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Host plant-dependent phenotypic reversion of *Ralstonia solanacearum* from non-pathogenic to pathogenic forms via alterations in the *phcA* gene

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Summary

Ralstonia solanacearum **is a plant pathogenic bacterium that undergoes a spontaneous phenotypic conversion (PC) from a wild-type pathogenic to a non-pathogenic form. PC is often associated with mutations in *phcA,* which is a key virulence regulatory gene. Until now, reversion to the wild-type pathogenic form has not been observed for PC variants and the biological significance of PC has been questioned. In this study, we characterized various alterations in *phcA* (eight IS element insertions, three tandem duplications, seven deletions and a base substitution) in 19 PC mutants from the model strain GMI1000. In five of these variants, reversion to the pathogenic form was observed *in planta*, while no reversion was ever noticed *in vitro* whatever culture media used. However, reversion was observed for a 64 bp tandem duplication *in vitro* in the presence of tomato root exudate. This is the first report showing a complete cycle of phenotypic conversion/reversion in a plant pathogenic bacterium.**

Introduction

Ralstonia solanacearum, the causal agent of bacterial wilt, affects several hundred plant species worldwide, including agronomically important hosts such as tomato, potato, tobacco, peanut and banana (Hayward, 1994). *R. solanacearum* is a soil-borne pathogen that infects plants mainly through the roots. It invades xylem vessels and reaches the upper part of the plant after active multiplication. The vascular dysfunction induced by this extensive colonization causes wilting symptoms and plant death (Vasse *et al*., 1995).

Biochemical, genetic and molecular approaches have shown that this bacterium possesses a wide array of virulence factors, such as Exopolysaccharide I (EPSI) (Orgambide *et al*., 1991; Denny, 1995; Araud-Razou *et al*., 1998; Saile *et al*., 1998), secreted plant cell wall-degrading exoenzymes, polygalacturonase (PG) and endoglucanase (EG) (Schell, 1987; Schell *et al*., 1988; Denny *et al*., 1990; Allen *et al*., 1991; Huang and Allen, 1997; Saile *et al*., 1998), a type III secretion machinery (Hrp machinery) that allows the secretion and the injection of effector proteins into plant cells (Arlat *et al*., 1994; Cornelis and van Gijsegem, 2000; Guéneron *et al*., 2000). Interestingly, all these virulence factors are differentially controlled by PhcA, a regulatory protein which plays a central role in a complex regulatory cascade, called the Phc confinement sensing system. The Phc network functions like a quorum sensing system mediated by the specific endogenous signal molecule, 3-hydroxypalmitic acid methyl ester (PAME) (Clough *et al*., 1994; Flavier *et al*., 1997). *phcA* activates the expression of genes coding for EPSI production, EG and several other exoproteins (Huang *et al*., 1995; Clough *et al*., 1997a; Schell, 2000) and represses the expression of other genes such as those involved in motility (Liu *et al*., 2001), PG production, siderophore production (Huang *et al*., 1993; Clough *et al*., 1997b; Garg *et al*., 2000; Schell, 2000) and the Hrp machinery (Schell, 2000; S. Genin and C. A. Boucher, pers. comm.). The regulation exerted by *phcA* is either direct or mediated through intermediary regulatory genes (Schell, 2000). When PAME accumulates at high concentrations, the repression of active PhcA exerted by the PhcS/PhcR two component system is removed, resulting in activation or repression of target genes.

The sequencing of the *R. solanacearum* strain GMI1000 genome, which is organized in two replicons, a 3.7 megabase (Mb) chromosome and a 2.1 Mb megaplasmid, has given a more extensive picture of potential pathogenicity determinants of this pathogen and revealed that this bacterium possesses many features suggesting that it can adapt to diverse ecological niches (Salanoubat *et al*., 2002). Moreover, the *R. solanacearum* replicons display a mosaic structure with numerous insertion

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sequence elements (Salanoubat *et al*., 2002). This strongly suggests some degree of plasticity and instability for the *R. solanacearum* genome and a potential propensity to rapid evolution and adaptation. DNA rearrangements affecting virulence have already been reported to occur naturally in this pathogen (Boucher *et al*., 1988; Brumbley *et al*., 1993).

Under certain growth conditions, such as still broth culture (Kelman and Hruschka, 1973) or prolonged culture on agar plates (Kelman, 1954; Buddenhagen and Kelman, 1964), *R. solanacearum* spontaneously undergoes a phenotypic conversion (PC), shifting from a mucoid to a non-mucoid colony morphology. PC-type mutants are unable to wilt host plants (Kelman, 1954). However, they still grow *in planta* (Denny and Baek, 1991) and cause some disease symptoms such as stunting, stem necrosis and adventitious root formation (Husain and Kelman, 1958a; Denny *et al*., 1988; Denny and Baek, 1991). Several traits linked to virulence are simultaneously affected in PC-type mutants: loss of EPS production, reduced EG activity, increased motility and increased endo-PG activity (Kelman, 1954; Husain and Kelman, 1958b; Denny *et al*., 1988; Brumbley and Denny, 1990). A genetic study, carried out by Brumbley and Denny (1990) with strain AW1 and 12 other representative strains of *R. solanacearum*, showed that PC generally results from mutations in *phcA*. Insertions (2, 200 bp and 1 kb), a deletion (132 bp) and an IS*Rso4* insertion were identified within *phcA* from independent PC mutants of strains AW1 (Brumbley *et al*., 1993) and ACH0158 (Jeong and Timmis, 2000). No reversion to a wild-type form has ever been observed for PC mutants.

In this paper, we have characterized a group of PC mutants from *R. solanacearum* strains GMI1000 and GMI1559, which carry new deletions, duplications and IS element insertions within *phcA*. We show that reversion from PC type to wild type can be detected in the presence of a susceptible host plant. This is the first report of a complete cycle of phenotypic conversion/reversion for a plant pathogenic bacterium.

Results

Selection of spontaneous PC mutants

Based on colony morphology, we isolated 225 independent spontaneous PC-like mutants from strain GMI1000, according to the procedure described by Kelman and Hruschka (1973). Among these variants, 112 maintained stable rough colony shape morphology and were further studied for coordinated change in traits associated with PC. They all exhibited reduced EG activity and increased PG activity as compared with parental strains (data not shown). The production of EPS was impaired as detected by immunofluorescence labelling using an anti-EPSI monoclonal antibody (data not shown). We also isolated two typical PC mutants from strain GMI1559, a derivative of GMI1000 which carries a Tn5-gus*A3* insertion and maintains pathogenicity.

The 114 PC mutants were root inoculated on susceptible tomato plants. No disease symptoms were visible 21 days post-inoculation, whereas strains GMI1000 or GMI1559 led to total wilting within 12 days (data not shown). Although non-pathogenic, the PC mutants could be isolated at the epicotyl level at densities of approximately 10^5 colony forming units (cfu) [g(dry weight)]$^{-1}$, 21 days post-inoculation. In comparison, the pathogenic strains were detected at the epicotyl level at densities around 10^8 cfu [g(dry weight)]$^{-1}$ of partially wilted tomato plants, 5 days after inoculation.

Thereafter, PC variants obtained from strains GMI1000 and GMI1559 will, respectively, be named 1000-PCX and 1559-PCX where X refers to the mutant number.

Identification of PC mutants affected in the phcA *gene*

To check whether the PC phenotypes were due to alterations in the *phcA* gene, this gene was amplified by PCR from the 114 PC mutants. PCR products of 11 PC mutants were clearly different in size from the corresponding PCR products of the parental strains (Fig. 1). Two PCR products were smaller and nine were larger as compared with GMI1000 or GMI1559, suggesting deletions or insertion of DNA sequences within the *phcA* locus (Fig. 1).

To identify smaller DNA alterations within *phcA*, PCR products of the 103 remaining PC mutant strains were then digested with *Alw*I. This enzyme generates seven restriction fragments from PCR products of the GMI1000 and GMI1559 parental strains. Restriction patterns of eight PC mutants differed from that of the wild-type strain,

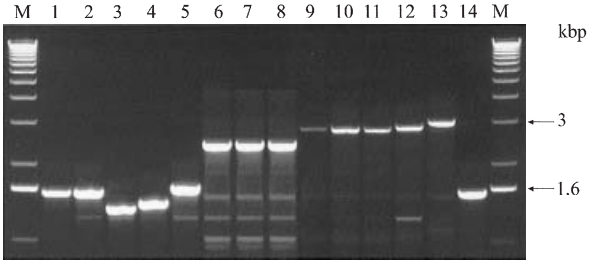

Fig. 1. Agarose gel electrophoresis of PCR products from parental strains and PC mutants using phcA1–phcA4 primers. M, molecular weight marker Invitrogen (1 kb ladder); lanes 1 and 14, wild-type strain GMI1000; lane 2, strain GMI1559; lane 3, strain 1000-PC3 (215 bp deletion); lane 4, strain 1000-PC72 (144 bp deletion); lane 5, strain 1559-PC1 (64 bp tandem duplication); lane 6, strain 1559-PC2 (884 bp IS*Rso1*); lane 7, strain 1000-PC99 (884 bp IS*Rso1*); lane 8, strain 1000-PC122 (884 bp IS*Rso1*); lane 9, strain 1000-PC13 (1287 bp IS*Rso8*); lane 10, strain 1000-PC19 (1287 bp IS*Rso8*); lane 11, strain 1000-PC80 (1287 bp IS*Rso8*); lane 12, strain 1000-PC7 (1335 bp IS*Rso10*); lane 13, 1000-PC11 (1467 bp IS*Rso13*).

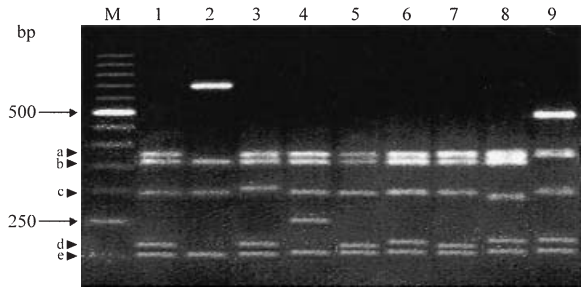

Fig. 2. Agarose gel electrophoresis of *Alw*I digested PCR products obtained with phcA1 and phcA4 primers from parental and PC mutant strains. M, molecular weight marker Roche (50 bp ladder); lane 1, wild-type strain GMI1000; lane 2, strain 1000-PC6 (2 bp deletion); lane 3, strain 1000-PC10 (7 bp tandem duplication); lane 4, strain 1000-PC12 (28 bp tandem duplication); lane 5, strain 1000-PC27 (10 bp deletion); lane 6, strain 1000-PC68 (4 bp deletion); lane 7, strain 1000-PC71 (10 bp deletion); lane 8, strain 1000-PC73 (9 bp deletion); lane 9, strain 1000-PC74 (1 bp substitution). The sizes of the restriction fragments generated from the PCR product of the GMI1000 strain are indicated by a black arrow: a, 376 bp; b, 355 bp; c, 295 bp; d, 219 bp and e, 197 bp. Two other fragments of 126 and 4 bp are also generated but are not visible on this figure.

showing the existence of DNA alterations within *phcA* (Fig. 2).

Thus, the PC phenotype of at least 19 of the 114 independent mutants was due to an alteration in the *phcA* gene. To confirm this and to assess whether the other PC variants could also be affected in *phcA*, we performed complementation experiments with the *phcA* gene. Plasmid pSB6, which carries the GMI1000 *phcA* gene, was conjugated into Sm^R derivatives of each of the 19 PC mutants shown to be affected in *phcA*. These complementation experiments were also carried out with 18 randomly chosen PC variants from the 114 which exhibited no obvious changes in fragment mobility (data not shown). As expected, the transconjugants of each of the 19 mutants showed a wild-type phenotype. Interestingly, 9 of the 18 other mutants were complemented by *phcA*, suggesting that they also carry alterations in *phcA*.

Sequence analysis of the phcA *locus of 19 PC mutants*

In order to further characterize the rearrangements in the *phcA* gene, we sequenced the PCR fragments of the 19 PC mutants described above. This analysis revealed four types of alterations within *phcA*: deletions, perfect tandem duplications, insertions of IS elements and a base substitution. The results are summarized schematically in Fig. 3.

Deletions and duplications within phcA

We identified six different deletions ranging from 2 to 215 bp in size within the *phcA* open reading frame (ORF) or straddling the promoter and the coding sequences (Table 1) in seven of the PC mutants (Tables 1 and 2). Most of them generate a frameshift, resulting in premature termination of translation, thus generating truncated PhcA proteins. For mutants 1000-PC72 and 1000-PC3, the deletions target the promoter region and the start codon of *phcA*.

Perfect tandem duplications of 7, 28 and 64 bp in size were identified in mutants 1000-PC10, 1000-PC12 and 1559-PC1 respectively (Tables 1 and 3). These duplications shift the reading frame and generate premature stop codons giving truncated PhcA proteins.

Disruption of phcA *by insertion sequences*

The IS elements of strain GMI1000 have been annotated and are presented on the GMI1000 Genome Database server (http://sequence.toulouse.inra.fr/R.solanacearum.html) and the IS Finder database server (http://www-IS.biotoul.fr/is.html). These data allowed us to readily identify insertions of four IS elements (IS*Rso1*, IS*Rso8*, IS*Rso10* or IS*Rso13*) within the *phcA* locus in eight PC variants (Table 1 and Fig. 3). IS*Rso1* and IS*Rso13* belong to the IS5 and IS4 families, respectively, whereas IS*Rso8* and IS*Rso10* are members of the IS3 family. The position and direct repeats (DRs) generated by insertions of these IS elements are presented in Fig. 3 and Table 1. Moreover, DNA sequences of IS copies inserted into *phcA* in the PC mutants were compared with those already annotated in the wild-type GMI1000 strain. Interestingly, this comparison allowed us to determine precisely which IS*Rso1* copy jumped into *phcA* for the three *phcA*::IS*Rso1* PC mutants (1559-PC2, 1000-PC99 and 1000-PC122). In these three independent mutants, the IS*Rso1* insertions occurred at the same position and in the same orientation and were all flanked by the same 3 bp DRs (Table 1 and Fig. 3). There are eight complete copies of this IS element in the GMI1000 genome. The DNA sequences of the IS*Rso1* elements inserted into *phcA,* in the three PC mutants, are identical to that of the megaplasmid copy, RS06002, suggesting that this copy transposed within *phcA*.

In contrast, for IS*Rso8*, IS*Rso10* and IS*Rso13*, such sequence comparisons did not allow us to identify which of the copies present in the GMI1000 genome transposed into *phcA* in PC mutants.

Base substitution within phcA

For mutant 1000-PC74, we identified a base substitution at nucleotide position 142 downstream of the *phcA* start codon, a G nucleotide is replaced by an A nucleotide. This substitution changes an Asp codon to a Gly codon.

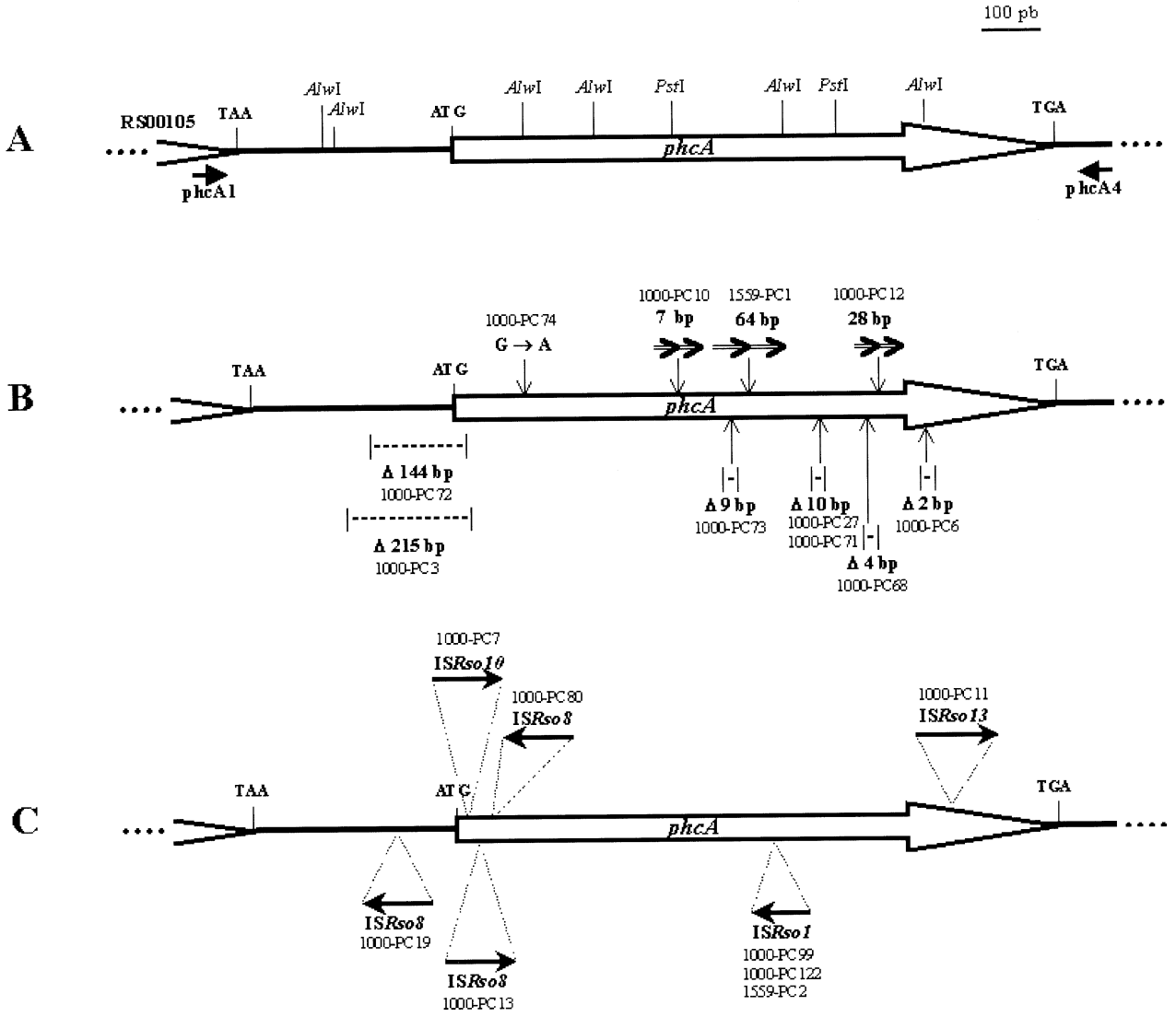

Fig. 3. Schematic representation of the *phcA* gene and the DNA rearrangements found in PC mutants.
A. Orientation of the *phcA* gene is shown by an open arrow, with the location of the start codon (ATG) and the stop codon (TGA). On the left, the truncated open arrow represents the ORF RS00105 upstream of *phcA*. Vertical bars indicate the position of *Alw*I, and *Pst*I restriction sites. Thick black arrows represent the position and orientation of the phcA1 and phcA4 primers.
B. Location of mutations is indicated by thin vertical arrows. The name of the PC mutant and the size of the corresponding mutation is shown. The symbols →, ⇒⇒, |---| Δ represent a substitution, and duplicated or deleted target sequences respectively.
C. Location of insertion sites of IS elements is shown by dotted lines. The name of the associated PC mutant is indicated. Thick black arrows represent the transcriptional direction of the IS transposase. The mutations shown are not drawn to scale.

Identification of revertants in root- or stem-inoculated soil-grown tomato plants

To further characterize the behaviour of PC mutants, we studied the invasiveness of the 19 PC variants affected in *phcA*, by measuring bacterial populations in tomato plants. For five of the 19 PC (*phcA*) mutants described above, WT-like colonies were isolated in association with PC colonies on BGT plates at the epicotyl level of symptom less root-inoculated tomato plants. For PC mutants, 1000-PC13 and 1559-PC1, which, respectively, carry an IS*Rso8* insertion and a 64-bp duplication in *phcA*, there were two orders of magnitude fewer WT-like colonies than PC colonies, giving an observed proportion of revertants of about 10^{-2} (Table 3). For the three other PC variants, 1000-PC99, 1000-PC122 and 1559-PC2, which carry an IS*Rso1* insertion at the same position in *phcA*, the proportion of revertants was much lower and ranged between 2×10^{-4} and 2×10^{-5}.

To further analyse the plant effect on reversion the 19 PC mutants were inoculated directly into stems of pot-grown tomato plants. No wilting was ever observed,

Table 1. Location, size and sequence of the diverse duplications, deletions, and IS direct repeats generated by IS insertion found within the *phcA* gene of PC mutants.

PC mutant strain	Position[a] of target for duplication, deletion or IS element	Size of duplication, deletion or IS element (bp)	Duplicated or deleted sequence in the *phcA* gene plus flanking sequence[b] and IS direct repeat sequence generated by IS insertion
Mutant with duplication			
1000-PC10	+356/+362	7	gtcaa**GCGCCTG<u>GCGCCTG</u>**gcgcc
1000-PC12	+670/+697	28	cggcc**TGAAGCGCCACTCCACCATGACTGTGCA<u>TGAAGCGCCACTCCACCATGACTGTGCA</u>**tgcat
1559-PC1	+422/+485	64	gcggc**GATGCGTTCCAATGAGCTGGACCTGGCG<u>TTCTCCCCGTTCCCGACGCGGCATCCGGAAAT CGTG</u><u>GATGCGTTCCAATGAGCTGGACCTGGCG TTCTCCCCGTTCCCGACGCGGCATCCGGAAAT CGTG</u>**gaaga
Mutant with deletion			
1000-PC6	+769/+770	2	gaagg[AT]ctggt
1000-PC68	+698/+702	4	gtgca[TGCA]tggcg
1000-PC73	+457/+465	9	ctccc[CGTTCCCGA]cgcgg
1000-PC27 and PC71	+636/+645	10	cgctg[ATTCCGCTCG]attac
1000-PC72	−126/+18	144	tgaaa[ATCGTCCGGCAATCCGTACACTGGACAATT ACTACATTTGTGACGCAGTCGCCGTATCCTGCTG CGTCTGGGCCGTTGCCTCGGCAACGTCTGCCTTT CCACCTTCTGTCTGAGCCAAAGCGTCCCATGGTC AACGTCGATACC]aagct
1000-PC3	−179/+36	215	cgaaa[ACGTTTGCGTCTAAGCAAATTTCGCAGACG GGCTGCTGGAATTCGCTTTGAAAATCGTCCGGCA ATCCGTACACTGGACAATTACTACATTTGTGACG CAGTCGCCGTATCCTGCTGCGTCTGGGCCGTTGC CTCGGCAACGTCTGCCTTTCCACCTTCTGTCTGA GCCAAAGCGTCCCATGGTCAACGTCGATACCAA GCTGTTGGTGATTTTT]gtaga
Mutant with IS			
1000-PC19 (IS*Rso8*)	−107	1287	TAC
1000-PC7 (IS*Rso10*)	+14	1335	GATAC
1000-PC13 (IS*Rso8*)	+49	1287	GCTG
1000-PC80 (IS*Rso8*)	+56	1287	TTC
1559-PC2, 1000-PC99 and PC122 (IS*Rso1*)	+527	884	TTA
1000-PC11 (IS*Rso13*)	+842	1467	GGCCTGAACAT

a. Nucleotide numbers are given considering the nucleotide A of the start codon of the *phcA* gene as 1.
b. Targets for duplication are in bold capitals, resulting tandem duplication in bold underlined and deleted regions are in capitals in square brackets. Nucleotides flanking target regions, tandem duplications and deleted regions are in lower case.

though each of these PC mutants induced noticeable necrosis of the central cylinder tissues surrounding the inoculation point (data not shown). For the 5 PC mutants which reverted in the previous conditions, isolation from the stem resulted in recovery of both PC and WT-like colonies. However, the proportion of revertants was highly variable for each PC mutant, ranging from zero up to 100% depending on the individual plant that was analysed (Table 3). This high variability may result from differences in the efficiency of the inoculation procedure, the kinetics of reversion and/or the physiological status of the plants. None of the 14 other PC mutants tested in these conditions gave rise to revertants.

For each of these five PC mutants, several independent WT-like strains, isolated from root- or stem-inoculated plants, were randomly chosen and tested for pathogenicity and other phenotypic traits. They were all fully pathogenic on tomato plants, produced EPSI and produced wild-type levels of endoglucanase and polygalacturonase activities (data not shown).

For the five PC mutants giving revertants in tomato, and the other PC mutants, no WT-like revertants were ever detected on BGT medium, despite screening more than 1.2×10^5 individual colonies from B liquid cultures for each mutant. This indicates that the potential spontaneous reversion rate of these PC mutant strains is lower than 4×10^{-5}.

Identification of revertants in axenic conditions

We conducted additional experiments with tomato plants grown in axenic conditions in Magenta boxes (see *Experimental procedures*). Twelve days after inoculation with the 19 PC mutants, roots of the plantlets or the M and S growth medium were rinsed with sterile water giving two kinds of rinsing: rinsing of tomato roots (RTR) and rinsing of inoculated M and S (RIMS). Interestingly, 1000-PC13

Table 2. Bacterial strains and plasmids.

Strain/plasmid	Other designation	Relevant phenotype/genotype	Source/reference
R. solanacearum			
GMI1000		wild-type (WT), race 1, biovar 4	Tomato, French Guyana Digat (1966)
GMI1559		GMI1000, RS01785::Tn5-gus3A	Etchebar *et al.* (1998)
PC mutants			
GMI1697	1000-PC10	7 bp *phcA* tandem duplication	This work
GMI1698	1000-PC12	28 bp *phcA* tandem duplication	This work
GMI1643	1559-PC1	64 bp *phcA* tandem duplication	This work
GMI1699	1000-PC7	*phcA*::IS*Rso10* (1335 bp)	This work
GMI1700	1000-PC13	*phcA*::IS*Rso8* (1287 bp)	This work
GMI1701	1000-PC19	*phcA*::IS*Rso8* (1287 bp)	This work
GMI1702	1000-PC80	*phcA*::IS*Rso8* (1287 bp)	This work
GMI1703	1000-PC11	*phcA*::IS*Rso13* (1467 bp)	This work
GMI1640	1559-PC2	*phcA*::IS*Rso1* (884 bp)	This work
GMI1704	1000-PC99	*phcA*::IS*Rso1* (884 bp)	This work
GMI1705	1000-PC122	*phcA*::IS*Rso1* (884 bp)	This work
GMI1706	1000-PC6	2 bp *phcA* deletion	This work
GMI1707	1000-PC68	4 bp *phcA* deletion	This work
GMI1708	1000-PC73	9 bp *phcA* deletion	This work
GMI1709	1000-PC27	10 bp *phcA* deletion	This work
GMI1710	1000-PC71	10 bp *phcA* deletion	This work
GMI1711	1000-PC72	144 bp *phcA* deletion	This work
GMI1712	1000-PC3	215 bp *phcA* deletion	This work
GMI1713	1000-PC74	*phcA* 1 bp substitution	This work
Revertants			
GMI1644	1559-PC1rev1		This work
GMI1645	1559-PC1rev2		This work
GMI1641	1559-PC2rev1		This work
GMI1642	1559-PC2rev2		This work
GMI1714	1000-PC99rev1		This work
GMI1715	1000-PC122rev1		This work
GMI1716 to GMI1727	1000-PC13rev1 to 1000-PC13rev12		This work
E. coli			
DH5α		*F-, recA, lacZ*ΔM15	Bethesda Research Laboratory
Plasmids			
pGEM-T Easy		3018 bp, Amp^R	Promega
pRK2013		SpR., *tra*RK2+, Δ*rec*RK2, *rep*E1$^+$, Km^R.:Tn7, *mob* ColE1	Figurski and Helinski (1979)
pLAFR6		Tc^R	Huynh *et al.* (1989)
pSB6		Tc^R, Gm^R, pLAFR6 carrying *phcA* of GMI1000	This work
pCZ428		pGEM-T carrying lacZ and *aprA*, Amp^R, Gm^R	C. Zischek (pers. comm.)

Table 3. Frequencies of reversion or proportion of revertants of three PC mutant strains as revealed by the susceptible tomato cv. Supermarmande in different combinations.

	Peat pots		Axenic conditions			BGT medium[d]	
Strain[g]	Root inoculation	Stem puncturing (RTR)[a]	Tomato root rinsings	Rinsing of inoculated M and S (RIMS)[b]	Non-supplemented media[c]	–TRE	+TRE
1559-PC1	2.10^{-2} to 8.10^{-2}	variable[f], up to 5.10^{-2}	5.10^{-2} to 5.10^{-3}	5.10^{-3}	ND[e] $< 10^{-4}$	ND $< 4.10^{-5}$	3.10^{-3}
1000-PC13	3.10^{-2}	3.10^{-3} to 1	5.10^{-2}	ND $< 10^{-4}$	ND $< 10^{-3}$	ND $< 4.10^{-5}$	ND $< 4.10^{-5}$
1559-PC2	2.10^{-5} up to 0.5	variable[f],	ND $< 10^{-4}$	ND $< 10^{-4}$	ND $< 10^{-3}$	ND $< 4.10^{-5}$	ND $< 4.10^{-5}$

a. Membrane-sterilized, rinsing of axenic tomato roots.
b. Membrane-sterilized, obtained after steeping the Murashige and Skoog culture medium in water.
c. Murashige and Skoog (M and S) medium alone, M and S complemented with sodium glutamate or with glucose.
d. BGT alone (–TRE) or complemented with tomato root exudate (+TRE).
e. ND: not detected; ND $< 10^{-3}$: no revertant detected among 5000 individual colonies; ND $< 10^{-4}$: no revertant detected among 50 000 individual PC colonies, ND $< 4.10^{-5}$ no revertant detected among 120 000 individual PC colonies.
f. Variable: frequency of reversion is dependent on the efficiency of individual PC clone injection in individual plants.
g. No revertant was ever isolated, whatever the technique used, from 1000-PC3, 6, 7,10, 11, 12, 19, 27, 68, 71, 72, 73, 74 and 80 mutants.

(*phcA*::IS*Rso8*) and 1559-PC1 (64 bp tandem duplication) variants gave WT-like colonies on BGT plates spread with RTRs. For both variants, the frequency for reversion was similar to that obtained in tomato plants grown in pots (Table 3). Under these conditions, no revertants were recovered for any of the other 17 PC mutants tested, even for mutants 1000-PC99, 1000-PC122 and 1559-PC2, which gave revertants in pot-grown tomato plants.

With RIMS, revertants were only observed for variant 1559-PC1 (64 tandem duplication). The frequency for reversion was one order of magnitude lower than that obtained with RTR (Table 3).

Identification of revertants ex planta in the presence of tomato root exudates

To assess whether the presence of plants was specifically required to see the reversion events, Magenta boxes containing M and S (supplemented or not with glucose or glutamate) were inoculated with the 19 PC mutant strains in the absence of tomato plants. No WT-like colonies were ever isolated from rinsings of the culture medium, even with the 1559-PC1 mutant (Table 3). Moreover, the 19 PC mutants were tested on minimal medium supplemented with glutamate (MMG), a medium which is known to specifically induce *R. solanacearum hrp* genes that seems to mimick apoplast conditions (Arlat *et al*., 1992). In these conditions, no revertants were ever observed (data not shown).

To see whether reversion could be induced by plant extracts, mutants 1000-PC13, 1559-PC2 and 1559-PC1 and the 16 other PC mutants were plated onto BGT medium supplemented or not with a sterile tomato root exudate (TRE) (see *Experimental procedures*). Under these conditions, WT-like colonies were identified at a frequency of 3×10^{-3} for mutant 1559-PC1 (among approximately 120 000 colonies of the PC-type). No revertant was identified for any other tested PC mutants on BGT+TRE.

All revertant strains recovered from RTR, RIMS or BGT+TRE plates were fully pathogenic and displayed WT levels of enzyme production.

Molecular analyses of revertants

For each of the five PC mutants giving revertants, we isolated several independent revertants obtained in different conditions and their *phcA* locus was analysed by PCR. The size of the *phcA* gene-specific products of all tested revertants and the WT strains were identical, as determined by agarose gel electrophoresis (data not shown). Direct sequence analysis of the PCR products of all revertants tested showed that the *phcA* wild-type sequence was restored, thus showing that the 64 bp direct duplication carried by the PC mutant 1559-PC1 had disappeared and that the IS elements and one DR copy had been completely excised from the *phcA* gene in revertants of PC carrying an IS insertion within *phcA* (data not shown).

To study whether the IS excision from *phcA* was followed by a new transposition event or not, we compared the distribution of IS*Rso1* and IS*Rso8* copies in the genome of the wild-type, PC variants and revertant strains by Southern hybridization. Genomic DNAs were digested with *Pvu*II or *Pst*I, and probed with internal DNA fragments of IS*Rso1*, IS*Rso8* or *phcA*. The *Pvu*II and *Pst*I restriction enzymes were chosen because no restriction sites are present in IS*Rso1* and IS*Rso8* respectively.

Using the *phcA*-specific probe, we observed the same hybridization pattern for the WT and revertant strains, whereas for all PC mutants tested, we obtained a larger band corresponding to IS*Rso*1 or IS*Rso*8 insertions within *phcA* (Fig. 4B and D).

Hybridization with the IS*Rso1*-specific probe gave similar results, which is the same profile for WT and revertant strains and an additional band corresponding to the insertion event into *phcA* for PC variants 1559-PC2, 1000-PC99 and 1000-PC122 (Fig. 4A, lanes 3, 5 and 7 respectively), thus showing that revertants were generated by excision of the IS element from *phcA.*

For strain1000-PC13 (*phcA*::IS*Rso*8), we identified two classes of revertants based on their hybridization patterns with the IS*RSo*8 probe. For the first class (two revertants out of 12), the hybridization pattern was identical to that of the WT strain (Fig. 4C, lane 4), whereas for the 10 remaining revertants the hybridization pattern was different from both the WT and 1000-PC13 patterns strain (Fig. 4C, lane 3). As expected, the band corresponding to the insertion into *phcA* disappeared, but a new band, corresponding to a 2.8 kb DNA fragment in size, appeared in these revertants (Fig. 4C, lane 3). The hybridization pattern with the *phcA* probe shows that this new band is not the result of a rearrangement in the *phcA* locus (Fig. 4D). Both classes of revertants were observed in axenic and pot-inoculated tomato plants.

Discussion

By responding to specific environmental signals, regulatory genes allow bacteria to adapt to changes in their habitat (Miller *et al*., 1989). Genetic variation leading to phenotypic conversion represents an alternative adaptive strategy, which by providing some degree of diversity, ensures survival in specific niches and/or adaptation to sudden changes in the environment. This phenomenon has been extensively studied in animal pathogenic bacteria (Henderson *et al*., 1999; Hallet, 2001). Reversibility of genetic variation is necessary for bacteria to maintain their adaptative features and to complete their life or infectious

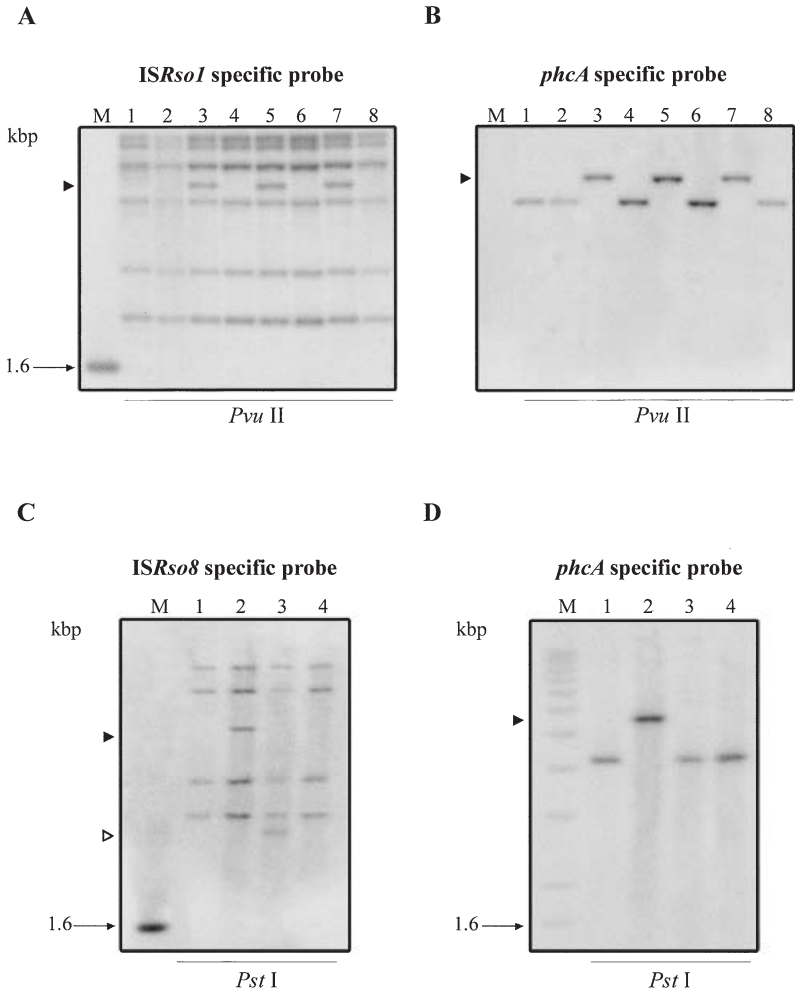

Fig. 4. Southern blot analysis of DNA of wild-type, PC mutants and revertant strains.
A, B. Total genomic DNA of the strains was digested with *Pvu*II and hybridized with IS*Rso1* (generated using ISRso1A–ISRso1B primers) (A) and *phcA* (generated using phcA1–phcA4 primers) (B) specific probes. M, molecular weight marker Invitrogen (1 kb ladder); lane 1, wild-type strain GMI1000; lane 2, strain GMI1559; lane 3, strain 1559-PC2; lane 4, strain 1559-PC2rev1; lane 5, strain 1000-PC99; lane 6, strain 1000-PC99rev1; lane 7, strain 1000-PC122; lane 8, strain 1000-PC122rev1.
C and D. Total genomic DNA was digested with *Pst*I and hybridized with IS*Rso8* (generated using ISRso8A–ISRso8B primers) (C) and *phcA* (generated using phcA1–phcA4 primers) (D) specific probes. M, molecular weight marker Invitrogen (1 kb ladder); lane 1, wild-type strain GMI1000; lane 2, strain 1000-PC13; lane 3, strain 1000-PC13rev1; lane 4, strain 1000-PC13rev2. The arrows indicate the positions of the additional bands in PC mutants (filled) or revertants (empty) strains.

cycles. This reversible alteration of gene expression, called phase variation or phenotypic conversion/reversion, is mediated by a variety of molecular mechanisms that modify the sequence and/or structure of DNA (Hallet, 2001).

In this study, we describe a complete phenotypic conversion/reversion cycle involving the key virulence regulatory gene *phcA* of the plant pathogenic bacterium *R. solanacearum*. Furthermore, we show that reversion events are specifically detected *in planta* or with plant extracts.

Is the frequency of reversion of tandem duplication PC mutants related to the size of the duplication?

We have isolated three PC mutants carrying direct tandem duplications of 7, 28 and 64 bp within *phcA*. Revertants were only obtained for the 1559-PC1 mutant, which carries the largest duplication. All 1559-PC1 revertants analysed carried a wild-type copy of the *phcA* gene, suggesting precise excision of the duplication. Phenotypic conversion/reversion linked to duplication events has been reported before in the mushroom pathogen *Pseudomonas tolaasii* (Han *et al*., 1997) and in the human pathogen *Streptococcus pneumoniae* (Waite *et al*., 2001). In type 3 pneumococci, the phenotype switching resulted from the presence of perfect tandem duplications, ranging from 11 to 239 bp in size, within the *cap*3A capsule gene. Capsular revertants, carrying a wild-type *cap3*A gene, formed spontaneously *in vitro*. Interestingly, a linear relationship between the (log) frequency of reversion and the (log) length of duplication was observed (Waite *et al*., 2001). Thus, the inability to detect revertants for the 7 and 28 bp duplication PC mutants of *R. solanacearum* might be explained by the existence of a relationship between the length of the duplication and the frequency of reversion, as is the case in *Streptococcus*. However, because the 64 bp duplication was obtained from strain GMI1559, whereas the two shorter duplications were derived from strain GMI1000, we cannot rule out that the mutation of ORF RS01785 carried by strain GMI1559 does not influence the reversion rate.

The mechanisms that may lead to sequence duplications in bacteria are not yet clearly understood. Three main mechanisms have been postulated, RecA-dependent recombination, RecA-independent replication misalignment and sister chromosome exchange-associated misalignment (Morag *et al*., 1999). In many organisms short repeats are stabilized by the mismatch-repair (MMR) system (Lovett and Fetchenko, 1996). Recently, it was shown that single-stranded DNA exonucleases play an additional, important role in stabilizing tandem repeats (Fetchenko *et al*., 2003). In *Escherichia coli*, rearrangements between tandem DNA repeats carried by plasmids, resulting either in expansion or deletion of the repeats have been deduced to occur primarily by slipped misalignment during DNA replication, in a RecA-independent manner (Bzymek and Lovett, 2001). In *P. tolaasii* reversion from the duplicated form is also RecA independent (Sinha *et al*., 2000). The sequence of strain GMI1000 genome should help us to assess whether similar mechanisms drive reversion of tandem duplications in *R. solanacearum.*

Reversion of PC mutants carrying IS elements within phcA

We show that four of the 17 IS elements identified in the GMI1000 genome are active mobile elements that are able to modify the expression of an important virulence regulatory gene. DNA analysis of the regions flanking the different IS element copies and of the *phcA* target sites identified in this study revealed no consensus sequence. It is therefore difficult to decide whether or not *phcA* nucleotide sequence represents a hot spot for the integration of these IS elements.

Among the PC mutants carrying an IS insertion in *phcA*, only those harbouring IS*Rso8* or IS*Rso1* gave revertants. Interestingly, for each of these two IS elements, three independent variants were obtained, whereas only one variant was obtained for IS*Rso10* and IS*Rso13*. This might suggest either that *phcA* sequences represent a hot-spot for IS*Rso1* and IS*Rso8* or that these two elements are more active than IS*Rso10* and IS*Rso13*. This latter hypothesis could suggest that the frequency of reversion of PCs might depend on the level of activity of IS elements.

We have isolated three independent PC mutants carrying identical IS*Rso1* insertions. These three PC mutants reverted to wild type with similar frequencies. In all revertants tested, the precise excision of the IS element restored the original IS*Rso1*-specific hybridization pattern of the wild-type strain. This indicates that excision entails loss of the IS*Rso1* copy and not its transposition to a new site.

Of the three IS*Rso8* insertions identified in this study, the copy carried by PC mutant 1000-PC13 was the only one that gave revertants. Interestingly, the transposase gene of the 1000-PC13 copy has the same transcriptional direction as *phcA*, whereas it is in the opposite orientation for the two other mutants. Although the *phcA* gene is autoregulated, it is constitutively expressed at a low level (Brumbley *et al*., 1993; Clough *et al*., 1997b). It might therefore be possible that the transposase gene of the IS element inserted into *phcA* is co-transcribed from its own promoter and from that of *phcA* in the 1000-PC13 mutant. Does this potential co-transcription control the reversion event? Further work is needed to clarify whether the orientation of the IS*Rso8* or the insertion context plays a role in the ability to generate revertants. The analysis of 12 independent revertants of 1000-PC13 suggested that a duplication of IS*Rso8* occurred in the course of the transposition process in the majority of revertants and that the new insertion took place in the same locus in these revertants.

Interestingly, reversible inactivation of virulence genes by an insertion sequence was recently reported for two human pathogens, *Neisseria meningitidis* (Hammerschmidt *et al*., 1996) and *Staphylococcus epidermis* (Ziebuhr *et al*., 1999). In both pathogens, spontaneous reversion was observed *in vitro* and was linked to complete excision of the IS element. Interestingly, in *S. epidermis* the excision event was accompanied by a new insertion event, as observed in our study.

Is reversion host-plant dependent?

Contrarily to what has been reported for phenotypic variants of *P. tolaasii* (Han *et al*., 1997), *S. pneumoniae* (Waite *et al*., 2001), *S. epidermis* (Ziebuhr *et al*., 1999) and *N. meningitidis* (Hammerschmidt *et al*., 1996), which reverse spontaneously, spontaneous reversion of *R. solanacearum* PC mutants to wild type was not observed *in vitro*. Revertants were detected after tomato root or stem inoculation for mutants carrying a 64 bp duplication, IS*Rso1* or IS*Rso8* insertions within *phcA*. Remarkably, the 64 bp tandem duplication mutant was the only one that reverted in the presence of tomato root exudates. This result suggests that the molecular mechanisms driving reversion differ with the nature of the mutation affecting *phcA*. It is important to address the potential specificity of the plant effect on the reversion events. Is the reversion event spontaneous but selected by the plant or does a specific plant factor(s) actively induce the reversion event by controlling specific bacterial functions? While phenotypic conversion/reversion seems to be mainly a random process, there are cases where the rate of phenotypic switching is controlled by environmental factors (Hallet, 2001). Recently, the existence of an environmental control of reversion frequency modulated by a regulatory protein (PvrR) was reported in *P. aeruginosa* (Drenkard and Ausubel, 2002).

Biological significance of phenotypic conversion/reversion in R. solanacearum

The potential biological significance of PC has been the source of considerable speculation. Our work gives a new insight into PC, by showing that reversion to a wild-type pathogenic phase can be detected *in planta*. In this study, about 50% of the PC mutants that were randomly tested were affected in *phcA*. Because this key regulatory gene both positively and negatively regulates two different sets of genes that might control two physiological statuses specific to different environmental niches (Schell, 2000), its phenotypic conversion/reversion most probably has great biological significance. This point is strengthened by the fact that under certain conditions PC mutants have a selective advantage over the wild type (Brumbley *et al*., 1993). It was proposed that PC-type bacteria may represent a form specifically adapted for survival in the soil, in plant debris or in starving conditions (Denny *et al*., 1994). Recently, it was shown that *phcA* negatively regulates *hrp* virulence genes (Schell, 2000; C. A. Boucher, pers. comm.) and twitching motility mediated by type IV pili (Liu *et al*., 2001). Therefore, PC (*phcA*) variants may have a greater spatial range, thus extending their nutrition capacity (Liu *et al*., 2001) and some increased ability to interact with plants. Our results and previous work (Denny *et al*., 1988) showing that PCs are able to invade and colonize plants to some degree, support the latter hypothesis. To further study the role of PC in the *R. solanacearum* life cycle it will be necessary to determine whether PC mutants are really better adapted to soil or plant debris conditions than the wild type.

phcA PC variants have been found in infected or wilted tomato plants (Brumbley *et al*., 1993). This suggests that the phenotypic conversion event itself can take place *in planta*. It will now be important to see whether PC mutants can also be directly detected from infected soils or water systems and to determine whether these 'natural' variants are able to reverse to the pathogenic form. This information would be useful for studies concerning detection, epidemiology and control of bacterial wilt.

This is, to our knowledge, the first report showing phenotypic conversion/reversion in a plant pathogenic bacterium. It reinforces the idea of common themes in animal and plant pathology by demonstrating that similar rearrangements drive phase variation in both animal and plant pathogens and by establishing that the host influences the structure of pathogen populations.

Experimental procedures

Bacterial strains, media and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 3. GMI1000 and GMI1559 are equally pathogenic on tomato and produce a large amount of exopolysaccharide as revealed by specific monoclonal immunofluorescence labelling (Araud-Razou *et al*., 1998). Strain GMI1559 is a derivative of GMI1000 that carries a Tn5-*gusA*3 insertion into ORF RS01785 which codes for a putative transcriptional regulator of the MarR family.

R. solanacearum strains were grown at 28°C on minimal medium, rich liquid B medium or solid BGT medium (Boucher *et al*., 1985). *E. coli* strains were grown in Luria–Bertani (LB) medium. When needed, antibiotics were added to the media at the following final concentrations (mg l^{-1}): streptomycin (Sm) 200, tetracycline (Tc) 10, ampicillin (Ap) (50) and gentamycin (Gm) 20.

Isolation and colony counting of *R. solanacearum* for PC selection, from inoculated plants, or from Magenta boxes (V8505, Sigma) was carried out using the Spiral Plate Maker method (Frey *et al*., 1994) on BGT in 14-cm-diameter Petri dishes. This method allowed a single specific colony type to be identified amongst thousands of colonies prior to confluent growth.

Pathogenicity tests

Pathogenicity tests on soil-grown plants were conducted as already described (Frey *et al*., 1994). Each tested strain was assessed for wilting ability in three independent 18 plant-experiments.

Selection and phenotypic characterization of spontaneous PC mutants

Spontaneous PC mutants were obtained according to the procedure of Kelman and Hruschka (1973). Two hundred and twenty-five independent still broth cultures were used, and from each a single PC colony was randomly selected. Colony morphology was the first characteristic that allowed selection of spontaneous PC mutants. Strains GMI1000 and GMI1559 exhibit ovoid to irregular-shaped smooth colonies with a pink centre when grown on rich medium. Spontaneous PC mutants derived from these pathogenic strains developed on the same medium and appeared as small, round, dark red colonies. Individual PC-like colonies were cloned and purified after three successive 8-days long subcloning steps, which allowed the stability of the colony morphology to be assessed. Tests for endoglucanase production and polygalacturonase production were carried out in agar-based methods (Andro *et al*., 1984). Deficiency in EPS production was assessed by immunofluorescence labelling with monoclonal antibodies that are specific for the EPS (Araud-Razou *et al*., 1998).

Obtention of revertants

The 19 PC mutants carrying an identified DNA rearrangement within *phc*A were further assessed for their invasiveness *in planta* (see above). Each PC was tested on 18 plants. Bacterial isolations were carried out 21 days post-inoculation from stem fragments sampled at the cotyledon level as previously described (Frey *et al*., 1994), and approximately

5×10^4 single colonies were tested with regard to their colony morphology on BGT plates. In addition, 10 individual colonies of each PC mutant strain were assessed for pathogenicity after stem puncturing with a needle containing 40 µl 3×10^8 cfu ml^{-1} inoculum. Stem fragments were sampled 2 cm above the inoculation point at 8 days post-inoculation.

Axenic cultivation of tomato plants was conducted as follows: tomato seeds were first surface sterilized (Etchebar *et al*., 1998) and allowed to germinate on BG rich medium. Germinated seeds were deposited onto 0.8% agar containing Murashige and Skoog (M and S) medium (ICN Biomedicals Inc.) in sterile Magenta boxes (V8505, Sigma). Tomato plants at the two leaf stage were inoculated by pipetting 2 ml of inoculum adjusted to 10^7 cfu ml^{-1} onto the surface of soft agar medium in Magenta boxes. Next, 5 ml of sterile water was used to rinse the soft culture medium off at 18 days post-inoculation. After 15 min soaking, 4 ml was pipetted off. This rinsing of tomato roots (RTR) was plated on BGT medium to assess the presence of WT revertants. Concurrently, 18 days after inoculation tomato seedlings were removed from Magenta boxes and the M and S medium was rinsed with 5 ml of sterile water. This rinsing (RIMS: rinsing of inoculated M and S) was spread on BGT medium. Controls consisted of bacteria inoculated into Magenta boxes in the absence of plant, or in Petri dishes containing M and S complemented with sodium glutamate (20 mM) or glucose (20 mM).

To obtain tomato root exudates (TRE), 30 sterile root systems taken from Magenta boxes were soaked in 12 ml of sterile water for 30 min on a rotary shaker and the solution was sterile-filtered through 0.45 and 0.22 µM membranes. Next, 2 ml of the freshly prepared, sterile TRE were poured onto individual 14-cm-diameter BGT-containing Petri dishes, and allowed to dry in a laminar sterile flow cabinet, prior to plating the PC suspension. Controls were performed with 2 ml of sterile water.

Frequencies of phenotypic reversion (*in vitro*) or proportion of observed revertants (*in planta*) revealed in the above experimental conditions were determined by plating serial dilutions of the bacterial suspension with a Spiral Plate Maker and counting the number of PC- and WT-like colonies. We assumed that there were only two mutually exclusive possibilities OFF or ON, and that the maximal probability P to detect one WT-like colony among a number N of PC colonies follows the Poisson distribution, i.e. $N \times Q = 5$, where Q is the reversion frequency and P = 99.33%.

DNA manipulation

Procedures used for DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, ligation, transformation of competent *E. coli* cells, Southern blotting and hybridization have been described previously (Sambrook *et al*., 1989).

Complementation experiments

The *phcA* gene was amplified by PCR from strain GMI1000 genomic DNA using primers phca1H and phca2X, which carry restriction sites for *Hin*dIII and *Xba*I respectively. The 1358 bp DNA fragment thus generated contains the coding sequences and the promoter region of *phcA* preceded by the putative transcription terminator of ORF RS00105 located upstream of *phcA*. This DNA fragment was cloned in a pGEMt derivative, named pCZ428, digested with *Hin*dIII and *Xba*I upstream the *lacZ* reporter gene and a gentamycin resistance cassette (*aprA* gene). The *Hin*dIII–*Kpn*I restriction fragment containing *phcA*, *lacZ* and *aprA* genes was then cloned into the pLAFR6 broad-host range vector. The resulting construct, named pBS6, was introduced into Sm^R derivatives of different PC mutants by triparental matings, as previously described (Arlat *et al*., 1992). As a control, the pLAFR6 vector was also introduced into Sm^R PC derivatives.

PCR amplification

PCRs were performed in a thermocycler (Mastercycler Gradient, Eppendorf) in a 20 µl (total volume) reaction mixture containing 10× buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4; Invitrogen), 2 mM $MgCl_2$, 250 µM each dNTP, 1 µM each primer, 1 U *Taq* polymerase (Invitrogen) and 50 ng template DNA. The following PCR profile was used: initial denaturation at 95°C for 3 min; 30 cycles consisting of 95°C for 30 s, 65°C for 30 s and 72°C for 1 min; and final elongation at 72°C for 5 min.

The primer sequences are listed in the *Supplementary material*. The *phcA* gene of the wild-type, PC mutants and revertants strains was amplified using the primers phcA1 and phcA4. The primers pairs ISRso1-A/ISRso1-B, ISRso8-A/ISRso8-B, ISRso10-A/ISRso10-B and ISRso13-A/ISRso13-B were used to amplify, respectively, specific internal fragments of the IS*Rso1*, IS*Rso8*, IS*Rso10* and IS*Rso13* elements inserted into the *phcA* gene of PC mutants. These amplified fragments were then used as probes in Southern hybridization experiments.

DNA sequencing and sequence analysis

Primers phcA1, phcA4, phcAS1H, phcAS2X, phcAS3, phcAS5, phcAS6 and phcAS7 were used to perform DNA sequencing of the *phcA* gene of the wild-type, PC mutants and revertant strains (*Supplementary material*). Primers ISRso1-C, ISRso1-D, ISRso8-C, ISRso8-D, ISRso10-C, ISRso10-D, ISRso13-C and ISRso13-D were used to determine the sequences of the ends of the IS elements inserted into the *phcA* locus of PC mutants. PCR product sequences were determined using the BigDye Terminator Cycle Sequencing Ready Reaction kit and an ABI Prism 3700 DNA analyser (Applied Biosystems). Sequence data were analysed with the GCG (Genetics Computer Group, version 10.0) software package and the BLAST search programs (Altschul *et al*., 1997).

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Supplementary material

The following material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/mmi/mmi3605/mmi3605sm.htm
Table S1. Primers used in this study.

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