Control of the *Ralstonia solanacearum* Type III secretion system (Hrp) genes by the global virulence regulator PhcA

Stéphane Genin[a,*], Belen Brito[a,1], Timothy P. Denny[b], Christian Boucher[a]

[a] *Laboratoire Interactions Plantes-Microorganismes, CNRS-INRA, UMR2594, BP52627, 31326 Castanet-Tolosan, France*
[b] *Department of Plant Pathology, The University of Georgia, Athens, GA 30602, USA*

Abstract **Expression of several virulence factors in the plant pathogen bacterium *Ralstonia solanacearum* is controlled by a complex regulatory network, at the center of which is PhcA. We provide genetic evidence that PhcA also represses the expression of *hrp* genes that code for the Type III protein secretion system, a major pathogenicity determinant in this bacterium. The repression of *hrp* genes in complete medium is relieved in a *phcA* mutant and two distinct signals, a quorum-sensing signal and complex nitrogen sources, appear to trigger this PhcA-dependent repression. This control of *hrp* gene expression by PhcA is realized at the level of the HrpG regulatory protein.**
© 2005 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

1. Introduction

Ralstonia solanacearum is a soil-borne pathogen that causes lethal wilt diseases of many plants around the world. The bacterium generally invades its host through wounds in the roots, colonizes the xylem vessels, and spreads rapidly up the stem and through the plant. The typical wilting symptoms result from an excessive production of extracellular polysaccharides (EPS) within the vascular system, thus altering water fluxes in the plant.

Expression of virulence factors in *R. solanacearum* is controlled by a complex regulatory network that responds to environmental conditions, the presence of host cells, and bacterial density [1]. At the center of this network is PhcA, a LysR family transcriptional regulator [2], which, directly or through intermediary regulatory genes, coordinates the expression of several virulence factors such as EPS, various plant cell wall-degrading enzymes, and bacterial motility [3]. The level of active PhcA is regulated in response to cell density by a quorum-sensing mechanism that involves the specific autoinducer molecule 3-hydroxy palmitic acid ester (3-OH PAME) [4]. At low cell density in culture, presumably corresponding to saprophytic life and early plant colonization, PhcA is not expressed, leading to expression of 'early' disease virulence factors, including several polygalacturonases and both twitching and swimming motility [5–7]. At a later stage of infection, at high cell density, the accumulation of 3-OH PAME leads to activation of PhcA and, subsequently, production of EPS and potent plant cell wall-degrading enzymes (cellulases and pectin methylesterase) will be activated (Fig. 1A).

Another key determinant of *R. solanacearum* pathogenicity is the *hrp*-encoded Type III secretion system (TTSS) that allows the translocation of effector proteins into plant cells [10]. *hrp* genes are required both for disease development on host plants and for the elicitation of a defensive hypersensitive response (HR), which is triggered by certain non-host or resistant plants after recognition of the pathogen. *hrp* gene expression in many Gram-negative plant pathogens is strongly influenced by growth environment. A general feature of *hrp* genes is that these genes are not expressed when the bacteria are grown in complete medium but are induced after growth in apoplast-mimicking minimal media [11,12]. In *R. solanacearum*, plant cell wall-derived signals perceived during the bacterium–plant cell contact were also shown to activate *hrp* gene expression through a six-gene regulatory cascade [13–15] (Fig. 1B). At the bottom of this cascade is HrpB, an AraC family regulator, which activates the *hrp* and TTSS-effector promoters via the hrp$_{II}$ box *cis*-element [16].

In this paper, we investigated whether the regulatory network controlled by PhcA and the Hrp regulatory pathway could be genetically connected. We show that PhcA negatively regulates expression of the TTSS in complete medium and that this PhcA-mediated repression is triggered by complex nitrogen sources. Our data also indicate that repression of *hrp* genes by PhcA is realized at the level of the HrpG regulatory protein, probably via a post-transcriptional mechanism.

2. Materials and methods

2.1. Bacterial strains, plasmids, and media

Plasmids were cloned in *Escherichia coli* strain DH5α (BRL). The plasmids and *R. solanacearum* strains used are listed in Table 1. *E. coli* was grown at 37 °C in LB broth and *R. solanacearum* strains were grown at 30 °C in complete B medium or minimal medium supplemented with glucose at 10 mM final concentration [17] or with Difco casamino acids (10 g/L). Antibiotics were used at the following concentrations (mg/L): ampicillin, Amp (100), spectinomycin, Spc (40), tetracyclin, Tc (10), gentamycin, Gm (10), kanamycin, Km (50).

[*]Corresponding author. Fax: +33 61 28 50 61.
E-mail address: sgenin@toulouse.inra.fr (S. Genin).

[1] Present address: Laboratorio de Microbiologia, Departamento de Biotecnologia-E.T.S. Ingenieros Agronomos, Universidad Politecnica de Madrid, 28040 Madrid, Spain.
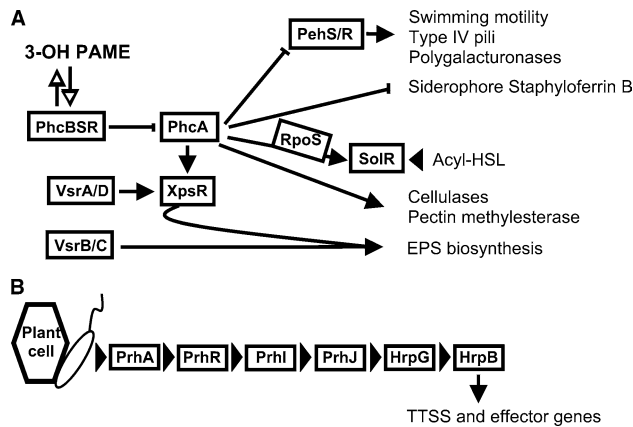
Fig. 1. Simplified model of the network regulating virulence factors and motility in *R. solanacearum*. (A) The PhcA-regulated pathways. (B) The Hrp Type III secretion system regulatory pathway induced in response to the bacterium–plant cell contact. Lines with solid arrowheads or bars represent positive or negative control, respectively, of gene expression. Lines with open arrowheads represent synthesis or sensing of the autoinducer molecule 3-OH PAME. PhcS/R, PehS/R, VsrA/D, and VsrB/C correspond to two-component regulator modules. Acyl-HSL: *N*-octanoyl and *N*-hexanoyl-homoserine lactone synthesized by *sol*I. Adapted from [1,8,9].

2.2. DNA manipulations

Standard recombinant DNA techniques were used. PCR amplifications were done with the Expand Long Template PCR System (Roche Molecular System) using genomic DNA of *R. solanacearum* GMI1000 as previously described [16].

2.3. Creation of phcA and hrpG mutants

A 1.3 kb DNA fragment encompassing the *phcA* open reading frame was PCR-amplified using the primers phc1 (5′-AAGCTTGG-TACGACAACGAGTGG-3′) and pCA2 (5′-CGGTGCCACAG-CATGTT-3′) and cloned into the pGEMT vector to give plasmid pGA. The Ω interposon [19] digested by *Sma*I was cloned into the unique *Eco*RV site of pGA, thus resulting in plasmid pGAΩ carrying an insertion of the Ω element 255 bp downstream of the *phcA* start codon. Plasmid pGAΩ was linearized by *Hin*dIII and ex-

changed into the genome as described previously [11]. Disruption of the *phcA* gene in GMI1000 and various *hrp*::*lacZ* mutant derivatives was verified by Southern analysis.

A *hrpG*::*aaaC1* deletion mutant was constructed by amplifying two DNA fragments flanking the *hrpG* coding sequence (using primers 5′-TCTAGACAACCGCACCAATGTGCTTG-3′ and 5′-GGTACC-GGACACATTCCACGTT-3′ for the left-flanking fragment and 5′-CCGCGGAACCTGTTCACGAACC-3′ and 5′-GTCGACTTGC-AGATGCTGGTGGAT-3′ for the right-flanking fragment). The *aaaC1* (Gm^r) cassette was then cloned between these two PCR fragments in the allelic exchange vector pCM351 [21]. The resulting construct, pSG393, was used to transform *R. solanacearum* to generate a Δ*hrpG*::*aaaC1* mutant. The deletion of *hrpG* in resulting strain GMI1755 was checked by PCR. GMI1755 elicited no HR after infiltration in tobacco leaves and the HR-inducing ability of the mutant strain was restored after introduction of pBBL12, a pLAFR6-based plasmid which carries a functional *hrpG* gene [14].

2.4. Cloning and trans-expression of phcA

The *phcA* gene was PCR-amplified as a 1.5 kb DNA fragment using the primers phc1 and pCA3 (5′-GGAGGAGCGCGGTGCTGG-3′). The PCR product was restricted by *Eco*RI and *Hin*dIII and the corresponding 1.3 kb fragment was cloned into pLAFR3 digested with the same enzymes. The resulting plasmid pSG318 therefore carries *phcA* downstream of the lac promoter which is constitutively expressed in *R. solanacearum*.

2.5. β-Galactosidase assays

β-Galactosidase assays were performed as described elsewhere [16]. Values are the mean of three independent experiments performed in duplicate.

2.6. HR tests

Ralstonia solanacearum strains were tested for HR ability by infiltration of bacterial cultures adjusted to 10^8 cells/mL in *Nicotiana tabacum* (cultivar bottom special) leaf parenchyma as described [14].

3. Results

3.1. The repression of hrp genes in complete medium is controlled by PhcA

A *phcA* mutant strain was constructed by disrupting the corresponding open reading frame with the Ω interposon. The resulting strain GMI1610 was non-mucoid and nearly

Table 1
List of the *R. solanacearum* strains and plasmids used in this study

Strain/Plasmid	Relevant characteristics	Reference
R. solanacearum		
GMI1000	Wild-type	[17]
GMI1425	*hrpG*::*lacZ* mutant (Km^r)	[14]
GMI1447	*hrpC*::*lacZ* mutant (Km^r)	[11]
GMI1475	*hrpB*::*lacZ* mutant (Km^r)	[18]
GMI1609	*hrpC*::*lacZ* *phcA*::Ω (Km^r, Spc/Sm^r)	This work
GMI1610	*phcA*::Ω (Spc/Sm^r) mutant	This work
GMI1611	*hrpB*::*lacZ* *phcA*::Ω (Km^r, Spc/Sm^r)	This work
GMI1612	*hrpG*::*lacZ* *phcA*::Ω (Km^r, Spc/Sm^r)	This work
GMI1756	Δ*hrpG*::*aaaC1* (Gm^r) mutant	This work
GMI1757	*hrpB*::*lacZ* *phcA*::Ω Δ*hrpG*::*aaaC1* (Km^r, Spc/Sm^r, Gm^r)	This work
Plasmids		
pGEMT	Cloning vector, Amp^r	Promega
pHP45Ω	Ω interposon (Spc/Sm^r)	[19]
pLAFR3	IncP1, Tc^r, cos$^+$, rlx$^+$	[20]
pLAFR6	pLAFR1 with *trp* terminators, Tc^r	[12]
pCM351	Allelic exchange vector with *aaaC1* cassette, Amp^r Tc^r Gm^r	[21]
pGA	pGEMT carrying a 1.3 kb *phcA* fragment	This work
pGAΩ	pGA with the Ω interposon cloned in *Eco*RV	This work
pSG315	pLAFR6 carrying a *hrpB*::*lacZ* transcriptional fusion	This work
pSG318	pLAFR3 carrying *phcA* on a 1.3 kb *Eco*RI–*Hin*dIII fragment	This work
pSG393	pCM351carrying a Δ*hrpG*::*aaaC1* construct	This work

Table 2
Effect of *phcA* on expression of chromosomal Tn5-*lacZ* fusions in the *hrpB* (*hrp* gene regulator) and *hrpC* (TTSS biogenesis) genes after growth of the bacterial cells in complete medium

Strains	Genotype	β-Galactosidase activity[a]	
		No plasmid	+pSG318[b]
GMI1447	*hrpC::lacZ*	11 (1)	6.2 (0.7)
GMI1609	*hrpC::lacZ phcA::Ω*	187 (11)	7.5 (0.5)
GMI1475	*hrpB::lacZ*	9 (1.5)	6.0 (1)
GMI1611	*hrpB::lacZ phcA::Ω*	629 (34)	8.9 (0.9)

[a]Cells were grown to an OD_{600} of ∼1.0, disrupted with sodium dodecyl sulfate–chloroform and assayed. Mean values are in Miller units with standard errors in brackets.
[b]pSG318 carries a wild-type copy of *phcA* constitutively expressed from plac on pLAFR3, a low-copy-number vector.

avirulent but was motile and overproduced cell-wall degrading polygalacturonases (data not shown), similarly to what was reported for *phcA* mutations in strain AW1 [2]. To evaluate the role of *phcA* and *hrp* gene expression, the *phcA::Ω* mutation was introduced in strains GMI1447 and GMI1475, which carry a *lacZ* transcriptional fusion in the *hrpC* and *hrpB* genes, respectively. β-Galactosidase activity of the corresponding strains, GMI1609 and GMI1611 (Table 1) was then monitored and compared to their respective parent. When bacterial cells were grown in complete medium, i.e., conditions in which *hrp* genes are normally expressed at background levels, the *phcA* mutation resulted in a dramatic increase (20- to 60-fold) of *hrp* gene transcription (Table 2). Expression of a functional *phcA* in *trans* on pSG318 restored expression to the low wild-type levels (Table 2). These results were confirmed by comparing the activity of a *hrpB::lacZ* fusion expressed in *trans* on the low-copy-number plasmid pSG315 in the wild-type and *phcA* mutant strains: here again inactivation of *phcA* led to a strong increase of *hrpB* expression in complete medium (802 Miller units) compared to that observed in the wild-type strain (62 Miller units).

3.2. Influence of metabolic conditions on the PhcA-mediated repression of hrp genes

β-Galactosidase activity of the strains GMI1447, GMI1475, and their *phcA* mutant derivatives were also measured after growth of bacterial cells in inducing minimal medium (with glucose as sole carbon source). Inactivation of *phcA* also resulted in enhanced transcription of *hrp* genes, although less than was observed in complete medium (2.5- to 6-fold) (Table 3). Growth of *R. solanacearum* with complex nitrogen sources (e.g., casamino acids) repressed *hrp* gene expression to <10%

Table 3
Effect of *phcA* on expression of chromosomal Tn5-*lacZ* fusions in the *hrpB* and *hrpC* genes after growth of the bacterial cells in minimal medium supplemented with different carbon sources

Strains	β-Galactosidase activity		
	Minimal medium + glucose	Minimal medium + glucose + casamino acids	Minimal medium + casamino acids
GMI1447	122 (5)	25 (2)	10 (0.9)
GMI1609	248 (21)	344 (30)	390 (32)
GMI1475	82 (7)	26 (3.5)	8 (1)
GMI1611	434 (30)	393 (18)	449 (20)

of that observed in minimal medium + glucose [11]. However, in the *phcA* mutant strain, the casamino acid-dependent repression was relieved and *hrp* genes were expressed to levels comparable to those observed in inducing conditions (Table 3). This observation indicates that the PhcA-dependent repression of *hrp* genes is directly linked to the metabolic status of the bacterial cells. Interestingly, the addition of glucose to minimal medium + casamino acids was able to trigger a weak induction of *hrp* genes in strains GMI1447 and GMI1475 (Table 3), suggesting that the PhcA-dependent repression exerted in presence of a complex nitrogen source could be relieved in presence of potent inducing signals.

3.3. Overexpression of phcA strongly reduces the ability of R. solanacearum to elicit the hypersensitive response on tobacco leaves

The ability of *R. solanacearum* GMI1000 to induce an HR on tobacco depends on a functional TTSS. Because *phcA* appeared to negatively regulate *hrp* gene expression, we investigated whether the constitutive expression of *phcA* on a low-copy-number was able to alter the ability of the wild-type strain to elicit the HR. Tobacco leaves were infiltrated with suspensions of 10^8 bacterial cells/mL of strains GMI1000/pSG318, which overexpresses *phcA*, and GMI1000/pLAFR3, a control strain carrying the empty vector. As shown in Fig. 2, GMI1000/pSG318 elicited no macroscopic reaction 24 h after inoculation, in contrast to the wild-type control. A partial HR could be observed only 48 h post-inoculation with the *phcA*-overexpressing strain, indicating that elevated amounts of PhcA have a negative effect on the TTSS-dependent translocation of the *R. solanacearum* HR-inducing factor.

3.4. PhcA-dependent repression of hrp genes is mediated through a post-transcriptional control of the regulatory gene hrpG

We were interested in determining at which step of the Hrp regulatory cascade the PhcA-dependent control of *hrp* gene expression takes place. First, we noticed that the *phcA* mutation had no effect on expression on *prhA*, the gene placed at the top of the cascade which encodes the TonB-dependent receptor involved in the sensing of plant signals (data not shown). Because the PhcA-dependent repression was influenced by the growth conditions of the bacteria, we then investigated the effect of *phcA* on *hrpG*, the regulatory gene placed just upstream of *hrpB* in the cascade (Fig. 1) and which was
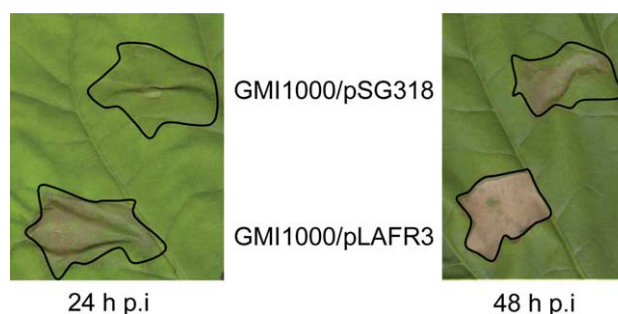

Fig. 2. Overexpression of *phcA* reduces the ability of *R. solanacearum* to elicit the HR on tobacco leaves. Tobacco leaf parenchyma was infiltrated with suspensions of 10^8 bacterial cells/mL of strains GMI1000/pSG318 (pLAFR3::*phcA*) and GMI1000/pLAFR3 (empty vector control). Areas infiltrated are circled. Photographs were taken 24 and 48 h post-inoculation.

Table 4
Expression of *hrpG* is not significantly altered by inactivation of *phcA* but *hrpG* is required for the *phcA*-dependent repression of the *hrpB* gene in complete medium

Strains	Genotype	β-Galactosidase activity	
		Complete medium	Minimal medium
GMI1425	*hrpG::lacZ*	247 (36)	70 (7)
GMI1612	*hrpG::lacZ phcA::Ω*	306 (29)	148 (14)
GMI1611	*hrpB::lacZ phcA::Ω*	629 (34)	434 (30)
GMI1757	*hrpB::lacZ phcA::Ω hrpG::Ω*	7 (1.5)	21 (2)

shown to integrate the nutrient/metabolic signals that trigger *hrp* gene transcription in minimal medium [14]. As previously noted, *hrpG* is constitutively expressed in complete and minimal media [14] and the *phcA::Ω* mutation had no significant effect on *hrpG* transcription in either conditions (Table 4). Since inactivation of *phcA* had a very strong effect on *hrpB* transcription, we then addressed the question of whether this activation of *hrpB* observed in the *phcA::Ω* mutant strain in complete medium was mediated through *hrpG*. For this purpose, a triple mutant strain carrying disruptions of both *phcA* and *hrpG* together with a *hrpB::lacZ* transcriptional fusion was constructed. Results presented in Table 4 clearly show that the enhanced expression of *hrpB* observed in complete medium conditions in the *phcA* mutant was abolished in the absence of a functional *hrpG* gene. Altogether, these results indicate that the activation of *hrpB* in the *phcA* mutant strain is dependent upon the activity of HrpG but is not mediated through a transcriptional activation at the level of the *hrpG* gene itself.

4. Discussion

In this paper, we provide genetic evidence that the two main regulatory pathways controlling pathogenicity functions previously characterized in *R. solanacearum* are coordinately regulated. Hence, the global virulence regulator PhcA represses the expression of TTSS genes and this repression is mainly exerted in the presence of complex growth environments. Consistent with this, the production of *R. solanacearum* Hrp pili appear to be repressed by *phcA* in rich medium-grown cells [6; unpublished observations] and the constitutive expression of *phcA* results in a delayed HR after infiltration of tobacco leaves.

Two distinct signals, high cell density and complex nitrogen sources, appear to be involved in the repression of the *R. solanacearum* TTSS through PhcA. Since the activity of PhcA is controlled by the autoinducer molecule 3-OH PAME [4], it implies that *hrp* genes are repressed at high cell density when this diffusible chemical signal accumulates (Fig. 1). This hypothesis is supported by the observation that expression of the *hrpB* gene in complete medium is induced by 6-fold at low cell density (10^6 cfu/mL) versus high cell density (10^8 cfu/mL). To date, a similar observation has only been reported for two pathogenic *Vibrio* sp. in which expression of TTSS genes is repressed by quorum-sensing signals at high cell density [22]. A possible scenario is that *R. solanacearum* perceives both high cell density and complex growth environment signals as indicators of nutrient sufficiency and in response represses use of its TTSS for acquisition of nutrients from plant cells. This model is consistent with the view that the deployment of the Hrp

TTSS is important for isolated bacterial cells during the early steps of plant infection, the inverse of what occurs for EPS, cellulases and other virulence factors which are massively produced when the bacterium reaches high cell density within the plant. Recent studies showing that the TTSS from phytopathogens is required for the suppression of basal plant defense responses during the first stages of interaction with their hosts [23] also support this hypothesis.

Our data indicate that the PhcA-dependent control of the Hrp regulatory pathway occurs at the level of HrpG, probably via a post-transcriptional mechanism. *hrpG* encodes an OmpR family response regulator that integrates different environmental signals, being required for *hrpB* gene activation in the presence of both plant cell-derived signals and stimuli from the metabolic state [14]. Plant cell signals activate transcription of *hrpG* via the Prh pathway (PrhARIJ, see Fig. 1) [13,15] but in minimal medium, the activation of *hrp* genes is mediated by *hrpG* but not the Prh pathway [14]. To accomodate the fact that transcription of *hrpG* is not activated in minimal medium while its gene product is required in these conditions, it was proposed that HrpG could be phosphorylated by an unidentified sensor kinase protein [14]. This hypothesis is also supported by the observation that point mutations near the putative phosphorylation site of the orthologous HrpG response regulator of *Xanthomonas campestris* cause constitutive expression of *hrp* genes [24]. Here we provide evidence that the PhcA-dependent repression observed when the growth environment gets enriched in complex nitrogen sources occurs through the control of the activity of HrpG. It is therefore tempting to speculate that PhcA somehow negatively regulates the phosphorylation state of HrpG in these conditions, probably through the control of its cognate sensor kinase(s). Recently, a yeast two-hybrid screen identified a candidate sensor histidine kinase that interacts with HrpG in *Xanthomonas axonopodis* [25]. In *R. solanacearum*, the isolation of a corresponding sensor-kinase gene remains a pre-requisite to consolidate our model on *hrp* gene activation.

In conclusion, HrpG appears to be the branching point of two 'input' signal pathways: the Prh plant-responsive pathway and an unidentified 'minimal medium' pathway that connect *hrp* gene regulation to the global virulence regulator PhcA. In such a model, the expression of *hrp* genes will be the result of the balance between these two pathways in response to environmental conditions (from complete repression in synthetic rich culture medium to full activation in the plant apoplast at low cell density).

References

[1] Schell, M.A. (2000) Control of virulence and pathogenicity genes of *Ralstonia solanacearum* by an elaborate sensory network. Annu. Rev. Phytopathol. 38, 263–292.

[2] Brumbley, S.M., Carney, B.F. and Denny, T.P. (1993) Phenotype conversion in *Pseudomonas solanacearum* due to spontaneous inactivation of PhcA, a putative LysR transcriptional regulator. J. Bacteriol. 175, 5477–5487.

[3] Huang, J., Carney, B.F., Denny, T.P., Weissinger, A.K. and Schell, M.A. (1995) A complex network regulates expression of *eps* and other virulence genes of *Pseudomonas solanacearum*. J. Bacteriol. 177, 1259–1267.

[4] Flavier, A.B., Clough, S.J., Schell, M.A. and Denny, T.P. (1997) Identification of 3-hydroxypalmitic acid methyl ester as a novel autoregulator controlling virulence in *Ralstonia solanacearum*. Mol. Microbiol. 26, 251–259.

[5] Liu, H., Kang, Y., Genin, S., Schell, M.A. and Denny, T.P. (2001) Twitching motility of *Ralstonia solanacearum* requires a type IV pilus system. Microbiology 147, 3215–3229.

[6] Kang, Y., Liu, H., Genin, S., Schell, M.A. and Denny, T.P. (2002) *Ralstonia solanacearum* requires type 4 pili to adhere to multiple surfaces and for natural transformation and virulence. Mol. Microbiol. 46, 427–437.

[7] Tans-Kersten, J., Brown, D. and Allen, C. (2004) Swimming motility, a virulence trait of *Ralstonia solanacearum*, is regulated by FlhDC and the plant host environment. Mol. Plant Microbe Interact. 17, 686–695.

[8] Brown, D.G. and Allen, C. (2004) *Ralstonia solanacearum* genes induced during growth in tomato: an inside view of bacterial wilt. Mol. Microbiol. 53, 1641–1660.

[9] Bhatt, G. and Denny, T.P. (2004) *Ralstonia solanacearum* iron scavenging by the siderophore staphyloferrin B is controlled by PhcA, the global virulence regulator. J. Bacteriol. 23, 7896–7904.

[10] Genin, S. and Boucher, C. (2004) Lessons learned from the genome analysis of *Ralstonia solanacearum*. Annu. Rev. Phytopathol. 42, 107–134.

[11] Arlat, M., Gough, C.L., Zischek, C., Barberis, P.A., Trigalet, A. and Boucher, C.A. (1992) Transcriptional organization and expression of the large *hrp* gene cluster of *Pseudomonas solanacearum*. Mol. Plant Microbe Interact. 5, 187–193.

[12] Huynh, T.V., Dahlbeck, D. and Staskawicz, B.J. (1989) Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity. Science 245, 1374–1377.

[13] Aldon, D., Brito, B., Boucher, C. and Genin, S. (2000) A bacterial sensor of plant cell contact controls the transcriptional induction of *Ralstonia solanacearum* pathogenicity genes. EMBO J. 19, 2304–2314.

[14] Brito, B., Marenda, M., Barberis, P., Boucher, C. and Genin, S. (1999) *prhJ* and *hrpG*, two new components of the plant signal-dependent regulatory cascade controlled by PrhA in *Ralstonia solanacearum*. Mol. Microbiol. 31, 237–251.

[15] Brito, B., Aldon, D., Barberis, P., Boucher, C. and Genin, S. (2002) A signal transfer system through three compartments transduces the plant cell contact-dependent signal controlling *Ralstonia solanacearum hrp* genes. Mol. Plant Microbe Interact. 15, 109–119.

[16] Cunnac, S., Boucher, C. and Genin, S. (2004) Characterization of the *cis*-acting regulatory element controlling HrpB-mediated activation of the type III secretion system and effector genes in *Ralstonia solanacearum*. J. Bacteriol. 186, 2309–2318.

[17] Boucher, C.A., Barberis, P., Trigalet, A.P. and Demery, D.A. (1985) Transposon mutagenesis of *Pseudomonas solanacearum*: isolation of Tn5-induced avirulent mutants. J. Gen. Microbiol. 131, 2449–2457.

[18] Genin, S., Gough, C.L., Zischek, C. and Boucher, C.A. (1992) Evidence that the *hrpB* gene encodes a positive regulator of pathogenicity genes from *Pseudomonas solanacearum*. Mol. Microbiol. 6, 3065–3076.

[19] Prentki, P. and Krisch, H.M. (1984) In vitro insertional mutagenesis with a selectable DNA fragment. Gene 29, 303–313.

[20] Staskawicz, B., Dahlbeck, D., Keen, N. and Napoli, C. (1987) Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. 169, 5789–5794.

[21] Marx, C.J. and Lidstrom, M.E. (2002) Broad-host-range cre-lox system for antibiotic marker recycling in gram-negative bacteria. Biotechniques 33, 1062–1067.

[22] Henke, J.M. and Bassler, B.L. (2004) Quorum sensing regulates type III secretion in *Vibrio harveyi* and *Vibrio parahaemolyticus*. J. Bacteriol. 186, 3794–3805.

[23] Alfano, J.R. and Collmer, A. (2004) Type III secretion system effector proteins: double agents in bacterial disease and plant defense. Annu. Rev. Phytopathol. 42, 385–414.

[24] Wengelnik, K., Rossier, O. and Bonas, U. (1999) Mutations in the regulatory gene hrpG of *Xanthomonas campestris* pv. *vesicatoria* result in constitutive expression of all *hrp* genes. J. Bacteriol. 181, 6828–6831.

[25] Alegria, M.C., Docena, C., Khater, L., Ramos, C.H., da Silva, A.C. and Farah, C.S. (2004) New protein–protein interactions identified for the regulatory and structural components and substrates of the type III secretion system of the phytopathogen *Xanthomonas axonopodis* pathovar *citri*. J. Bacteriol. 186, 6186–6197.