Twitching and Swimming Motility Play a Role in *Ralstonia solanacearum* Pathogenicity

Jordi Corral,a Pau Sebastià,b Núria S. Coll,b Jordi Barbé,a Jesús Aranda,a Marc Vallsb,c

aDepartament de Genètica i Microbiologia, Facultat de Biociències, Universitat Autònoma de Barcelona, Cerdanyola del Vallès (Barcelona), Catalonia, Spain
bCentre for Research in Agricultural Genomics (CSIC-IRTA-UAB-UB), Cerdanyola del Vallès (Barcelona), Catalonia, Spain
cGenetics Section, Universitat de Barcelona, Barcelona, Catalonia, Spain

Jesús Aranda and Marc Valls contributed equally to this article as senior authors. Author order was determined on the basis of seniority.

**ABSTRACT** *Ralstonia solanacearum* is a bacterial plant pathogen causing important economic losses worldwide. In addition to the polar flagella responsible for swimming motility, this pathogen produces type IV pili (TFP) that govern twitching motility, a flagellum-independent movement on solid surfaces. The implication of chemotaxis in plant colonization, through the control flagellar rotation by the proteins CheW and CheA, has been previously reported in *R. solanacearum*. In this work, we have identified in this bacterium homologues of the *Pseudomonas aeruginosa* pilI and chpA genes, suggested to play roles in TFP-associated motility analogous to those played by the cheW and cheA genes, respectively. We demonstrate that *R. solanacearum* strains with a deletion of the pilI or the chpA coding region show normal swimming and chemotaxis but altered biofilm formation and reduced twitching motility, transformation efficiency, and root attachment. Furthermore, these mutants displayed wild-type growth *in planta* and impaired virulence on tomato plants after soil-drench inoculations but not when directly applied to the xylem. Comparison with deletion mutants for pilA and fliC—encoding the major pilin and flagellin subunits, respectively—showed that both twitching and swimming are required for plant colonization and full virulence. This work proves for the first time the functionality of a pilus-mediated pathway encoded by pil-chp genes in *R. solanacearum*, demonstrating that pilI and chpA genes are bona fide motility regulators controlling twitching motility and its three related phenotypes: virulence, natural transformation, and biofilm formation.

**IMPORTANCE** Twitching and swimming are two bacterial movements governed by pili and flagella. The present work identifies for the first time in the Gram-negative plant pathogen *Ralstonia solanacearum* a pilus-mediated chemotaxis pathway analogous to that governing flagellum-mediated chemotaxis. We show that regulatory genes in this pathway control all of the phenotypes related to pili, including twitching motility, natural transformation, and biofilm formation, and are also directly implicated in virulence, mainly during the first steps of the plant infection. Our results show that pili have a higher impact than flagella on the interaction of *R. solanacearum* with tomato plants and reveal new types of cross-talk between the swimming and twitching motility phenotypes: enhanced swimming in bacteria lacking pili and a role for the flagellum in root attachment.

**KEYWORDS** *Ralstonia solanacearum*, pilI, chpA, pilA, fliC

*Ralstonia solanacearum* is a soilborne Gram-negative bacterium that causes a plant disease known as bacterial wilt mainly in tropical and subtropical climates (1). *R. solanacearum* exhibits an unusually broad host range comprising more than 200 plant species.

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Address correspondence to Marc Valls, marc.valls@ub.edu.

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species from over 50 families, including potato, tomato, tobacco, peanut, and banana, among other crops (2). These facts have contributed to the ranking of *R. solanacearum* as among of the most destructive plant-pathogenic bacterial species worldwide (3).

Plant colonization begins with the attachment of *R. solanacearum* on roots and entry into the host plant through wounds, at sites of secondary root emergence or elongation (4). The bacterium subsequently colonizes the root cortex and moves to the xylem, where it spreads systematically, grows extensively, and produces large amounts of exopolysaccharides (EPS) that cause vascular obstruction. This blockage results in wilting of the stem and leaves and, eventually, plant death (1).

In order to reach different plant tissues and get inside the vascular system, *R. solanacearum* uses different types of movement strategies. The first is swimming motility, an individual cell movement powered by rotating flagella and produced in aqueous environments. In *R. solanacearum*, this kind of motility is mediated by one to four polar flagella and mutants lacking either FliC (the flagellar subunit protein) or FliM (the flagellar motor switch protein) are nonmotile and present a reduction of virulence in tomato after soil-soak inoculation (5). Chemotaxis enables bacterial cells to sense specific chemicals and depends on the presence of several proteins, which ultimately interact with the flagellar motor to move toward more-favorable conditions. This complex behavior begins in cell membrane-associated receptors, called MCPs (methyl-accepting chemotaxis proteins), which detect environmental stimuli and respond to them by changing their conformation. These changes trigger autophosphorylation of the cytoplasmic histidine autokinase CheA, which forms a complex with the receptor through the coupling protein CheW (Fig. 1A). CheA transfers its phosphate group to CheY, a diffusible cytoplasmic response regulator that interacts with the flagellar motor to switch its direction of rotation. Both *R. solanacearum* cheA and cheW null mutants are motile but nonchemotactic, and their virulence is as low as that of a completely nonmotile fliC knockout mutant (6).

The other movement used by *R. solanacearum* is twitching motility, a coordinated multicellular movement driven by the extension, attachment, and retraction of the type IV pilus (TFP) appendages in solid surfaces or viscous media. In Gram-negative bacteria, the TFP system requires at least 35 pil genes for the synthesis, display, and function of polar and retractable TFP (7). *R. solanacearum* also possesses TFP-mediated motility, which plays a role in natural transformation, biofilm formation, and virulence (8). The genes pilA, pilQ, and pilT, whose products are a monomer of the major pilin protein, the secretin involved in the pilus extrusion, and the protein required for pilus retraction, respectively, have been identified in *R. solanacearum*, and inactivation of any of them reduces both twitching motility and virulence (8, 9). In addition, the pilA mutant was reduced in virulence on tomato plants, in attachment to roots, and in biofilm formation as well as being not naturally competent for transformation (8).

In the Gram-negative nosocomial pathogen *Pseudomonas aeruginosa*, which bears both flagella and pili, a hypothetical pilus-mediated chemotaxis pathway encoded by the pil-chp genes in so-called cluster IV has been proposed to exist based on homology to the flagellar chemotaxis system (10, 11). In a manner analogous to that seen with flagellum-mediated chemotaxis, in this pathway the molecular signal generated by the cell membrane-associated receptor (PilJ) is expected to trigger autophosphorylation of the cytoplasmic CheA-like histidine autokinase called ChpA, which may form a complex with two CheW homologues called PilII and ChpC (Fig. 1A). The control of movement of pili in this hypothetical system in *P. aeruginosa* is likely performed by two CheY homologues (PilG and PilH), which would interact with the putative TFP motor to control twitching motility after their phosphorylation by ChpA (10, 11).

In this report, we describe a new gene cluster in *R. solanacearum* with strong similarities to *P. aeruginosa* cluster IV, including genes encoding PilII and ChpC homologues. We have constructed pilII and chpC knockout mutants and mutants in well-described twitching (pilA) and swimming (fliC) motility genes in *R. solanacearum* and have studied the role of these genes in *R. solanacearum* motility and plant colonization.
and in other related processes such as chemotaxis, biofilm formation, and natural transformation.

RESULTS

Analysis of the *R. solanacearum* GMI1000 genome reveals the presence of single *pilI* (CheW-like) and *chpA* (CheA-like) orthologues clustered in a *pil-chp* operon. Orthologous analysis revealed that both TFP-associated protein domains, the CheW-like and CheA-like domains, were found in two genes located in a putative
operon in the GMI1000 genome sequence, which includes in total five pil-chp homologues: R. solanacearum RSc0668 (pilG), RSc0669 (pilH), RSc0670 (pilI), RSc0671 (pilJ), and RSc0672 (chpA) (Fig. 1B). This cluster is syntenic to that previously described in P. aeruginosa, except that it lacks the chpB and chpC genes downstream of chpA. CheW-like and CheA-like domains were found in the pil and chpA genes, respectively, whereas no other CheW-like homologues—such as the mentioned orthologue of P. aeruginosa chpC—were found in the R. solanacearum GMI1000 genome. Compared to its P. aeruginosa counterpart (PAO1 protein WP_003114893), the R. solanacearum PilI homologue presents an identity level of 68%, with fragment coverage of 31%, whereas the R. solanacearum ChpA homologue shows an identity level of 39.73% with 33.33% coverage with respect to the P. aeruginosa ChpA (WP_003114893) protein. To determine whether the five pil-chp genes are part of the same transcriptional unit, reverse transcription-PCRs (RT-PCRs) were performed (Fig. 1C). The resulting bands confirmed that the pil-chp gene cluster is transcribed as a single polycistronic unit, transcriptionally independent of the surrounding RSc0667 and RSc0673 genes (Fig. 1C), predicted to encode a rubredoxin protein and a hypothetical protein, respectively.

The R. solanacearum PilI and ChpA proteins are involved in twitching but not in swimming motility or chemotaxis. To determine the role of pil and chpA in R. solanacearum motility and chemotaxis, we created null mutants by replacing their protein-coding sequences with a kanamycin cassette. Strains with an inactivated pilA, fliC, or cheA gene were also constructed to be used as controls: the pilA mutant was described previously as impaired in twitching (8), the fliC mutant as deficient in swimming (5), and the cheA mutant as nonchemotactic (6). All mutants obtained were confirmed by PCR (see Fig. S1 in the supplemental material) and subsequent sequencing (Macrogen) using specific primers (see Table S1 in the supplemental material). Furthermore, none of the constructed knockout mutants exhibited macroscopic changes in colonial shape, EPS production (Fig. S2), or growth rate in vitro (Fig. S3).

After growth in the appropriate solid medium, colonies of the wild-type (WT) R. solanacearum GMI1000 strain exhibited a normal twitching phenotype characterized by irregular colony edges with multiple projections easily observed under light microscopy (Fig. 2A). In contrast, the pil mutant presented round-shaped colony margins without projections, a phenotype identical to that of the nontwitching motility control pilA mutant (Fig. 2A). The chpA mutant strain also showed impaired twitching movement, but unlike the pil and pilA mutants, the reduced twitching motility in the chpA mutant was characterized by smaller projections in the colony margins, indicating some residual movement (Fig. 2A). As expected, the fliC flagellum mutant control strain presented a twitching phenotype similar to that of the WT GMI1000 strain (Fig. 2A). It is worth noting that the complementation of the pil mutant restored twitching motility, discarding polar effects on downstream genes caused by the pil disruption or by secondary mutations (Fig. S4A).

It was reported previously that inactivation of TFP genes might modify the motility controlled by flagella and vice versa (12–14). Thus, we analyzed the swimming capacity in pil and chpA mutants, including again as controls the pilA and the fliC knockouts, known to be affected only in twitching and swimming motility, respectively (5, 8). After growth in the appropriate semisolid medium, the pil and chpA mutants exhibited a typical swimming halo around the inoculated area, similar to that of the WT strain, whereas the fliC control mutant was completely impaired in this type of motility, as expected (Fig. 2B). Surprisingly, the pilA mutant strain displayed an increased swimming halo compared to that of the WT parental strain (Fig. 2B). Bacterial swimming was more accurately quantified by measuring the dispersion halo at 72 h (Fig. 2C). Significant (P < 0.05) differences—more remarkable over time—between the pilA mutant and the rest of strains (the WT strain and the pil and chpA mutants) showing a normal swimming phenotype were recorded (Fig. 2C). As previously described, the fliC control strain showed a significantly reduced swimming halo (Fig. 2C).

In order to determine whether these motility patterns affected bacterial chemotaxis, capillarity assays were performed using Casamino Acids as a chemoattractant (6). Since
the fliC mutant lacked swimming motility, a cheA mutant strain—whose chemotactic response was abolished (6)—was constructed and included in the assays as a more appropriate control. In the parental strain and TFP-related mutants, an approximately 20-fold increase in bacterial counts was observed in capillaries filled with Casamino Acids relative to those containing only chemotaxis buffer (Fig. 3). Thus, with the exception of the motile but nonchemotactic cheA knockout, no significant differences in chemotaxis were observed in any TFP-related knockout compared to the WT strain (Fig. 3).

The *R. solanacearum* pilI and chpA mutants present reduced natural transformation abilities. The TFP are essential for bacteria to carry out natural transformation (8). Thus, in order to examine their natural transformation abilities, the WT strain and the corresponding pilI, chpA, pilA, and fliC knockout mutant counterparts were exposed to DNA containing a gentamicin cassette flanked by ~1-kb-long sequences homologous to a noncoding region of the *R. solanacearum* genome (15) and the frequencies of recovery of gentamicin-resistant colonies were calculated. The results obtained showed that the transformation frequencies of the pilI and chpA mutants were reduced by approximately 20-fold and 6-fold, respectively, compared to the WT strain (Table 1). As expected, the pilA mutant, which lacks TFP, was totally unable to take up DNA naturally (8), whereas the fliC mutant was transformed with a level of efficiency comparable to that of the WT strain (Table 1).
TFP- and flagellin-associated genes are involved in biofilm formation and attachment of *R. solanacearum* to plant roots. Alongside roles in motility, TFP and flagella are required for biofilm formation and initial bacterial adsorption to plant roots (16). We thus measured the capacity of our bacterial mutants to produce biofilm in polystyrene microplate cultures. The pilA control mutant was previously shown to produce less-developed biofilms than a WT strain (8), which was quantified here as a significant (*P* < 0.05) reduction (~70%) in biofilm formation. The chpA and fliC mutants also displayed comparable ~70% reductions in their ability to produce biofilm compared to the GMI1000 strain (Fig. 4A). Interestingly, the pilI mutant, which exhibited an abolishment of twitching motility similar to that shown by the nonmotile pilA mutant, presented a significant (*P* < 0.05) increase of ~25% in biofilm formation with respect to the WT parental strain (Fig. 4A). Furthermore, the complemented pilI mutant showed a restored ability to produce biofilm (Fig. S4B).

Next, we incubated each bacterial strain with isolated tomato roots and quantified their capacity to attach to the root surface. The results of these experiments showed that all TFP mutants (pilI, chpA, and pilA) presented a statistically significant (*P* < 0.05) 10-fold-lower level of root attachment than the WT GMI1000 strain (Fig. 4B). Furthermore, the aflagellated fliC mutant also displayed a statistically significant (*P* < 0.05) 5-fold decrease compared with the WT strain (Fig. 4B), denoting that both TFP and flagella promote adhesion between *R. solanacearum* cells and tomato roots.

**The pilI and chpA mutants show reduced virulence in soil-soak inoculations but not when directly inoculated in the stem of tomato plants.** To determine the effect of PilI and ChpA on *R. solanacearum* pathogenicity, tomato plants were infected by drenching the soil with the collected bacterial solution without wounding the roots, which mimics the natural infection process of this soilborne pathogen. The four mutants analyzed (pilI, chpA, pilA, and fliC) exhibited a significant (*P* < 0.05) reduction in their ability to develop plant wilting compared to the parental WT strain, but to

### TABLE 1 Natural transformation frequencies of the indicated *R. solanacearum* strains

| Strain      | Natural transformation frequency<sup>a</sup> |
|-------------|--------------------------------------|
| GMI1000 WT  | 1.05 (±1.2) × 10<sup>-5</sup>         |
| pilI        | 5.07 (±4.9) × 10<sup>-8</sup>         |
| chpA        | 1.78 (±1.5) × 10<sup>-7</sup>         |
| pilA        | <2.57 × 10<sup>-9</sup>              |
| fliC        | 1.66 (±2.1) × 10<sup>-6</sup>         |

<sup>a</sup>Each experiment was carried out in triplicate in five independent assays.

<sup>b</sup>Natural transformation frequency data are represented as means ± standard deviations and were calculated as the number of recombinant colonies by the total number of viable cells. At least 10% of the recombinant colonies obtained for each strain were confirmed by sequencing.
differing degrees (Fig. 5A). Statistical analysis classified the mutants into four groups: the pilA deletion mutant was the least virulent, followed by the pilI and chpA mutants, with both showing an intermediate phenotype, and the fliC mutant, with a small but significant decrease in apparent wilting compared to the WT parental strain (Fig. 5A). Bacterial counts obtained from 3-cm stem cuts from tomato plants infected using the same procedure were also carried out at 4, 8, and 12 dpi (days postinoculation). Only the pilA mutant exhibited a significant (2 log) reduction at days 4 and 12 (P < 0.05) with respect to the rest of strains, whose numbers in stem tissues were similar to those seen with the WT strain (Fig. 5B). To discard any potential fitness effects resulting from the growth of the knockout strains in tomato plants, all strains were infiltrated in tomato leaves. The results showed no differences in bacterial growth of any of the mutants with respect to the WT (Fig. S5). In contrast, when tomato plants were infected by direct petiole injection, pilI and chpA knockout strains showed no statistically significant reduction in their capacity to wilt plants (Fig. 6A), while the control pilA mutant exhibited significant (P < 0.05) differences in disease index in comparison to the WT, as previously described (8). Finally, the virulence of the flagellum-deficient fliC mutant was also comparable to that of the WT strain (Fig. 6A). Bacterial counts measured over time in infected plant stems were similar to those reached by the WT strain for all mutants except for the pilA mutant, which presented a significant (P < 0.05) reduction in plant colonization (Fig. 6B). Interestingly, the fliC mutant exhibited a reduction in stem numbers only at 3 dpi, reaching values similar to those shown in the pilI and chpA mutants and the WT strains at later infection times (Fig. 6B).

Deletion of pilA but not pilI, chpA, and fliC limits bacterial spread in plant tissues. In order to study the distribution of pilI, chpA, pilA, and fliC R. solanacearum knockout mutants along tomato plants, reporter strains were constructed through the insertion into their genome of the luxCDABE operon under the control of the hrpB promoter (Fig. S6). Tomato plants grown in pots were soil-inoculated with the reporter
strains, and luminescence in different stem sections was recorded at 3 and 6 dpi. At 3 dpi, no significant differences were observed between any of the knockouts and the WT strain at any stem height (Fig. 7). However, at 6 dpi, the pilA mutant carrying the luxCDABE operon exhibited a significant ($P < 0.05$) reduction of its luminescence compared to the WT reporter strain (Fig. 7). No significant differences in colonization between the stem sections from internodes 2 and 3 were observed for any other strain at 6 dpi (Fig. 7). To confirm that expression of the reporter operon was not affected by any of the gene disruptions, bacterial growth and luminescence were both measured over time in in vitro cultures (Fig. S7). In these experiments, all mutants showed comparable levels of luminescence and indistinguishable differences in growth from the WT strain, ruling out an inhibition of the reporter in the PilA mutant (Fig. S7).

**DISCUSSION**

**A conserved cluster involved in twitching motility.** In this study, we identified a new gene cluster in *R. solanacearum* strain GMI1000 (Fig. 1B) presenting synteny with respect to *P. aeruginosa* and *Lysobacter enzymogenes* cluster IV (17–19) and the *Xylella fastidiosa pil-chp* operon (20). With the construction of pilus- and ChpA-deficient strains, we demonstrated the involvement of these genes in *R. solanacearum* twitching but not
in swimming (Fig. 2). Whereas the chpA mutant presented reduced twitching, PilI inactivation produced a total abolition of this movement, comparable to that seen with the nontwitching pilA control (Fig. 2A). Decreased twitching motility has been similarly observed in chpA (CheA-like) knockout mutants in other bacteria such as P. aeruginosa (21) and X. fastidiosa (20) and in the pilI (CheW-like) null mutant of P. aeruginosa (22). However, the pilI knockout remains twitching proficient in L. enzymogenes (23), indicating differences in TFP gene function that depend on the bacterial species. In contrast to our results seen in R. solanacearum revealing that the chpA mutant showed some residual twitching motility, both pilI and chpA/pilL mutants in Acidovorax citrulli lacked twitching motility and did not produce TFP (24). However, a pilJ mutant in the A. citrulli pil-chp operon retained the ability to produce TFP (13).

TFP influence swimming motility. Since the inactivation of specific genes associated with one type of appendage may affect the movement controlled by others (12–14, 25), swimming motility assays were also performed with the R. solanacearum mutants that had been constructed. Surprisingly, results obtained showed swimming hypermotility performed by the pilA mutant (Fig. 2B and C), a phenomenon observed in other R. solanacearum knockouts such as those lacking the transcriptional regulators phcA (26) and motN (27), the latter being chemotaxis proficient like the R. solanacearum
pilA mutant (Fig. 3). Cross-effects between the two appendage-dependent movements are likely underestimated due to the comparative paucity of studies in which both swimming and twitching assays have been carried out to evaluate the effect of single mutants. One example of this connection is found in the PilS-PilR two-component system of *P. aeruginosa*, which regulates TFP expression and whose inactivation also causes a reduction in swimming motility (25). Similarly, the inactivation of genes encoding PilA, the pilus assembly protein PilO, or two predicted minor pilins, FimU and FimT, caused reduced twitching but complete abolition—or, in the case of the *fimT* mutant, impairment—of swimming in *P. syringae*, suggesting that TFP are involved not only in twitching but also in swimming (14).

**R. solanacearum** PilI and ChpA play a role in all known TFP-related functions. As previously reported in *R. solanacearum* studies of the pilA mutant, TFP are required for natural transformation and biofilm formation (8). Our results demonstrate that PilI and ChpA also contribute to natural competence (Table 1), presumably through proper regulation of TFP. Regarding biofilm formation, our data showed that PilI and ChpA play contrary roles (Fig. 4A). Interestingly, knockouts lacking either PilA or PilQ in *Xanthomonas* spp. displayed reduced twitching motility, but biofilm formation was affected only in the pilQ mutant (28, 29). On the other hand, in the *A. citrulli* *fliR*-null mutant, whose swimming and twitching movements remained impaired, no effect on biofilm development was observed (12). Besides their roles in natural transformation and biofilm formation, TFP are also key for bacterial virulence (7). In *R. solanacearum*, virulence processes during plant colonization have been investigated in some TFP-related genes such as pilA (8) and pilQ (30), highlighting the relationship between twitching motility and virulence (31–33). Our data demonstrate that PilI and ChpA proteins are required for early pathogenic stages that result in effective plant colonization and wilting. This is shown by the fact that strains deleted of these genes caused reduced wilting in response to a naturalistic infection method (soil drenching; Fig. 5A) but behaved like the WT when applied directly by petiole inoculation, a procedure that overcomes all initial steps of plant colonization until the bacterium reaches the xylem (Fig. 6A). Similarly, our data corroborate the idea that PilA plays a role in the pathogenesis of *R.
**Novel roles of flagella in *R. solanacearum* GMI1000.** This work demonstrates that adhesion to roots via TFP is crucial for optimal plant colonization and disease development but also that flagella are involved in these processes (Fig. 4B). Implication of flagella in attachment to both animal and plant cells has been recently reported in bacterial pathogens at the early stage of infection (36–38). Although flagella have not been reported to play a role in attachment of members of the Xanthomonadaceae (39), in other bacterial species such as *Azospirillum brasilense*, flagellin-deficient mutants are impaired in attachment to wheat roots, and the purified polar flagellum binds directly to the wheat root surface (40).

In addition, our results shown that *fliC* inactivation produces a reduction in biofilm formation (Fig. 4B), indicating that flagella also contribute to this process in *R. solanacearum* GMI1000, as had been observed in *P. aeruginosa* and other bacteria (41, 42). In contrast, inactivation of either *fliC* or genes involved in aerotaxis—an active cell movement along oxygen gradients—in *R. solanacearum* strain K60 caused increased biofilm production (43), indicating strain-specific TFP functions.

The flagellar protein FliC was previously shown to play a role in pathogenesis of *R. solanacearum*, especially during the first steps of the interaction (5, 8). Here, we also observed that the *fliC* deletion mutant caused reduced wilting only when inoculated by root drenching and not by direct petiole inoculation (Fig. 5A and 6A). However, this flagellum-deficient mutant was affected only slightly in its capacity to multiply in planta (Fig. 6B) and colonize the plant stem (Fig. 7), in contrast with the stronger phenotypes shown by the nonpilliated *pilA* strain. We conclude that TFP are more important than flagella for the interaction of *R. solanacearum* with tomato plants.

**Conclusion.** In this work, we have demonstrated that the virulence of *R. solanacearum* *pilII*, *chpA*, *pilA*, and *fliC* deletion mutants is impaired in the first stages of plant colonization and that the *pilA* mutant shows decreased growth after drenching or petiole inoculation. This is the first report on the putative *R. solanacearum* type IV pilus regulators PilII and ChpA, where we clearly demonstrate their role in twitching motility, biofilm formation, natural transformation, and virulence. Additionally, a hypermotile swimming phenotype in GMI1000 strain lacking PilII and the role of FliC in root attachment and biofilm formation have been described here for the first time. Our work suggests that further research in *R. solanacearum* should be addressed to elucidate putative connections between swimming and twitching motilities in both well-documented genes and genes with unknown function.
**TABLE 2** Bacterial strains and plasmids used in this work

| Strain or plasmid | Relevant characteristics | Source or reference |
|-------------------|-------------------------|---------------------|
| **Strains**       |                         |                     |
| *E. coli* DH5α    | *E. coli* supE4 ΔlacU169 (80 ΔlacZΔM15) hsdR17 recA1 endA1 gyr96 thi-1 relA1 | Clontech            |
| *R. solanacearum* GMI1000 | Wild-type strain (phytotype I, race 1 biovar 3) | 45                  |
| *R. solanacearum* GMI1000 PhB-lux | GMI1000 strain with *PhpB*-lux from pRCGent-PhB-lux, Gm' | This work          |
| *R. solanacearum* pilP | GMI1000 strain with Δ*pilP*-loxP-Km from pCM184, Km' | This work          |
| *R. solanacearum* pilP PhB-lux | GMI1000 strain with pilP::loxP-Km from pCM184, Km' | This work          |
| *R. solanacearum* chpA | GMI1000 strain with ΔchpA::loxP-Km from pCM184, Km' | This work          |
| *R. solanacearum* pilA PhB-lux | GMI1000 strain with pilA::loxP-Km from pCM184, Km' | This work          |
| *R. solanacearum* pilA PhB-lux | PI4 strain with *PhpB*-lux from pRCGent-PhB-lux, Km', Gm' | This work          |
| *R. solanacearum* fic | GMI1000 strain with Δ*ficC*-loxP-Km from pCM184, Km' | This work          |
| *R. solanacearum* fic PhB-lux | FI4 strain with *PhpB*-lux from pRCGent-PhB-lux, Km', Gm' | This work          |
| *R. solanacearum* cheA | GMI1000 strain with Δ*cheA*-loxP-Km from pCM184, Km' | This work          |
| **Plasmids**      |                         |                     |
| pCM184            | Allelic exchange vector, carrying kanamycin cassette flanked by *loxP*, *Ap*, Km' | Addgene            |
| pGEMT             | Cloning vector, *Ap*    | Promega             |
| pRCGent-Pps-GWY   | Vector carrying gentamicin cassette flanked by regions homologous to the GMI1000 genome, *Ap*, Gm' | 15                 |
| pRCGent-PhB-lux   | Vector carrying *luxCDABE* operon from pMU1 cloned in KpnI–NotI in pRCGent-PhB, *Ap*, Gm' | This work          |
| pBT4              | Broad-host-range vector carrying the pBBR1 replicon, Tc' | Addgene            |
| pDSK-GFPuv        | Vector carrying the PpsbA promoter, Km' | LiveScience        |

*Km', Gm', *Ap*, and Tc' stand for resistance to kanamycin, gentamicin, ampicillin, and tetracycline resistance, respectively.

**MATERIALS AND METHODS**

Bacterial strains, plasmids, plant material, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 2. *Escherichia coli* DH5α was grown at 37°C in Luria-Bertani (LB) (44) agar or in LB broth with shaking at 180 rpm. The *R. solanacearum* GMI1000 WT strain and derive mutants were routinely grown at 28°C in rich B medium, Boucher’s minimal medium (MM) (45), and CPG (Casamino Acids-peptone-glucose) medium (46) agar or broth with shaking at 180 rpm. When necessary, rich B medium was supplemented with 0.5% glucose and 0.005% 2,3,5-triphenyltetrazolium chloride (final concentration) in agar plates, and MM broth was supplemented with 2% glycerol or 20 mM glutamate (final concentration). When needed, ampicillin (50 mg/liter), kanamycin (50 mg/liter), gentamicin (10 mg/liter), or tetracycline (5 mg/liter) was added in growth media. For phytopathogenesis assays, tomato plants (*Solanum lycopersicum* cultivar Marmande) were used to evaluate virulence of *R. solanacearum*. Plants were routinely grown in pots in a mixed soil of Substrate 2 (Klasmann-Deilmann GmbH, Geeste, Germany), perlite, and vermiculite at a proportion of 30:1:1 for 1 to 4 weeks at 22°C and 60% relative humidity under long-day light regimen conditions (16 h light and 8 h darkness). Before the infectious assays, tomato plants were acclimated at least 3 days by transferring them to a growth chamber at 27°C under the same humidity and photoperiod conditions.

RNA extraction and RT-PCR. Total RNA was extracted by the use of an RNeasy minikit (Qiagen, Hilden, Germany) from 5-ml cultures at an absorbance level of 0.4 (optical density at 600 nm [OD600]) that had previously been pelleted and treated with lysozyme (50 mg/ml) and resuspended in Tris-EDTA (TE) buffer for 10 min at 37°C. Once extracted, the RNA was incubated with DNase Turbo Ambion (Thermo Fisher, Waltham, MA) to remove DNA contaminants, and PCR analysis of the mixtures performed using RNA samples without reverse transcriptase confirmed the absence. Reverse transcription of RNA was performed through the use of a first-strand cDNA synthesis kit (Nzytech, Lisbon, Portugal) and selected in ampicillin- and kanamycin-containing LB plates.

Gene identification and construction of *R. solanacearum* knockouts and of complemented and reporter strains. The *P. aeruginosa* PilP and ChpA homologues in *R. solanacearum* were identified through mapping coded proteins to nonsupervised orthologous groups (NOGs) using the eggNOG v4.0 Web service (47) and complete *R. solanacearum* GMI1000 chromosome (NC_003295.1) and megaplasmid (NC_003296.1) sequences. Knockout deletion mutants *pilP* (WP_011000625), *chpA* (WP_011000627), *pilA* (WP_011000517), *fic* (WP_011003694), and *cheA* (WP_011004658) were generated by replacing the coding sequence of each target gene by a kanamycin resistance cassette as described previously (48). To this end, DNA fragments corresponding to ~1 kb of the upstream and downstream flanking regions of each gene and a kanamycin cassette flanked with *loxP* regions were PCR amplified from the *R. solanacearum* GMI1000 genome and plasmid pCM184 (Addgene, respectively). The oligonucleotides used (Table S1) included overlapping regions enabling the three fragments to be fused in two amplification rounds. All PCRs were performed using proofreading Phusion High-Fidelity DNA polymerase (Thermo Fisher). Next, 3'-A overhangs were added to the final PCR products using Taq polymerase (Invitrogen, Waltham, MA) and the fragments were cloned into pGEM-T plasmid (Promega, Madison, Wisconsin), transformed in *E. coli* DH5α, and selected in ampicillin- and kanamycin-containing LB plates.
Sequenced constructs (Macrogen, Seoul, South Korea) were amplified with appropriate primers (Table S1), and the final product was purified (NZYgelPure; NZYtech), naturally transformed in *R. solanacearum* strain GMI1000, and plated in kanamycin B rich medium for mutant selection (49). For complementation, the *PpsbA* promoter and the corresponding gene were amplified from the pOSK-GFPuv (LifeScience) plasmid and the *R. solanacearum* GMI1000 genome, respectively. Then, both fragments were cloned into the pBT4 vector (Addgene) using Gibson assembly master mix (New Engand Biolabs, Ipswich, Suffolk, United Kingdom) and the appropriate primers (Table S1). Knockouts were then electroporated with the constructed plasmid and selected in tetracycline-containing medium plates. To construct luminescent reporter strains, integration of the luxCDABE operon under the *hrpB* promoter was carried out as previously described (49). Briefly, the reported operon was amplified from the pRCGent-Ph8-lux plasmid using the appropriate primers and electroporated to each strain. Transformants were selected on gentamicin-containing B rich plates. All knockouts and reported strain verifications were carried out by PCR amplification and sequencing (Macrogen) with the corresponding primers indicated in Table S1.

**Twitching and swimming assays.** Modified CPG plates with 0.3% and 1.6% Difco agar were used on the day of their preparation for analysis of swimming and twitching motilities, respectively (50). Both motility tests were carried out by the inoculation of a 2-μl drop of a bacterial suspension into Milli-Q (MQ) water at an absorbance level of 0.1 (OD600) in the middle of the appropriate plate. After inoculation, both CPG-type plates were incubated at 30°C and 24 h for twitching (until layered edges with multiple irregular projections were observed in the WT strain) and 72 h for swimming (measuring the bacterial halo daily). A Zeiss Axiosmager M2 microscope (Carl Zeiss Microscopy, Oberkochen, Germany) was used to obtain representative images of twitching motility at 40× increases. A ChemiDoc XRS+ system (Bio-Rad, Hercules, CA) was used to obtain swimming motility images.

**Chemotaxis capillary assays.** To establish chemotactic effects in *R. solanacearum* knockouts, capillary assays were carried out as described previously (51), with some modifications. Briefly, three V-shaped bent needles (Nipro; Kita-ku, Osaka, Japan) covered with a 24-by-65 mm microscope coverslip (Menzel-Glässer; VWR, Radnor, PA) were placed on the surface of aseptic 140-mm-diameter petri dishes (Deltalab; Rubí, Barcelona, Spain) to form the chemotaxis chambers. Sealed and autoclaved 1-ml capillaries (Microcaps, Drummond Scientific Co., Broomall, PA) (3 cm in length) were filled with chemotactic buffer (CB) as a negative control or with CB with 2% Casamino Acids as a chemoattractant (6). Bacterial suspensions were prepared from overnight cultures in B rich medium with the appropriate antibiotics, washed twice with MQ water, and adjusted to an OD600 of 0.1. Chemotaxis chambers were filled with the corresponding bacterial suspensions and incubated at 30°C during 2 h. Once incubated, the tip plate reader were washed twice to remove any external attached cell and then broken off, and the content was poured into a microcentrifuge tube containing 1 ml of MQ water. Proper dilutions were plated in rich B medium, and the CFU counts per milliliter obtained under each set of conditions were normalized between the capillaries treated with 2% Casamino Acids and those left untreated.

**Natural transformation assays.** Natural transformation efficiencies were determined as previously described (52). Briefly, 100 ng of DNA containing a gentamicin cassette with homologous regions amplified from pRCG-Pps-GWY (53) was added to each 50-μl volume of the corresponding strains grown in MM broth supplemented with 2% glycerol. Transformed clones were selected on gentamicin-containing B rich medium plates and verified by PCR and sequencing (Macrogen) with the corresponding primers indicated in Table S1. The same cultures were also plated in B rich medium without antibiotic to obtain the total number of viable bacteria (CFU per milliliter). Transformation frequencies were calculated as the number of recombinant colonies divided by the total number of CFU per milliliter.

**Biofilm quantification.** Quantitative analysis of *R. solanacearum* biofilm formation was carried out through crystal violet assay by the use of a method adapted from previous work (54). Briefly, CPG overnight cultures were collected, washed, and adjusted in CPG to an OD600 of 0.1. Next, 95 μl of CPG broth and 5 μl of each bacterial suspension were added onto 96-well polystyrene microplates (Greiner, Kremsmünster, Austria) and incubated without shaking at 30°C during 24 h. After incubation, biomass growth in wells was measured as an OD600. Subsequently, wells were washed twice with MQ water, incubated with 100 μl of 0.1% crystal violet stain, and incubated at room temperature for 30 min. Wells were then washed with MQ water three times, and the stained biofilm immobilized on the wells was solubilized with 100 μl of 95% ethanol and measured at OD580. Measurements were performed using a multiplate reader (Sunrise, Tecan, Zürich, Switzerland), and results were normalized according to previously obtained biomass absorbance (OD600/OD580).

**Root attachment quantification.** Attachment to tomato roots was carried out as described previously (55), with slight modifications. One- or 2-week-old plants, grown as described above without previous acclimation, were collected and washed. Roots were submerged in 1 ml of bacterial culture (approximately 108 CFU), obtained from a diluted culture at an OD600 of 0.1 (≈104 CFU/ml) in MQ water. Submerged root plants were incubated at 25°C with gentle shaking at 50 rpm during 4 h to promote bacterium-root contact. After incubation, roots were placed in tubes with 30 ml sterile 0.88% NaCl, shaken during 1 min at 200 rpm to remove unattached bacteria, and gently dried to remove liquid excess. Attached bacteria were then collected by immersing 2-cm-long dried roots in tubes with 1 ml sterile 0.88% NaCl, followed by 1 min of vortex mixing. Appropriate dilutions were plated in B rich medium.

**Bacterial leaf multiplication assays.** Bacterial growth in planta was measured as previously described (56), with some modifications. A 3-liter inoculum of each bacterial strain was prepared, starting from a culture maintained at an OD600 of 0.1 in MQ water (approximately 106 CFU/ml) to obtain a final concentration of ≈1010 CFU/ml. Infections were performed using a vacuum machine, and to reduce the...
surface tension of the water, 150 μl of Silwet L-77 was added to the 3-liter inoculum. Four-week-old tomato plants were submerged in the respective bacterial inocula, and vacuum was applied during 1 min to promote bacterial infiltration in leaves. After inoculation, plants were retrieved, dried, and incubated under standard conditions (27°C, 60% relative humidity, and 16-h light/8-h darkness). At specific dpi, four 5-mm² leaf disks were recovered from the infiltrated plants, collected in tubes containing 200 μl of MQ water, and subjected to a grinding process. The collected dilutions were plated on B medium to obtain the final concentration of each strain on leaf surface (expressed as CFU count per square centimeter).

**Bacterial virulence experiments.** For drenching assays, 4-week-old unwounded plants were soil-soak inoculated by watering with 40 ml of bacterial culture, at an OD₆₀₀ of 0.1 (~10⁸ CFU/ml) in MQ water, per plant (56). Once infected, plants were incubated under standard conditions and wilting signs were recorded daily on a disease index scale from 0 (no wilt) to 4 (death). At 4, 8, and 12 dpi, bacterial multiplication in tomato plants was measured by collecting 3 cm of the stem, 0.5 cm above the petiole of the first true leaf, into tubes containing 300 μl of MQ water. After 30 min of incubation at room temperature, serial dilutions were plated in corresponding rich B medium to measure viable bacteria.

For petiole inoculation assays, 4-week-old plants were directly infected by inoculation with a 100-μl drop (approximately 10³ CFU of a sample obtained from a culture at OD₆₀₀ of 0.1 (~10⁸ CFU/ml) diluted in MQ water. Bacterial injections into the stem, above the petiole of the first true leaf, were performed using a sterile needle, and plants were incubated under standard conditions (56). Disease index scale values were assigned daily during 9 days, and at 3, 6, and 9 dpi, bacterial multiplication in tomato plants was measured as previously described by collection of 3 cm of the stem, 0.5 cm above the inoculation point, and plating in corresponding rich B plates.

**Luminescence assays.** Overnight cultures of all strains carrying PhrpB::lux were inoculated in MM supplemented with glutamate and gentamicin at absorbance of 0.3 (OD₆₀₀), and culture aliquots were collected at 0, 4, 6, and 8 h postinoculation to measure luminescence and absorbance (57). Expression of the hpb promoter was represented as relative light units (RLU) per second. A FB-12 luminometer (Berthold Detection Systems, Pforzheim, Germany) and a V-1200 spectrophotometer (VWR, Radnor, PA) were used for each measurement, respectively.

To evaluate bacterial distribution through plant stem of reporter strains, 4-week-old plants were infected as previously described in a petiole inoculation assay but with slight modifications. Drops (10 μl) of cultures of each luminescent strain (approximately 10⁵ CFU) were injected into the plant stem, above the petiole of the second true leaf. Once infected, plants were incubated under standard conditions and at 3 and 6 dpi, luminescence in tomato plants stem was measured by collecting 0.5 cm of internodes 2 and 3 into tubes containing 200 μl of MQ water. After 30 min of incubation, luminescence was measured from different stem cuts.

**Statistics.** All data were analyzed in a two-tailed, one-way analysis of variance (ANOVA) followed by the Tukey test for post hoc multiple-group comparisons. Results were considered statistically significant when a P value of <0.05 was obtained.

**Data accessibility.** The data that support the findings of this study are available from the corresponding author upon request.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**FIG S1**, TIF file, 1.3 MB.

**FIG S2**, TIF file, 2.5 MB.

**FIG S3**, TIF file, 0.5 MB.

**FIG S4**, TIF file, 1.5 MB.

**FIG S5**, TIF file, 0.5 MB.

**FIG S6**, TIF file, 1.1 MB.

**FIG S7**, TIF file, 0.5 MB.

**TABLE S1**, DOCX file, 0.01 MB.

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M.V., J.A., N.S.C., and J.B. conceived the study. J.A. and J.C. performed the in vitro bacterial experiments. J.C. and P.S. performed all the bacterial infections in planta. M.V., J.A., N.S.C., and J.C. supervised the study. M.V., J.A., J.C., and P.S. analyzed the results. M.V., J.A., N.S.C., J.B., J.C., and P.S. wrote the article with contributions from all of us. We declare that we have no conflict of interests.

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