter/*lacZ* fusion then was cloned into the *HindIII-KpnI*-digested pLAFR6 plasmid (Huynh et al. 1989), giving the pGMI3622 plasmid.

Plasmid pGMI3623 is a pLAFR6 derivative (Huynh et al. 1989) in which a 2.08-kb fragment containing a *popP1* gene driven by its own promoter has been inserted between the *HindIII* and *KpnI* cloning sites. This insert was generated by PCR using primers P1-*HindIII* and P3-*KpnI* (5'-GGTACCTCAGACTCCAGGGCATG-3') and strain GMI1000 genomic DNA as template.

β -Galactosidase assays.

β -Galactosidase activities in bacterial cultures grown in minimal medium at 28°C for 18 h were assayed as previously described by Arlat and associates (1992). Activities also were measured upon coculture with *A. thaliana* At-202 cell suspensions following the procedure described by Marenda and associates (1998).

Plant cell cultures and bacterial-plant cell co-cultures.

A. thaliana At-202 cell suspensions were grown in Gamborg B5 (Flow Laboratories) as previously described by Marenda and associates (1998).

Plant tests.

HR tests on tobacco leaves and pathogenicity assays on *Arabidopsis* ecotypes Col-5 and Nd-1, *Petunia*, or tomato plants were performed as previously described by Marenda and associates (1998). Wilting symptoms were scored daily using a disease index scale ranging from 0 to 4 according to the percentage of wilted leave: (0 = no wilt, 1 = 1 to 25%, 2 = 26 to 50%, 3 = 51 to 75%, 4 = >75%) (Arlat and Boucher 1991).

Production of antibodies.

The *popP1* gene was amplified from the genomic DNA of *R. solanacearum* strain GMI1000 by PCR using primers 5'-GCTCT-AGAATGAAAAGACTATTTCAGA-3' and 5'-CGCGAAGCTTTCACGACTCCAGGGCATG-3'. The PCR fragment was digested with *XbaI-HindIII* and ligated into similarly cleaved pRDS vector, a modified pET28 expression vector that furnished an N-terminal His₆ tag. *E. coli* BL21(DE3)-RIL (Stratagene, La Jolla, CA, U.S.A.) cells were transformed and these produced recombinant PopP1 after induction with 1 mM IPTG. PopP1 was expressed at a very high level as an insoluble protein. Cells were lysed by sonication in 50 mM Tris, pH 8.0, 1% Triton X-100, 10 mM imidazole, and 50 mM NaCl (TIN buffer). Inclusion bodies were harvested by centrifugation (15,000 $\times$ g) and solubilized with 6 M urea in TIN buffer. After centrifugation, the lysate was

loaded onto a 5-ml Ni²⁺ affinity column (Pharmacia) preequilibrated with 6 M urea in TIN buffer. After extensive washing, PopP1 was eluted with 300 mM imidazole in 50 mM Tris, pH 8, 6 M urea, and 50 mM NaCl. Fractions containing the eluted protein were pooled, diluted 20 times in 20 mM Tris, pH 7.5, 0.1% sodium dodecyl sulfate (SDS), and concentrated to 0.5 mg/ml, using Centrprep 10 (Amicon). This preparation was used to immunize rabbits according to Ausubel and associates (1990).

Western Blots.

Strain GMI1000 and derivatives were grown at 28°C for 20 h in MMG supplemented with Congo red (100 μ g ml⁻¹). Concentrated supernatant preparations (CSP) and cell-free lysates were obtained from these cultures as described by Guéneron and associates (2000), except that one-fourth of these CSP were used for SDS-polyacrylamide gel electrophoresis.

The PopA-specific antiserum used in this study has been described previously (Arlat and associates 1994). A commercial polyclonal antiserum (Sigma Aldrich, St. Louis) was used for the detection of β -galactosidase.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Ralstonia solanacearum strain GM1100 annotated complete genome sequence: <http://sequence.toulouse.inra.fr/R.solanacearum>.