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# PrhG, a Transcriptional Regulator Responding to Growth Conditions, Is Involved in the Control of the Type III Secretion System Regulon in *Ralstonia solanacearum*▽†

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**The ability of *Ralstonia solanacearum* to cause disease in plants depends on its type III secretion system (T3SS). The expression of the T3SS and its effector substrates is coordinately controlled by a regulatory cascade, at the bottom of which is HrpB. Transcription of the *hrpB* gene is activated by a plant-responsive regulator named HrpG, which is a master regulator of a wide array of pathogenicity functions in *R. solanacearum*. We have identified in the genome of strain GMI1000 a close paralog of *hrpG* (83% overall similarity at the protein level) that we have named *prhG*. Despite this high similarity, the expression pattern of *prhG* is remarkably different from that of *hrpG*: *prhG* expression is activated after growth of bacteria in minimal medium but not in the presence of host cells, while *hrpG* expression is specifically induced in response to plant cell signals. We provide genetic evidence that *prhG* is a transcriptional regulator that, like *hrpG*, controls the expression of *hrpB* and the *hrpB*-regulated genes under minimal medium conditions. However, the regulatory functions of *prhG* and *hrpG* are distinct: *prhG* has no influence on *hrpB* expression when the bacteria are in the presence of plant cells, and transcriptomic profiling analysis of a *prhG* mutant revealed that the PrhG and HrpG regulons have only one pathogenicity target in common, *hrpB*. Functional complementation experiments indicated that PrhG and HrpG are individually sufficient to activate *hrpB* expression in minimal medium. Rather surprisingly, a *prhG* disruption mutant had little impact on pathogenicity, which may indicate that *prhG* has a minor role in the activation of T3SS genes when *R. solanacearum* grows parasitically inside the plant. The cross talk between pathogenicity regulatory proteins and environmental signals described here denotes that an intricate network is at the basis of the bacterial disease program.**

In order to successfully colonize plants, bacterial pathogens must deploy a genetic program to express virulence genes, which enable adaptation to living conditions inside the host (25). A set of genes that is highly expressed upon contact with the eukaryotic host is the type III secretion system (T3SS) present in many plant and animal bacterial pathogens. This system is a major genetic determinant of disease development, as it translocates bacterial proteins—called effectors—into host cells to interfere with cellular defense responses and facilitate bacterial colonization (reviewed in references 12, 20, and 39). In bacterial plant pathogens, the T3SS is encoded by a cluster of some 20 *hrp* genes named after the inability of mutants deficient in them to cause a hypersensitive response in resistant plants or pathogenicity in susceptible hosts (21). In all of the species studied, the transcription of the T3SS and most of its effector substrates is tightly controlled by a succession of consecutively activated regulators.

*Ralstonia solanacearum* is the agent that causes bacterial wilt in more than 200 plant hosts, including important crops such as potato and tomato, and has long been studied as a model plant vascular pathogen (10, 14). In *R. solanacearum* GMI1000, the regulatory cascade driving T3SS gene expression has been very well characterized (see references 6, 32, and 38 for a review). In this bacterium, the transcription of type III secretion genes is regulated by the HrpB regulator, which belongs to the AraC family (16). HrpB may directly bind to a specific $hrp_{II}$ box found in the promoters of most of its target genes (8). The transcription of the *hrpB* gene is activated by the HrpG transcriptional activator, which belongs to the OmpR/PhoB subfamily of two-component response regulators (7, 13). The key role of the HrpB and HrpG regulators is illustrated by the fact that mutant strains deficient in any of them are nonpathogenic (7, 16, 35). Activation of the T3SS regulatory cascade in *R. solanacearum* is controlled by the outer membrane receptor protein PrhA. It has been shown that PrhA senses an unidentified, nondiffusible, plant-specific signal and transfers the activating signal through PrhR, PrhI, and PrhJ to drive HrpG transcription (1, 6).

Transcription of *hrp* genes is much higher when bacteria are cultured in defined minimal media rather than complete media (3, 30). This induction pattern seems to be general in all of the plant pathogens studied, and it has been attributed to the fact that minimal media somehow mimic the conditions encountered in the apoplastic environment (30, 32). In *R. solanacearum*, this metabolic regulation is superimposed on the induction caused by plant cell contact and seems to be integrated into the cascade by HrpG, HrpB, or both (7, 15, 16). This conclusion is based on the observation that only *hrpB* and *hrpG*, and not the upstream components of the *hrp* regulatory

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TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristic(s) | Reference or source |
|---|---|---|
| *E. coli* DH5α | F$^-$ *recA lacZ*ΔM15 | Bethesda Research Laboratories |
| *R. solanacearum* | | |
| GMI1000 | Wild-type strain | 31 |
| GMI1425 | *hrpG*::Tn*5*-B20 mutant | 7 |
| GMI1421 | *hrpY*::Tn*5*-B20 mutant | 33 |
| GMI1475 | *hrpB*::Tn*5*-B20 mutant | 16 |
| GMI1525 | *hrpB*::Ω mutant | 16 |
| GMI1613 | *popA*::Tn*5*-B20 mutant | 4 |
| GMI1755 | GMI1000 Δ*hrpG* mutant | 34 |
| GRS404 | *prhG*::*lacZ* mutant | This work |
| GRS445 | GMI1000 Δ*prhG* mutant | This work |
| GRS491 | GMI1000 Δ*hrpG* Δ*prhG* mutant | This work |
| GRS497 | RSp0201::*lacZ* mutant | This work |
| Plasmids | | |
| pBluescript KS(+) | Cloning vector, Amp$^r$ | Stratagene |
| pLAFR6 | pLAFR1 with trp terminators, Tc$^r$ | 18 |
| pBBL12 | pLAFR6 with a 1.5-kb PstI fragment carrying *hrpG* | 7 |
| pCZ367 | pUC18-derived vector used for insertional mutagenesis, Ap$^r$ Gm$^r$ | 9 |
| pCM184 | Allelic exchange vector, Km$^r$ | 23 |
| pGG15 | pLAFR6 carrying the *cre* recombinase gene | This work |
| pSG371 | pCZ367 carrying a 0.5-kb HindIII-XbaI *prhG* fragment | This work |
| pLP2 | PCR fragment containing the *prhG* gene and 500 bp upstream the predicted coding sequence cloned in pLAFR6, Tc$^r$ | This work |
| pLP5 | PCR fragment containing the *prhG* gene and 500 bp upstream the predicted coding sequence cloned in pBBL12, Tc$^r$ | This work |

cascade, are required for *hrp* gene induction when the bacteria are grown in minimal medium.

Bacterial plant pathogens of the genus *Xanthomonas* share part of the *hrp* regulatory cascade of *R. solanacearum*, in contrast to other bacterial plant pathogens, where T3SS transcriptional control involves $\sigma^{54}$-dependent enhancer-binding regulators (32). Indeed, the HrpG and HrpB (named HrpX) regulators and their functions have been found to be conserved in *Xanthomonas campestris* pv. *vesicatoria*, although the upper components of the cascade have not yet been identified (36, 37). More generally, HrpX and HrpG orthologs have been found in all of the genome sequences of *Xanthomonas* T3SS-harboring species (29). The two different regulatory systems clearly coincide with the two subgroups into which the *hrp* gene clusters have been classified on the basis of gene conservation (2).

Recent transcriptomic profiling analyses have determined the HrpB and HrpG regulons and provided evidence that, besides activating HrpB transcription (and thus, T3SS expression), HrpG also controls the expression of other virulence determinants (33). From these studies, control of T3SS gene expression is now viewed as a regulatory network connected with other virulence determinants, rather than a linear cascade. HrpG is located at a central node in this network, integrating diverse environmental signals and controlling most of the bacterial functions that promote disease (33). In this work, we have identified in the genome sequence of *R. solanacearum* GMI1000 a new regulatory gene encoding a protein highly similar to HrpG. We have characterized this novel regulatory gene, named *prhG*, and we provide evidence that it is a new player in the complex network controlling T3SS gene expression. PrhG appears to be specifically involved in the control of the *hrpB* gene in response to environmental signals encountered by the bacteria when they are grown under minimal medium conditions.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The relevant characteristics of the plasmids and bacterial strains used in this work are listed in Table 1. *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium (5). *R. solanacearum* strains were grown in complete BG medium or in MP minimal medium supplemented with glutamate at a 20 mM final concentration. The composition of BG medium is as follows (g liter$^{-1}$): Bacto peptone, 10; Casamino Acids, 1; yeast extract, 1. For agar plates, BG medium was supplemented with glucose (5 g liter$^{-1}$) and triphenyltetrazolium chloride (0.05 g liter$^{-1}$). The composition of MP medium is as follows (g liter$^{-1}$): $FeSO_4 \cdot 7H_2O$, $1.25 \times 10^{-4}$; $(NH_4)_2SO_4$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.05; $KH_2PO_4$, 3.4. The pH was adjusted to 7 with KOH. When needed, antibiotics were added to the media at the following final concentrations (mg liter$^{-1}$): kanamycin, 50; spectinomycin, 40 for *R. solanacearum*; gentamicin, 10; tetracycline, 10; ampicillin, 100 for *E. coli*.

**DNA manipulations and genetic constructs.** Standard recombinant DNA techniques were performed as described previously (5). Restriction enzymes, DNA ligase, and other DNA enzymes were used according to the manufacturers' recommendations. PCR amplification of *prhG* with 500 bp of its promoter region was performed with *Pfx* polymerase (Invitrogen) using genomic DNA of *R. solanacearum* from strain GMI1000 and primers Amo2360 (5′-TCCCACATCGTGTCGACTT-3′) and Aval2360H3 (5′-AAGCTTTGCCTGCGCCGTCCCG-3′).

An RSp0201 disruption mutant was created as previously described (9), using integrative plasmid pCZ367, into which an internal fragment of the gene was cloned after PCR amplification using primers 0201H3 (5′-GAAGCTTCGTTA TCGAGCAAC-3′) and 0201Xba (5′-ATCTAGATCCGCCGGCTGAAG-3′). Plasmids (Table 1) were introduced into *R. solanacearum* strains by electroporation (2.5 kV, 200 W, 25 mF, 0.2-cm cuvette gap).

**Creation of an unmarked Δ*prhG* mutant strain.** A *prhG* deletion mutant was generated by using allelic exchange vector pCM184 for antibiotic marker recycling (23). Two DNA fragments flanking the *prhG* sequence were amplified by using primers 2360R1 (5′-GAATTCTCTCGCCCGGTCGGC-3′) and 2360NotI (5′-GCGGCCGCAAAGCTGGAGGGAGAGG-3′) for the left flanking frag-

ment and 2360Sc2 (5′-CCGCGGCGCATGTCGAATGGATGAA-3′) and 2360Sc1 (5′-GAGCTCTTACCCGCGTTGCAACG-3′) for the right flanking fragment.

Both fragments were cloned into pCM184, and the resulting construct was used to transform GMI1000 to generate a Δ*prhG*::*nptII* mutant. The kanamycin resistance cassette was then excised *in vivo* using plasmid pGG15, which carries a copy of the *cre* recombinase in a pLAFR6 backbone vector. The deletion of *prhG* in resulting strain GRS445 was then checked by PCR.

**Transcriptome analysis.** RNA extraction and probe labeling were performed as already described (26). The *R. solanacearum* whole-genome DNA microarray with 65/70-mer oligonucleotides specific to the annotated open reading frames (ORFs) of GMI1000 was used for all of the transcriptomic profiling experiments. Hybridizations were conducted manually under the following conditions. Microarray slides were prehybridized for 1 h at 42°C in DIG Easy buffer (Roche) containing 10 mM salmon sperm DNA. Hybridizations were carried out for 13 h under the same conditions, except that the heat-denatured and labeled probes were added to the reaction mixture. Following hybridization, slides were washed once in 1× SSC (0.15 mM NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 60°C for 10 min and once in 0.1× SSC for 1 min at room temperature. Slides were finally rinsed in isopropanol at 37°C and dried by 3 min of centrifugation at 1,000 × *g*.

Hybridized slides were scanned using a GenePix 4000B dual-channel confocal laser scanner (Axon Instruments, Union City, CA).

Signal quantification and data analysis were achieved using GenePix Pro (version 3.0; Molecular Devices, Sunnyvale, CA) and GeneSight software (version 4.1.6; Biodiscovery Inc., El Segundo, CA). For each array, following local background subtraction, the signal for each spot was normalized on the basis of the mean value of the intensity of all of the spots corresponding to *R. solanacearum* ORFs. The ratio of expression of the wild-type strain to the *prhG* deletion mutant or of the wild-type strain to the *prhG*-overexpressing mutant was calculated. The two ratios corresponding to the two replicates present on the same slide were averaged, and the averaged ratio was converted to its $\log_2$ value.

For each experimental condition (wild-type strain versus *prhG* deletion mutant and wild-type strain versus *prhG*-overexpressing mutant), at least two independent RNA extractions from independent cultures were used and a minimum of six hybridizations were performed with dye swap labeling. In order to minimize false positives, only genes with high levels of significance ($P < 0.05$ by Student's *t* test) and an absolute $\log_2$ value ratio of greater than 1.3 were considered in this study.

All of the primary data from transcriptome experiments, as well as the experimental protocols used, are available from the ArrayExpress depository (accession number E-MEXP-2456).

**Pathogenicity tests.** Pathogenicity tests by soil inoculation were performed by watering 4-week-old tomato plants (*Lycopersicum esculentum* cv. Marmande) with 50 ml of a suspension containing $10^7$ or $10^8$ CFU ml$^{-1}$. Pathogenicity tests by stem injection were performed by injecting 20 μl of a $10^6$ CFU ml$^{-1}$ bacterial suspension into the stems of 4-week-old plants. Disease tests on *Arabidopsis thaliana* accession Col-0 were performed by immersing plants with cut or uncut roots into bacterial suspensions of $10^7$ and $10^8$ CFU ml$^{-1}$.

Disease development was scored daily by using a disease index scale ranging from 0 for no symptoms to 4 for completely wilted plants (27). Within-group Kaplan-Meier survival estimates (the total number of plants surviving [i.e., with disease index scores below 3] out of the total number inoculated for each group) were computed for each time interval in order to build Kaplan-Meier survival curves for each group. The log rank test was used to perform between-group comparisons, testing the equivalence of the Kaplan-Meier survival curves for a pair of groups.

**Creation of *lacZ* reporter fusion strains.** The *prhG*::*lacZ* fusion was created by using integration plasmid pCZ367 (9). An internal fragment of the *prhG* gene was PCR amplified by using primers 2360FW (5′-GTAAGCTTCCACACGGC CGGTCAGC-3′) and 2360REV2 (5′-GTCTAGATGTGCTGCTCGATG-3′) and cloned as a 0.5-kb HindIII-XbaI fragment into pCZ367 to generate plasmid pSG371. This plasmid was used to transform *R. solanacearum* GMI1000, and a single integrative event was selected by using pCZ367 gentamicin resistance (9). Recombinant clones were then checked by PCR.

*lacZ* reporter fusions with the *hrpG*, *prhG*, *hrpB*, *popA*, and *hrpY* genes were introduced into the different genetic backgrounds (Δ*hrpG*, Δ*prhG*, and Δ*hrpG* Δ*prhG*) by transformation with the genomic DNA of the donor strains. Recombinant strains were selected on media with adequate antibiotics, and the correct genomic insertion of the *lacZ* fusion was checked by PCR.

**Plant cell cultures and bacterium-plant cell cocultures.** The *A. thaliana* At-202 (accession Col-0) and tomato Msk8 cell suspension lines were grown in Gamborg B5 (Flow Laboratories) and T-MSMO (Sigma) media, respectively (22). For the bacterium-plant cell cocultures, 10-ml samples of *Arabidopsis* and tomato cell suspensions were inoculated with *R. solanacearum*. After 16 h of incubation at 28°C, the coculture was filtered and the bacterial cells were recovered for β-galactosidase assays.

**β-Galactosidase assays.** β-Galactosidase assays were performed as described previously (4). β-Galactosidase activity was measured according to Miller (24), and values were expressed in Miller units or as a percentage of the activity of the strain carrying a *lacZ* fusion in a wild-type genetic background.

**Protein sequence analysis.** The analysis was performed with the MEGA versus 3.1 software (http://www.megasoftware.net/) using a neighbor-joining algorithm with 1,000 replications to build the tree and calculate the bootstrap values. The accession numbers of the 10 protein sequences compared in this analysis are as follows: PrhG from *R. solanacearum* GMI1000, NP_522584; PrhG from *R. solanacearum* Molk2, YP_002255075; PrhG from *R. solanacearum* UW551, ZP_00945868; HrpG from *R. solanacearum* GMI1000, CAA07190; HrpG from *R. solanacearum* Molk2, YP_002254969; HrpG from *R. solanacearum* UW551, ZP_00944901; HrpG from *Xanthomonas oryzae* pv. *oryzae* PXO99A, AAK92203; HrpG from *X. campestris* pv. *vesicatoria* 85-10, YP_363045; HrpG from *X. campestris* pv. *campestris* ATCC 33913, NP_636540; OmpR from *E. coli* K-12, AAA58202.

## RESULTS

**Identification of *prhG*, a close paralog of *hrpG*, the master regulator of pathogenicity functions in *R. solanacearum*.** Annotation of the genome of strain GMI1000 revealed the existence of a gene, RSp1023 (hereafter named *prhG*), homologous to the T3SS regulatory gene *hrpG*. A comparison of the gene products reveals that the two proteins are 72% identical (83% similar). The main difference between the two proteins is that PrhG is slightly longer, as it contains an ~50-amino-acid C-terminal extension which is absent in HrpG (Fig. 1A). Like HrpG, the PrhG protein belongs to the OmpR/PhoB family of two-component response regulators (13) and possesses a highly conserved helix-turn-helix motif DNA-binding domain (residues 173 to 205). The relatedness between PrhG and HrpG in this predicted DNA-binding domain reaches 96% amino acid identity. Also similarly to *hrpG*, no predicted sensor histidine kinase-encoding gene was found in the vicinity of *prhG*. Although *prhG* is located 200 kb away from the *hrp* gene cluster, the two immediate flanking genes (RSp1022 and RSp1024) are predicted to encode type III-dependent effectors (28), which could be suggestive of a functional link of this genomic region with the T3SS.

Analysis of the phylogenetic relationships with homologous sequences in the databases revealed that *prhG* is conserved in *R. solanacearum* strains taxonomically distant from GMI1000, such as Molk2 or UW551 (Fig. 1B). Secondly, this cladogram showed that the PrhG sequence group is closer to the *R. solanacearum* HrpG group than to the *Xanthomonas* sp. HrpG clade. This observation and the fact that the amino acid sequence identity between HrpG and PrhG is significantly higher than that between HrpG and its *Xanthomonas* orthologues (37 to 40%) support the view that *prhG* might be the result of an ancient *hrpG* duplication event that took place in *R. solanacearum* before the divergence of its various strains. Interestingly, none of the sequenced *Xanthomonas* sp. genomes was found to harbor a close paralog of HrpG, as observed in all of the *R. solanacearum* genomes investigated.

**A *prhG* mutant is slightly reduced in pathogenicity on tomato plants.** To evaluate the role of *prhG* in *R. solanacearum* pathogenicity, an unmarked deletion mutant derivative was generated from strain GMI1000. The resulting strain, GRS445,

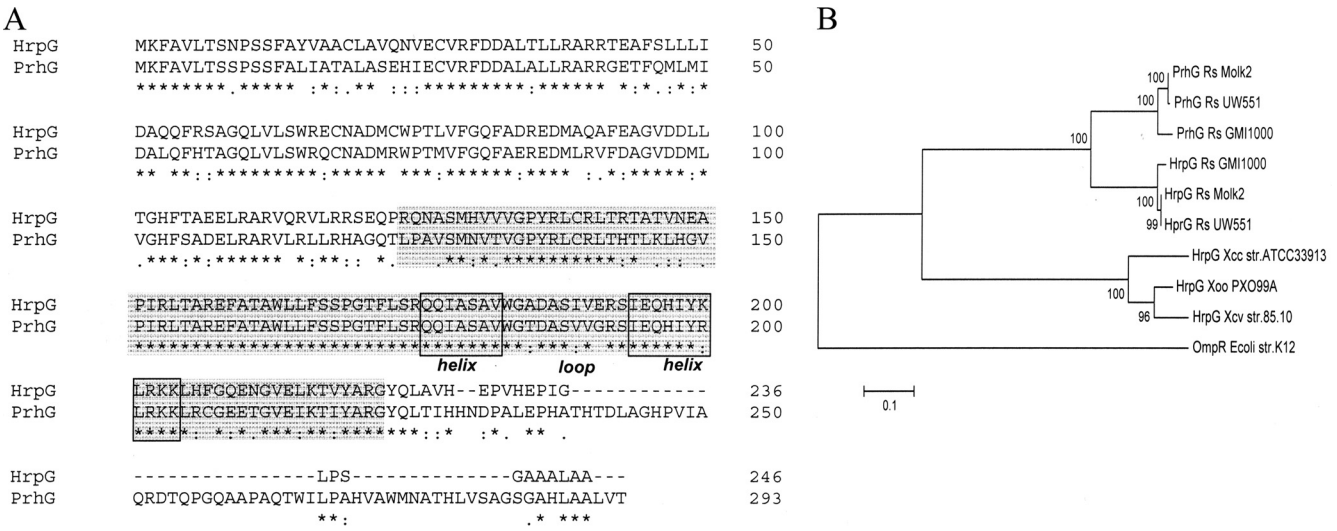


FIG. 1. (A) ClustalW analysis of homologous proteins HrpG and PrhG (72% global identity and 96% in the helix-loop-helix domain). The predicted DNA-binding and transactivation domain is highlighted in gray, and the helix-loop-helix domain is boxed. (B) Neighbor-joining analysis showing the relationships among the PrhG and HrpG protein sequences of three *R. solanacearum* strains (GMI1000, Molk2, and UW551) and three *Xanthomonas* sp. strains (*X. oryzae* pv. *oryzae* strain PXO99A, *X. campestris* pv. *vesicatoria* strain 85-10, and *X. campestris* pv. *campestris* strain ATCC 33913). The *E. coli* K-12 OmpR protein sequence was added as an outgroup. The tree was constructed and the bootstrap values were calculated by using the neighbor-joining algorithm with 1,000 replications. The asterisks, periods, and colons below the sequences in panel A indicate identical residues, conserved substitutions, and semiconserved substitutions, respectively.

was used for inoculation assays on tomato, the natural host of GMI1000. Routine infection tests were performed either by injection of bacteria into the stem or by drenching the plants with a bacterial suspension, an inoculation method which better mimics natural infection conditions.

The pathogenicity assays done by drenching tomato plants showed no significant difference in the appearance of symptoms due to the mutant compared to the wild-type strain (data not shown). A difference in virulence was observed when a bacterial suspension of $10^4$ CFU was injected directly into the stems. Under these conditions, symptoms appeared with a slight delay when the plants were inoculated with the *prhG* mutant strain compared to the wild-type strain (Fig. 2). We applied a log rank test to determine whether the disease curves obtained with the wild-type strain and the *prhG* mutant derivative were significantly different under these conditions. A score of 3 was used to discriminate between live plants (score below 3) and dead plants (score of 3 or above). With these criteria, the *P* value is 0.00997, showing that the difference between the two disease curves is indeed significant and that *prhG* is required for full pathogenicity on tomato.

Pathogenicity assays were also performed on another host plant, *A. thaliana* ecotype Columbia, which is susceptible to GMI1000. No significant difference in pathogenicity was observed between the *prhG* mutant and the wild-type strain when *A. thaliana* plants with uncut or cut roots were inoculated (data not shown).

***prhG* and *hrpG* respond to different environmental signals.** Since *R. solanacearum hrpG* was known to display a distinct expression pattern, depending on the environmental conditions used (7), we determined the expression profile of *prhG* under the same conditions. In these assays, bacteria carrying a chromosomal *lacZ* fusion to *hrpG* (strain GMI1425) or *prhG* (strain GRS404) were grown in complete medium, in minimal medium supplemented with glutamate as the sole carbon source, or in the presence of *A. thaliana* or tomato host cell suspensions. Figure 3 shows the levels of β-galactosidase activity monitored for each strain under each set of conditions. It appears that the *prhG*::*lacZ* fusion was induced in minimal medium (fivefold with respect to complete medium). However, in contrast to the specific and strong expression of the *hrpG*::*lacZ* fusion (strain GMI1425) detected in the presence of plant cells, the *prhG*::*lacZ* fusion was not induced under this

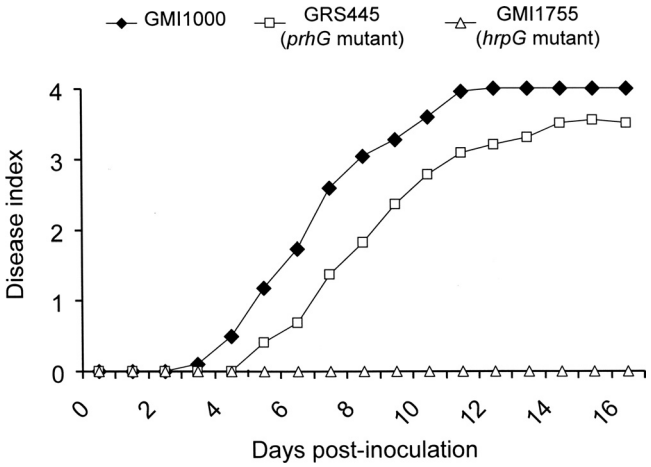


FIG. 2. Disease progress curves of *R. solanacearum* wild-type strain GMI1000 and the *prhG* and *hrpG* deletion mutant strains on tomato plants. An equivalent number of bacterial cells ($10^4$ CFU) was injected into the stems of susceptible tomato seedlings, which were monitored for 18 days and rated on a disease index scale where 0 indicates healthy plants and 4 indicates 100% wilted. All of the assays had a minimum of 24 plants per treatment per assay. Results are representative of three independent experiments.

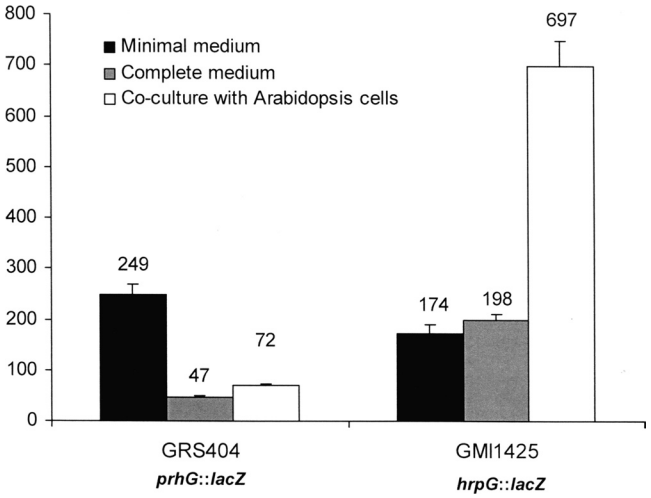


FIG. 3. Expression of *hrpG* and *prhG* in different media and coculture with plant cells. Strains GRS404 (*prhG*::*lacZ*) and GMI1425 (*hrpG*::*lacZ*) were grown for 16 h in minimal medium supplemented with glutamate at a 20 mM final concentration in B medium (complete medium) and in coculture with *Arabidopsis* At-202 cells in Gamborg medium. β-Galactosidase activity is expressed in Miller units. Each measurement corresponds to the average of four replicates, and bars indicate standard deviations.

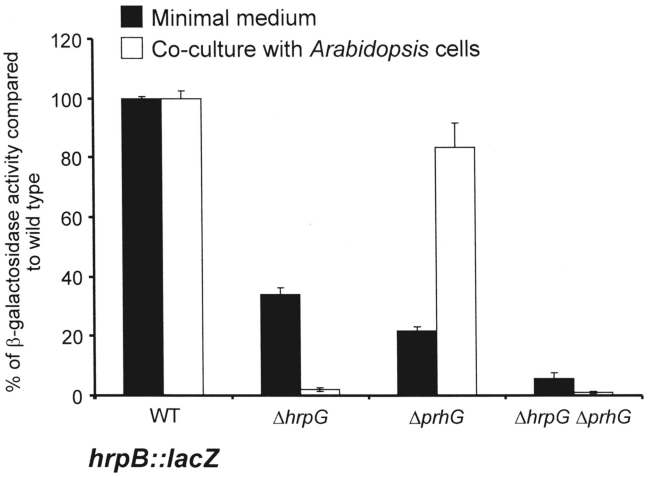


FIG. 4. Expression of *hrpB* in different genetic backgrounds after 16 h of growth in minimal medium supplemented with glutamate at a 20 mM final concentration and in coculture with *Arabidopsis* At-202 cells in Gamborg medium. β-Galactosidase activity is expressed as a percentage of the expression level measured in the wild-type (WT) genetic background. Each measurement corresponds to the average of four replicates, and bars indicate standard deviations.

condition. In the presence of *Arabidopsis* plant cells, *prhG* expression levels were even lower than those detected in minimal medium and ~10 times lower than those of *hrpG*. Similar observations were made when both strains were grown in tomato cell suspensions (data not shown). These data show that the two regulatory genes respond differently to environmental signals: transcription of *hrpG* is specifically enhanced in response to plant signals, while transcription of *prhG* appears to be mainly induced under minimal medium conditions.

**Transcriptomic profiling of a *prhG* mutant reveals that the PrhG regulon significantly differs from the HrpG regulon.** In order to determine the complete PrhG regulon, a GMI1000 whole-genome microarray (26) was used to compare the transcriptomes of *R. solanacearum* GMI1000 and its *prhG* deletion mutant derivative. Both strains were grown to late exponential phase in minimal medium, which has been shown to induce the expression of *hrp* genes (3), as well as *prhG*. Using >1.3 as the threshold of the absolute $\log_2$ ratio (an approximately threefold difference from the wild-type RNA level), we identified 95 genes as activated by PrhG and 1 gene (RSc1863) as repressed. The PrhG regulon largely overlaps the previously described HrpB regulon: nearly 75% of the genes which are activated by PrhG have already been proven to be activated by HrpB, and the gene repressed by PrhG is also repressed by HrpB (26) (see Table S1 in the supplemental material).

The comparison of the transcriptomes of *R. solanacearum* GMI1000 and a *prhG*-overexpressing strain (GMI1000/pLP2) enabled us to identify 21 additional genes upregulated by PrhG, 17 of which also belong to the HrpB regulon, and 22 additional genes repressed by PrhG, 15 of which are also repressed by HrpB. Finally, in order to avoid possible heterologous complementation of PrhG by HrpG, we also compared gene expression in the Δ*hrpG* Δ*prhG* double mutant with that in the Δ*hrpG* Δ*prhG* mutant expressing a *prhG* copy on a plasmid (pLP2). Here again, almost all of the differentially expressed genes were found to be part of the *hrpB* regulon (data not shown).

In conclusion, the transcriptomic profiling analyses performed showed that PrhG mainly controls the expression of *hrpB* and thus of the genes regulated by HrpB. Besides the genes belonging to the HrpB regulon, 39 genes appeared to have a transcription level specifically altered in the *prhG* mutant, 32 of which are under positive control and only 7 of which are under negative control (see Table S1 in the supplemental material). Except for one gene, none of the genes known to be regulated by HrpG independently of HrpB (33) was found to be regulated by PrhG. The exception is the RSp0201 gene, which encodes a conserved hypothetical protein of unknown function. We generated a disruption mutant of RSp0201 in GMI1000 and found that its pathogenicity was not altered on tomato plants (data not shown). Therefore, rather surprisingly, the overlap between the PrhG and HrpG regulons is essentially reduced to the HrpB regulon.

**PrhG controls the expression of *hrpB* and HrpB-regulated genes.** To confirm the control of *hrpB* expression by PrhG, the *hrpB*::*lacZ* fusion from strain GMI1475 (16) was introduced into the *prhG* deletion mutant. The level of transcription of *hrpB* in this strain was measured by β-galactosidase activity and compared to that measured in a wild-type genetic background. The results showed a threefold decrease in *hrpB* expression in the *prhG* deletion mutant after growth of the strains in minimal medium compared to that in the wild-type genetic background (Fig. 4). In contrast, no decrease in *hrpB* expression was measured in the *prhG* deletion mutant compared to its expression in the wild-type strain upon coculture of these strains with tomato or *A. thaliana* cell suspensions. The comparison of *hrpB* expression in the mutant strains indicates that both HrpG and PrhG are required for full induction of *hrpB* in minimal me-

TABLE 2. β-Galactosidase activities obtained with *hrpY*::*lacZ* and *popA*::*lacZ* fusions in different genetic backgrounds

| Genetic background | Avg (SD) β-galactosidase activity (Miller units)[a] with reporter fusion: | |
|---|---|---|
| | *hrpY*::*lacZ* | *popA*::*lacZ* |
| Wild type | 741.0 (14.10) | 1,677.0 (181.00) |
| Δ*hrpG* | 590.5 (4.90) | 44.0 (2.80) |
| Δ*prhG* | 193.0 (15.60) | 73.0 (2.80) |
| Δ*hrpG* Δ*prhG* | 9.0 (1.40) | 11.0 (5.70) |

[a] Measurements of four replicates were made after 16 h of bacterial growth in minimal medium supplemented with glutamate at a 20 mM final concentration.

dium but that in the presence of plant cells, only HrpG is involved in the induction of *hrpB* expression.

We also monitored the expression of two *hrpB*-regulated genes, *popA*, which codes for an *R. solanacearum* harpin (4), and *hrpY*, which encodes the structural component of the Hrp pilus (34). As shown in Table 2, expression of a *popA*::*lacZ* fusion decreased 23-fold in a *prhG* deletion mutant compared to that in the wild-type genetic background after the growth of these strains in minimal medium. For the *hrpY*::*lacZ* fusion, only a fourfold decrease was observed under the same conditions, but the impact was more important than that observed in the *hrpG* mutant. The transcription of both *popA* and *hrpY* was almost completely abolished in the double-deletion mutant.

Taken together, these results show that both HrpG and PrhG are necessary for full induction of *hrpB* and its targets in minimal medium.

**Evidence that PrhG and HrpG can control *hrpB* expression independently.** To get a better understanding of the regulation mechanism involving HrpG and PrhG in minimal medium, we conducted a complementation analysis of *R. solanacearum* mutants with *hrpG*, *prhG*, or both regulatory genes deleted, all bearing an *hrpB*::*lacZ* fusion. Three plasmid constructions were used: a plasmid carrying a copy of *prhG* (pLP2) under the control of its promoter, a plasmid with a copy of *hrpG* (pBBL12) under the control of its promoter, and a plasmid containing both regulators (pLP5), each under the control of its own promoter. Plasmids were introduced into the different *hrpB*::*lacZ* strains (the wild type and the Δ*hrpG*, Δ*prhG*, and Δ*hrpG* Δ*prhG* mutants), and the expression of *hrpB* was measured by β-galactosidase assays.

The results presented in Fig. 5 indicate that both HrpG and PrhG are able to induce *hrpB* expression on their own. In the absence of the chromosomal copy of *hrpG* and *prhG*, plasmid pBBL12 or pLP2 carrying a copy of *hrpG* or *prhG*, respectively, is able to restore a significant and comparable expression level of the *hrpB*::*lacZ* reporter fusion. These results also show that under the same growth conditions and expressed on the same backbone vector, *prhG* and *hrpG* have similar *hrpB* promoter induction strengths. The complementation of the double mutant strain with pLP5, carrying both regulators, also restored *hrpB* expression, but not to a significantly higher level than the plasmids carrying a single regulatory gene. In conclusion, these complementation experiments show that HrpG and PrhG act independently to control the expression of their common target, *hrpB*, and suggest that no cooperation mechanism is involved in this control.

***prhG* does not control the expression of *hrpG* and vice-versa.** We then evaluated whether *prhG* could influence the transcription of its paralog *hrpG* or if, on the other hand, it could depend on *hrpG* for its own expression. The interdependency of each regulator was studied by using *R. solanacearum* strains carrying either a *hrpG*::*lacZ* or a *prhG*::*lacZ* fusion in a *prhG* or *hrpG* deletion background, respectively. β-Galactosidase assays proved that *prhG* transcription is independent of HrpG under minimal medium conditions, as the activity measured in the *hrpG* mutant is similar to that measured in the wild-type genetic background (Fig. 6B). Concerning the transcription of *hrpG*, the results showed a twofold decrease in β-galactosidase activity in the *prhG* deletion mutant and a twofold increase when an extra copy of *prhG* is introduced on a plasmid into the *hrpG*::*lacZ* strain. Because, it is known that HrpB exerts a positive but relatively modest impact on *hrpG* expression (16, 26), we investigated whether this slight effect of PrhG on *hrpG* expression is indirectly mediated through *hrpB*. As shown in Fig. 6A, this influence of PrhG on *hrpG* expression is abolished in an *hrpB* mutant, thus showing that the observed twofold factor maybe due to a positive feedback effect of HrpB on *hrpG*. Altogether, these data support the view that HrpG and PrhG do not exert direct transcriptional control on each other and belong to different signaling pathways.

## DISCUSSION

In this study, we have identified PrhG, a novel component of the regulatory network controlling the expression of pathogenicity genes in *R. solanacearum*. The determination of the *prhG*

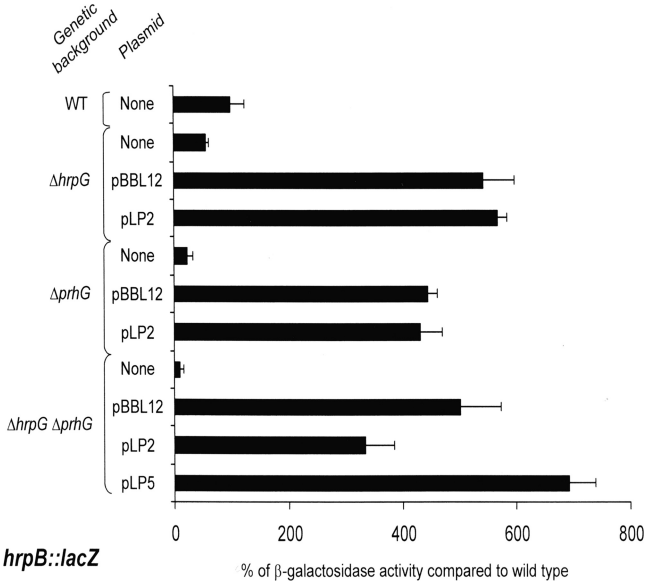


FIG. 5. Expression of *hrpB* in different genetic backgrounds after 16 h of growth in minimal medium supplemented with glutamate at a 20 mM final concentration. pBBL12, pLP2, and pLP5 are low-copy-number plasmids carrying, respectively, a copy of *hrpG*, a copy of *prhG*, and both *hrpG* and *prhG*, all under the control of their own promoters. β-Galactosidase activity is expressed as a percentage of the level of expression measured in the wild-type (WT) genetic background devoid of any plasmids. Each measurement corresponds to the average of three replicates, and bars indicate standard deviations.

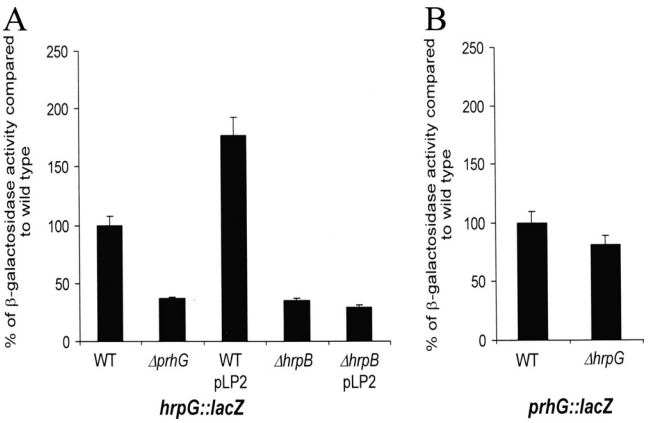


FIG. 6. Expression of *hrpG* (A) and *prhG* (B) in different genetic backgrounds after 16 h of growth in minimal medium supplemented with glutamate at a 20 mM final concentration. β-Galactosidase activity is expressed as a percentage of the level of expression measured in the wild-type (WT) genetic background. Each measurement corresponds to the average of four replicates, and bars indicate standard deviations.

regulon through transcriptomic profiling after growth of the bacteria in minimal medium revealed that a large proportion (75%) of the *prhG*-dependent genes are part of the previously described *hrpB* regulon (26). We confirmed by gene reporter fusion assays that expression of *hrpB* and some *hrpB*-controlled genes is indeed under the transcriptional control of *prhG* specifically under minimal medium conditions. PrhG is encoded by an OmpR/PhoB family regulatory gene which is the closest paralog to *hrpG*, a master regulator of pathogenicity genes in *R. solanacearum* (33).

Both PrhG and HrpG control the expression of T3SS and type III effector genes through *hrpB*. It is therefore rather surprising that a *prhG* mutant is only slightly affected in its pathogenicity and is not completely avirulent, as *hrpG* (or *hrpB*) deletion mutants have been shown to be (7). This marked difference in the *prhG*/*hrpG* mutant phenotypes on plants clearly reveals that the corresponding proteins have distinct roles, despite their strong relatedness and their having in common a major pathogenicity target (*hrpB*). The most probable hypothesis to explain the low impact of a *prhG* mutation on pathogenicity is based on the striking difference observed in the transcription pattern of the *prhG*/*hrpG* genes (Fig. 3). In the presence of plant signals, *hrpG* is much more strongly expressed in the bacterial cell and this low level of *prhG* expression in a *hrpG* mutant is probably not sufficient to restore pathogenicity. It is also possible that, in a plant environment, HrpG integrates specific signals or posttranslational modifications that do not impact PrhG so that specific activation of HrpG would bypass any requirement for PrhG. In this scenario, *prhG* seems to be dispensable for the expression of T3SS genes in plants as *hrpB* activation is mainly achieved through *hrpG*. This is supported by the data presented in Fig. 4 showing that the disruption of *prhG* has nearly no impact on *hrpB* expression after growth of bacteria in the presence of plant cells while disruption of *hrpG* abolishes *hrpB* expression.

The comparison of the defined PrhG and HrpG regulons reveals that they overlap only for the *hrpB* regulon. HrpG was shown to control the expression of 184 genes independently of *hrpB* in minimal medium (33), and—with a single exception (RSp0201)—none of these genes appears to be a regulatory target of *prhG*, thus illustrating the specificity of these regulatory proteins. This specificity raises intriguing questions about the very high level of identity observed between PrhG and HrpG, especially in the helix-turn-helix domain which is predicted to bind DNA operator sequences. This suggests that the homologous PrhG and HrpG proteins are able to discriminate between specific target promoter sequences and that the *hrpB* promoter is unique in being a confirmed target of both regulators.

How is *hrpB* control jointly achieved by PrhG and HrpG? The fact that several OmpR/PhoB family regulators bind to DNA as dimers (13, 19) raised the possibility that an active PrhG/HrpG heterodimer controls *hrpB* transcription in minimal medium. Our complementation data (Fig. 5) do not fit this hypothesis: both regulators provided in *trans* on the same backbone vector are individually able to activate *hrpB* expression at similar levels. The use of the pLP5 construct containing *prhG* and *hrpG* on the same vector also revealed that there may be no cooperative binding on DNA since in the Δ*hrpG* Δ*prhG* mutant background, complementation experiments with pLP5 did not lead to a significantly higher level of *hrpB* expression. The slight increase observed compared to complementation with only one of the regulators probably corresponds to an additive effect due to the presence of multiple copies of both regulatory genes. Although the mechanism of action and the DNA-binding specificities of PrhG and HrpG are still unknown, our data suggest that these two related regulators are able to activate *hrpB* independently. Simultaneous control of key T3SS promoters by distinct regulatory proteins has also been reported in other bacterial pathogens, such as *Salmonella* (11).

Because HrpG is the regulator acting just upstream of HrpB in the Hrp regulatory cascade, we determined whether PrhG could influence *hrpG* transcription (and could therefore indirectly control *hrpB*) or if, on the other hand, it was itself under the transcriptional control of HrpG. Our data show that the expression of each regulator is independent of that of the other. Interestingly, in the course of this investigation, we confirmed that *hrpG* expression is partially dependent on *hrpB* under minimal medium conditions. This had been previously observed through the transcriptomic profiling analysis of an *hrpB* deletion mutant, which showed decreased *hrpG* transcription (26). This retropositive regulatory loop illustrates the complex interplay between T3SS gene regulators in *R. solanacearum* (Fig. 7).

Since *prhG* is not crucial during plant pathogenesis, the biological relevance of this novel *hrp* regulatory component remains elusive. Surprisingly, transcriptomic profiling analyses revealed that, besides the *hrpB* regulon, *prhG* controls very few specific targets compared to *hrpG* (39 versus 184). Most of these *prhG*-specific genes were under positive control (32 out of 39), which makes unlikely a role for PrhG as a direct transcriptional repressor. The examination of the specifically activated genes does not provide any clue about the role of *prhG* during the *R. solanacearum* life cycle. In particular, none of these genes are clustered or organized in defined operons. Although eight of them (RSc0695, RSc1853, RSc2942,

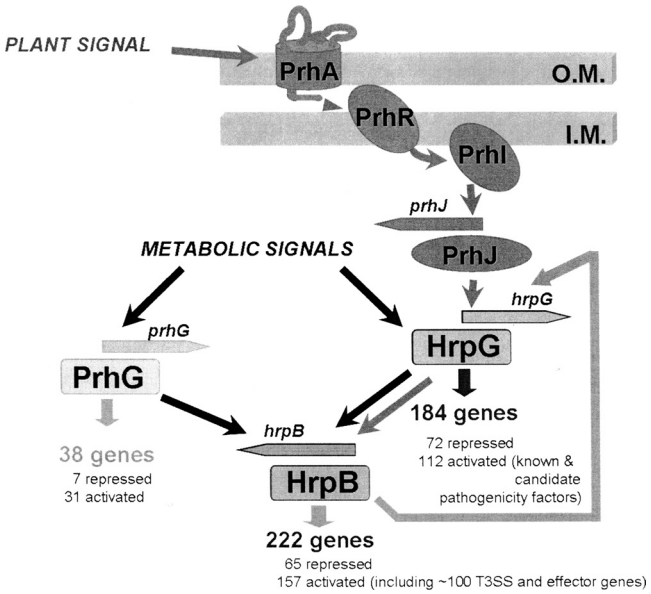


FIG. 7. Model describing the regulation network involved in the control of *R. solanacearum* pathogenicity. HrpB is the regulator mainly devoted to the control of the T3SS and effector gene transcription. PrhG and HrpG both regulate the expression of *hrpB* but belong to different signaling pathways and integrate at least two distinct inducing signals. The nature of the activating plant signal(s) is unknown, but it requires physical contact between bacteria and plant cells (1). The metabolic signals indicated here are those perceived by bacteria grown under minimal medium conditions. HrpG regulates, independently of HrpB, the expression of a subset of genes that includes several virulence and pathogenicity factors (33). The specific subset of genes regulated by PrhG is smaller and contains only one other target regulated by HrpG, RSp0201. The feedback loop by which HrpB induces *hrpG* (see text) is also shown. O.M., outer membrane; I.M., inner membrane.

RSc3114, RSp0142, RSp0312, RSp0934, and RSp1577) are predicted to be involved in type I or II secretion processes, the significance of this is unknown.

The fact that *prhG* is an ancestral gene broadly conserved among the taxonomically diverse *R. solanacearum* phylotypes is an argument supporting the view that this gene is not an evolutionary remnant of the *hrp* regulatory system and may have conserved a specific function. The *prhG* gene was indeed detected through comparative genomic hybridization in a selection of 18 strains representative of the biodiversity of the species (17), also suggesting that *prhG* is not the result of a recent duplication of *hrpG*. It is interesting that, unlike *hrpG*, which is universally conserved among *Xanthomonas* spp. and *R. solanacearum* as a T3SS regulator component, *prhG* appears to be specific to *R. solanacearum*, as no direct counterpart could be detected in any of the *Xanthomonas* sp. genomes sequenced to date. The analysis of phylogenetic relationships suggests that the orthologous *hrpG* genes from *R. solanacearum* and *Xanthomonas* spp. have diverged before the *prhG*/*hrpG* separation in *R. solanacearum* (Fig. 1B), thus raising the possibility that *Xanthomonas* HrpG proteins could combine the specificities of both the HrpG and PrhG proteins of *R. solanacearum*.

The findings that *prhG* expression is specifically induced under minimal medium conditions and that the *prhG*-dependent regulation of *hrpB* follows this *prhG* expression profile strongly suggest that *prhG* has an important role in activating the T3SS before contact with host cells, which is the signal triggering the activation of *hrpG* (1). In the case of *hrp* gene expression, minimal medium conditions have been proposed to mimic the plant apoplast environment (3, 30, 32), and this probably corresponds to an early phase of the plant-bacterium interaction before the translocation of type III effectors inside plant cells. It is tempting to speculate that minimal medium conditions reproduce the conditions under which the bacteria first perceive some host physicochemical signals necessary to induce the setting up of a functional T3SS before a second, stronger, plant cell wall-specific signal specifically transduced through *hrpG* would act as a type III effector translocation-triggering signal. Since a *prhG* mutant is not affected or very slightly affected in pathogenicity on tomato and *Arabidopsis* plants, this scenario implies that the first step could also be mediated through *hrpG*. It is possible that *prhG* requirement may be more important under other conditions such as on other host plants (considering the very wide host range of the bacterium) or respond specifically to some unknown signals in the environment that were absent under the experimental conditions of the present study. Further work is necessary to define the mechanistic basis of *hrpB* regulation by PrhG and obtain a better definition of the environmental signals involved.

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