Twitching motility of *Ralstonia solanacearum* requires a type IV pilus system

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Twitching motility is a form of bacterial translocation over firm surfaces that requires retractile type IV pili. Microscopic colonies of *Ralstonia solanacearum* strains AW1, K60 and GMI1000 growing on the surface of a rich medium solidified with 1.6% agar appeared to exhibit twitching motility, because early on they divided into motile 'rafts' of cells and later developed protruding 'spearheads' at their margins. Individual motile bacteria were observed only when they were embedded within masses of other cells. Varying degrees of motility were observed for 33 of 35 strains of *R. solanacearum* in a selected, diverse collection. Timing was more important than culture conditions for observing motility, because by the time wild-type colonies were easily visible by eye (about 48 h) this activity ceased and the spearheads were obscured by continued bacterial multiplication. In contrast, inactivation of PhcA, a transcriptional regulator that is essential for *R. solanacearum* to cause plant disease, resulted in colonies that continued to expand for at least several additional days. Multiple strains with mutations in regulatory genes important for virulence were tested, but all exhibited wild-type motility. Many of the genes required for production of functional type IV pili, and hence for twitching motility, are conserved among unrelated bacteria, and *pilD*, *pilQ* and *pilT* orthologues were identified in *R. solanacearum*. Colonies of *R. solanacearum pilQ* and *pilT* mutants did not develop spearheads or rafts, confirming that the movement of cells that had been observed was due to twitching motility. Compared to the wild-type parents, both *pilQ* and *pilT* mutants caused slower and less severe wilting on susceptible tomato plants. This is the first report of twitching motility by a phytopathogenic bacterium, and the first example where type IV pili appear to contribute significantly to plant pathogenesis.

Keywords: pili, fimbriae, bacterial wilt, bacterial cell movement

INTRODUCTION

Ralstonia solanacearum (previously *Pseudomonas solanacearum*) is a soil-borne pathogen that causes lethal wilt symptoms in > 200 plant species around the world, including tomato, potato, peanut and banana (Hayward, 2000). A part-time soil inhabitant, *R. solanacearum* enters plant roots via wounds or where secondary roots emerge, invades xylem vessels, and rapidly spreads throughout the vascular system. Wilting is due

to vascular dysfunction caused by high bacterial cell densities (> 10⁹ c.f.u. per g fresh weight) and the attendant production of plant-cell-wall-degrading enzymes and copious amounts of a high-molecular-mass extracellular polysaccharide (EPS1) (McGarvey *et al.*, 1999; Schell, 2000).

Efficient colonization of a host plant requires both EPS1 and multiple extracellular proteins (Araud-Razou *et al.*, 1998; Kang *et al.*, 1994; Saile *et al.*, 1997; Schell, 2000). Some extracellular proteins made by *R. solanacearum* are delivered to host cells via a type III secretion system encoded by a cluster of > 20 *hrp* genes (Cornelis & van Gijsegem, 2000; van Gijsegem *et al.*, 1995). These

Abbreviations: EPS, extracellular polysaccharide; HR, hypersensitive reaction; Tfp, type IV pili.

'effector' proteins promote bacterial multiplication in hosts and trigger defence responses in nonhosts. Other extracellular proteins, like the plant-cell-wall-degrading enzymes, transit the general secretory pathway (type II secretion) (Hueck, 1998; Pugsley, 1993). Individual enzymes enhance virulence of *R. solanacearum* (e.g. the rate and severity of wilting) (Denny *et al.*, 1990; Huang & Allen, 1997; Tans-Kersten *et al.*, 1998), but together they may be essential for disease (Kang *et al.*, 1994). Expression of virulence factors, plus other genes not obviously required for virulence, is controlled by a complex, environmentally responsive regulatory network (Schell, 2000). Central to this network is the Phc confinement-sensing system, which employs the PhcA global transcriptional regulator to control multiple genes directly and indirectly. Some PhcA-regulated genes, like the *egl* endoglucanase and the *eps* gene cluster, are expressed strongly only at high cell density, whereas others, such as the *pglA* endo-polygalacturonase, are more highly expressed at low cell density.

To understand better the process of systemic colonization by *R. solanacearum*, we are investigating what role the cell surface components of the pathogen may play during interactions with plant tissues. We are particularly interested in surface molecules that would be exposed when EPS1 is not produced, a condition that occurs when cells are at low density (e.g. early in colonization) (Kang *et al.*, 1999). The obvious candidates are the lipopolysaccharide and pili (fimbriae). However, biosynthesis of lipopolysaccharides is complex, requiring multiple enzymic steps, and the *R. solanacearum* LPS-deficient mutants described to date also have defects in EPS1 synthesis or membrane function (Cook & Sequeira, 1991; Titarenko *et al.*, 1997). In contrast, pili consist primarily of a single structural protein (pilin), so they are more amenable to reverse genetics to evaluate their role during pathogenesis.

Numerous Gram-negative bacteria, including some animal and plant pathogens, produce type IV pili (Tfp) (Ojanen-Reuhs *et al.*, 1997; Roine *et al.*, 1998; Strom & Lory, 1993; Su *et al.*, 1999; van Doorn *et al.*, 1994; Wall & Kaiser, 1999). Depending on the organism, Tfp can be important for adhesion, aggregation, biofilm formation, horizontal gene transfer, multicellular development, pathogenesis and twitching motility (Fussenegger *et al.*, 1997; Merz *et al.*, 2000; Strom & Lory, 1993; Wall & Kaiser, 1999). Twitching motility, which is a form of flagella-independent translocation of bacteria over solid surfaces, now appears to be somewhat misnamed, because cell movements can be intermittent and jerky or smooth and prolonged (Henrichsen, 1983; Semmler *et al.*, 1999; Wall & Kaiser, 1999). The amount of movement necessary to qualify as twitching also varies from almost nonobservable to quite large (e.g. ≥ 0.6 mm h⁻¹ for *Pseudomonas aeruginosa*) (Alm & Mattick, 1997; Henrichsen, 1972, 1983; Semmler *et al.*, 1999; Strom & Lory, 1993). Retraction of Tfp, probably by filament disassembly, has long been considered the likely mechanism for twitching motility (Bradley,

1980; Wall & Kaiser, 1999; Wolfgang *et al.*, 2000). Very recently, sophisticated microscopic techniques have provided direct evidence that Tfp are the actuators of cell movement in *P. aeruginosa* and *Myxococcus xanthus* (Skerker & Berg, 2001; Sun *et al.*, 2000) and shown that *Neisseria gonorrhoeae* Tfp exert sufficient retractile force to move bacterial cells (Merz *et al.*, 2000). Tfp and twitching motility have been studied most in *P. aeruginosa*, where at least 35 genes are required for synthesis, display and function of polar, retractable Tfp (Alm & Mattick, 1997; Wall & Kaiser, 1999). Many genes for production of Tfp, including that for the pilin subunit protein, are well conserved in a variety of bacteria.

We became suspicious that *R. solanacearum* exhibits twitching motility when we noted that young, microscopic colonies growing on the surface of agar media had margins that were serrated and layered in appearance, resembling colonies of twitching *P. aeruginosa* pictured in the literature (Bradley, 1980; Darzins, 1993; O'Toole & Kolter, 1998; Semmler *et al.*, 1999). Our suspicion was reinforced when a subsequent search of the preliminary genomic DNA sequence of *R. solanacearum* strain GMI1000 revealed ORFs that were predicted to encode proteins very similar to many of those that are essential for biosynthesis of Tfp, and hence twitching, in *P. aeruginosa* and other bacteria. In this paper we present strong evidence that *R. solanacearum* exhibits twitching motility using a Tfp system, and that Tfp contribute to pathogenesis on tomato plants.

METHODS

Strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2. *R. solanacearum* AW1-gfp38, which expresses a modified green fluorescent protein (GFP-mut2) from a constitutive pathogen promoter, was created by randomly mutagenizing AW1 using pUTmini-Tn5gfp as described by Matthysse *et al.* (1996). The selected strain was chosen based on its bright fluorescence, wild-type multiplication and colony morphology in culture, and wild-type virulence on tomato plants. *Escherichia coli* strain DH5 α (Gibco-BRL) was used as the host for maintenance of plasmids. *R. solanacearum* strains were cultured routinely at 30 °C in BG broth (1% Bacto peptone, 0.1% Casamino acids, 0.1% yeast extract, 0.5% glucose) or BG agar (BG broth + 1.6% agar). Other media used were EG broth (Brumbley & Denny, 1990) and a liquid minimal medium (MM) (Clough *et al.*, 1994) that was solidified with 1.6% agar when desired. When they were to be used for observing twitching motility, freshly poured plates were dried by temporarily removing their lids and placing them either inverted in a 37 °C incubator for 10 min or upright at room temperature in a laminar-flow hood for 15 min. After inoculation, plates were incubated in a gravity convection incubator (i.e. without a fan). *E. coli* and *P. aeruginosa* strains were cultured routinely at 37 °C in Luria-Bertani (LB) broth or LB agar (Miller, 1972). Antibiotics used were ampicillin (Ap, 100 μ g ml⁻¹), gentamicin (Gm, 5–10 μ g ml⁻¹), kanamycin (Km, 50 μ g ml⁻¹), nalidixic acid (Nx, 20 μ g ml⁻¹), spectinomycin (Sp, 50 μ g ml⁻¹) and tetracycline (Tc, 15 μ g ml⁻¹).

Assay methods. To quantify EPS1, endoglucanase activity or endo-polygalacturonase activity, *R. solanacearum* was cul-

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristics*	Reference or source
Strains		
<i>R. solanacearum</i>		
AW1	Nx ^r derivative of wild-type AW, Tfp ⁺ Twt ⁺ HR ⁺	Denny <i>et al.</i> (1988)
AW1-Q	<i>pilQ</i> :: <i>nptI</i> , Twt ⁻ Nx ^r Km ^r	This work
AW1-T	<i>pilT</i> :: <i>nptI</i> , Twt ⁻ Nx ^r Km ^r	This work
K60	Wild-type, Tfp ⁺ Twt ⁺	J. S. Huang
K60-Q	<i>pilQ</i> :: <i>nptI</i> , Twt ⁻ Km ^r	This work
K60-T	<i>pilT</i> :: <i>nptI</i> , Twt ⁻ Km ^r	This work
GMI1000	Wild-type Tfp ⁺ Twt ⁺ HR ⁺	van Gijsegem <i>et al.</i> (2000)
<i>P. aeruginosa</i>		
PAK	Wild-type, Tfp ⁺ Twt ⁺	S. Lory
PAK-DΩ	<i>pilD</i> ::Ω, Tfp ⁻ Twt ⁻ Sp ^r	S. Lory
Plasmids		
pGEM-T easy	Cloning vector, LacZ ⁺ Ap ^r	Promega
pKS-Gm	pBluescript KS II+ with <i>aacC1</i> replacing the <i>SspI</i> fragment, Ap ^r Gm ^r	This work
pKSL	pKS-Gm Δ <i>SmaI</i> - <i>HincII</i> , Ap ^r Gm ^r	This work
pTOK2	Integration vector, Cole1 Tc ^r	Kitten & Willis (1996)
pRK415	Broad-host-range vector, Tc ^r	Keen <i>et al.</i> (1988)
pLAFR3	Broad-host-range vector, Tc ^r	Staskawicz <i>et al.</i> (1987)
pPILQ1	1.3 kb PCR fragment internal to <i>pilQ</i> in pGEM-T easy, Ap ^r	This work
pPILQ2	2.2 kb PCR fragment with <i>pilQ</i> in pGEM-T easy, Ap ^r	This work
pKSLQ1	1.3 kb <i>XbaI</i> fragment with <i>pilQ</i> segment from pPILQ1 in pKSL, Ap ^r Gm ^r	This work
pKSLQ1::npt1	pKSLQ1 with <i>nptI</i> replacing the 96 bp <i>PstI</i> fragment of <i>pilQ</i> , Ap ^r Gm ^r Km ^r	This work
pTOQ1::npt1	<i>NotI</i> fragment with <i>pilQ</i> :: <i>nptI</i> from pSLQ1::npt1 in pTOK2, Tc ^r Km ^r	This work
pRQ2+, pRQ2-	2.2 kb <i>XbaI</i> fragment with <i>pilQ</i> from pPILQ2 in pRK415 (+, aligned with <i>lac</i> promoter; -, opposite to <i>lac</i> promoter), Tc ^r	This work
pPILT	1.2 kb PCR fragment with <i>pilT</i> in pGEM-T easy, Ap ^r	This work
pKSLT	1.2 kb <i>XbaI</i> fragment with <i>pilT</i> from pPILT in pKSL, Ap ^r Gm ^r	This work
pKSLT::npt1	pKSLT with <i>nptI</i> in the unique <i>EcoRI</i> site of <i>pilT</i> , Ap ^r Gm ^r Km ^r	This work
pTOT::npt1	<i>XbaI</i> fragment with <i>pilT</i> :: <i>nptI</i> from pKSLT::npt1 in pTOK2 Tc ^r Km ^r	This work
pRT+, pRT-	1.2 kb <i>XbaI</i> fragment with <i>pilT</i> from pPILT in pRK415 (+, aligned with <i>lac</i> promoter; -, opposite to <i>lac</i> promoter), Tc ^r	This work
pLAD1, pLAD2	AW1 genomic library clones in pLAFR3, restore twitching to PAK-DΩ Tc ^r	This work

* Twt, twitching motility; Tfp, type IV pili; HR, hypersensitive response on tobacco. Ap^r, Gm^r, Km^r, Nx^r, Tc^r, Sp^r, resistant to ampicillin, gentamicin, kanamycin, nalidixic acid, tetracycline and spectinomycin, respectively.

tured in EG broth, and culture supernatants assayed as described previously (Brumbley & Denny, 1990; Clough *et al.*, 1994). The method used to monitor *P. aeruginosa* extracellular lipase activity on agar plates was similar to that described previously (Kagami *et al.*, 1998; Roine *et al.*, 1998) except that we used MM agar modified by omitting citric acid and adding olive oil (0.5%, v/v) and bromothymol blue (40 µg ml⁻¹). This medium selects for cells that produce lipase, because they can grow on the fatty acids released from the olive oil as their sole carbon source; the resulting colonies are surrounded by a yellow halo due to acidification of the medium.

To test the virulence of *R. solanacearum* on tomato plants, seeds (*Lycopersicon esculentum* Mill., cultivar Marion) were germinated in vermiculite and 2-week old seedlings were transplanted into 4 inch (10 cm) plastic pots containing a mixture of composted pine bark and vermiculite (3:1) amended with lime and fertilizer. Plants were incubated in a growth chamber (constant 30 °C, 13 h photoperiod) for 4–5 weeks before inoculation. Unwounded roots were inoculated

by drenching the soil with 40 ml of a bacterial suspension in water, containing $\leq 2 \times 10^8$ c.f.u. ml⁻¹, prepared as described by Saile *et al.* (1997). Leaf petioles were inoculated by excising the first leaves above the cotyledon 0.5 to 1 cm from their base, and immediately applying to the cut surfaces a 2 µl droplet of a water suspension containing 8×10^4 c.f.u. of *R. solanacearum* cells taken from a 2-d-old BG agar plate. To test for elicitation of the defensive hypersensitive reaction (HR), water suspensions with 1×10^7 c.f.u. ml⁻¹ of *R. solanacearum* taken from BG agar plates were infiltrated into leaves of tobacco (a nonhost plant) by making a hole in the leaf with a 25-gauge hypodermic needle, pressing the blunt end of a filled tuberculin syringe against the leaf surface while supporting the other side of the leaf with a finger, and gently injecting a small volume of inoculum (Carney & Denny, 1990).

Microscopy. Colonies were examined for twitching motility by placing a Petri dish without its lid on the stage of an upright light microscope (Nikon Labophot) equipped with 4× and 10× objectives. Digital images were acquired using a colour

CoolSnap CCD camera (Roper Scientific) and saved as uncompressed TIFF files. Image processing, which was limited to cropping, conversion to greyscale, and adjustment of brightness and/or contrast, was done with CoolSnap or Photoshop (Adobe) software.

Isolation of an *R. solanacearum* cosmid clone with PILD-like activity. A pre-existing, en masse cosmid library of total DNA from strain AW1 (Carney & Denny, 1990) was introduced into *P. aeruginosa* strain PAK-D Ω (*pilD*:: Ω) by triparental mating, and Sp- and Tc-resistant transconjugants were selected on lipase activity agar plates. Colonies that acidified the surrounding medium were identified and restreaked on the same medium to confirm restoration of lipase activity and to check for twitching motility.

Plasmid construction. To create pKS-Gm, the 0.85 kb *aacC1* gentamicin-resistance gene cartridge was released from pUCGM (Schweizer, 1993) by digestion with *Sma*I and was cloned into pBluescript KS II+ (Stratagene) that had been digested with *Ssp*I (which releases a 131 bp fragment outside of the multiple cloning site). pKSL was created by deleting the *Sma*I–*Hinc*II fragment from within the multiple cloning site of pKS-Gm.

Cloning of *R. solanacearum* *pil* genes and creation of *pil* mutants. *R. solanacearum* genes involved in Tfp production were assigned names based on homology and the naming scheme used for *P. aeruginosa* (Alm & Mattick, 1997). The putative *pilQ* and *pilT* genes in *R. solanacearum* were identified in the genome sequence of strain GMI1000 using tBLASTn (Altschul *et al.*, 1997) with the *P. aeruginosa* PilQ and PilT protein sequences as probes. PCR primers were designed to amplify the complete *pilQ* gene (2178 bp fragment: forward 5'-TACCTCTAGAGACCCTGAAAGTTCAGGAGGGCGG-3', reverse 5'-TACCTCTAGACTTCAGCGACAGCTGGTCGGACAG-3'), an internal portion of *pilQ* (1336 bp fragment: forward 5'-TACCTCTAGAGCACCCGCGTCGTGCTGGATCTGG, reverse 5'-TACCTCTAGATCGGTACCCCTGCTCGATCAGCGCC-3') and the complete *pilT* gene (1190 bp fragment: forward 5'-GGTACCTCTAGACATCGTGGCACTCCGGAGC-3', reverse 5'-GGTACCTCTAGACAAGTCCGAGCCACGGCTG-3'). Each primer had an *Xba*I site incorporated into it (nucleotides underlined above) for use in subsequent cloning steps. PCR was performed using standard conditions (94 °C for 4 min; 30 cycles of 94 °C for 1 min, 60 °C for 1.5 min, 72 °C for 2 min; 72 °C for 10 min) with AW1 genomic DNA as the template and the reaction products were resolved by agarose gel electrophoresis. Amplified fragments were purified from the gel using the QIAquick Gel Extraction Kit (Qiagen), cloned onto pGEM-T easy, and sequenced using vector primer sites. The *Xba*I fragments with the 1.3 kb *pilQ* internal fragment and the *pilT* gene were subcloned separately onto the *Xba*I site in pKSL to create pKSLQ1 and pKSLT, respectively. Inactivated alleles of each gene were made by inserting the 0.9 kb nonpolar *nptI* cartridge (encoding Km resistance) (Galan *et al.*, 1992) to create pKSLQ1::nptI and pKSLT::nptI. The *pilQ*::*nptI* and *pilT*::*nptI* constructs were released by digestion with *Not*I or *Xba*I, respectively, and cloned into similarly digested pTOK2 to create pTOQ1::nptI and pTOT::nptI. The inactivated alleles were introduced into AW1 and K60 wild-type strains by electroporation of purified plasmid DNA. pTOK2 cannot replicate in *R. solanacearum*, so selection for Km-resistance followed by screening for Tc-sensitivity returned colonies where the wild-type gene on the chromosome had been site-specifically replaced with the inactivated allele. Fidelity of replacement was checked by: (1) determining whether

genomic DNA from a mutant simultaneously transformed wild-type strains to Km resistance and the mutant's phenotype; and (2) PCR amplification of the wild-type or inactivated allele using primers flanking the gene and analysis of the fragment sizes by agarose-gel electrophoresis. The complete *pilQ* and *pilT* genes also were subcloned in both orientations in pRK415 (Keen *et al.*, 1988) to create plasmids pRQ2+, pRQ2–, pRT+ and pRT–, respectively.

Recombinant DNA techniques and DNA sequence analysis. Standard protocols were used for cloning, conjugation, electroporation, preparation of competent cells and Southern blotting (Ausubel *et al.*, 1989; Carney & Denny, 1990). Hybridization to Southern blots used the DIG High Prime labelling and detection kit (Roche Molecular Biochemicals) and followed the manufacturer's protocols. Plasmid DNA was isolated using a Qiagen mini kit, and genomic DNA by the method of Chen & Kuo (1993). Natural transformation of *R. solanacearum* with genomic DNA was similar to that described by Bertolla *et al.* (1997), except that BG agar plates were used and the mixture of bacteria and DNA was incubated directly on the agar plate for 18–24 h. Restriction enzymes were purchased from New England Biolabs and the MasterAmp *T*/DNA polymerase PCR kit was from Epicentre Technologies. DNA primer synthesis and DNA sequencing were performed at the University of Georgia Molecular Genetics Instrumentation Facility.

RESULTS

R. solanacearum exhibits twitching motility in culture

Light microscopy revealed that young colonies (about 20 h old) of the wild-type strains AW1, K60 and GMI1000 growing on the surface of BG agar plates had various micromorphologies, which included separate irregular rafts or tendrils (Fig. 1a), a diffuse growth over much of a circular area (Fig. 1b), and a ring with mostly empty centres (Fig. 1c). Colonies that were 24 h old sometimes had a reticulate appearance (Fig. 1d) or were confluent, with ragged edges (Fig. 1e). By the time colonies were 30 h old they typically had thin or layered edges with multiple irregular projections ('spearheads') (Fig. 1f, g). Colonies of an EPS1[–] mutant were indistinguishable from those of the wild-type throughout these early stages of development. By the time the *R. solanacearum* colonies became visible by eye (about 42 h), the spearheads were becoming obscured by overgrowth of cells from the centre of the colony and also by EPS1 production in the wild-type (Fig. 1h). By 50 h, the spearheads were almost or completely obscured (Fig. 1i). Colony margins of twitching-positive *P. aeruginosa* strains PAO1 and PAK growing on the surface of BG plates had very irregularly shaped spearheads, and often there were rafts of bacteria separated from the colony (results not shown). Unlike wild-type *R. solanacearum*, isolated *P. aeruginosa* colonies continued to expand for > 3 d, and spearheads at the margins did not become obscured by overgrowths of cells from the centre of the colonies.

To prove that the remarkable *R. solanacearum* colony micromorphology is the result of cells migrating over the agar surface rather than being due to multiplication

Fig. 1. Typical micromorphology of *R. solanacearum* colonies. Strains were cultured using standard procedures (see Methods) on BG agar plates. Strain AW1 is shown 20, 24, 30, 42 and 50 h after streaking (a, d, f, h and i, respectively). Strain K60 is shown after 20 h (b and c), 24 h (e) and 30 h (g). Different colonies are shown in each panel. Bars, 0.2 mm (a–e); 0.5 mm (f and g); 1.0 mm (h and i).

Fig. 2. Time course of *R. solanacearum* micro-colony development. A single colony of K60 growing on BG agar was imaged at hourly intervals from 12 to 25 h after streaking, and then again at 36 h; selected images are shown. Standard incubation conditions and observation methods were used. Repeated observation retarded colony development relative to that shown in Fig. 1. Bar, 0.2 mm (with 10 nm subdivisions).

oriented away from the centre of the colony, we looked for individual cells or rafts of cells leaving an area that they had previously occupied. Migration was documented for K60 during development of a single colony by taking images at hourly intervals starting 12 h after a BG plate was streaked (Fig. 2). When first observed, the colony consisted of a small irregular cluster of cells, but by 19 h the colony had split into three irregular rafts of cells, leaving what had been the middle of the colony empty. By 25 h the colony had further

expanded and divided into ≥ 10 rafts, leaving much of the space within the roughly circular zone of the colony unoccupied. Colonies at a similar developmental stage were observed routinely for K60 and other *R. solanacearum* strains in many additional experiments. We did not observe migration of single bacteria away from a raft or spearhead. By 36 h the rafts had merged, probably by a combination of movement and multiplication (Semmler *et al.*, 1999), to give a thin colony with multiple spearheads at its margin.

Fig. 3. Movement of single *R. solanacearum* cells. A 10:1 mixture of AW1 (nonfluorescent) and AW1-gfp38 (fluorescent) cells was spread on a BG agar plate, which was subsequently incubated for 24 h. (a) A tendrill of an AW1-gfp38 colony at the upper right had fused with the advancing edge of an AW1 colony. Single fluorescent cells were observed deep within the bulk of the nonfluorescent cells. (b, c) The same tendrill of a colony containing both types of cells imaged 90 min (about one generation) apart. Numerous fluorescent cells moved into the tendrill during this interval. Images were made using combined epifluorescent and low-level transmitted light. Bars, 10 nm.

Movement of individual bacteria was observed within spearheads and rafts by culturing a mixture of fluorescent (AW1-gfp38) and nonfluorescent (AW1) wild-type cells together on a BG agar plate. Where spearheads or rafts from the two strains met during colony development, the cells intermixed to varying degrees. It was common to find isolated fluorescent cells within rafts of nonfluorescent cells (results not shown) or deep within a nonfluorescent colony (Fig. 3a), which could only have got there by migration. In addition, serial images revealed the movement of single fluorescent

cells within a raft or spearhead of nonfluorescent cells (Fig. 3b,c). These observations show clearly that *R. solanacearum* can migrate over an agar surface, and the genetic evidence presented below shows that this is due to twitching motility.

Factors that affect expression of twitching motility in culture

To optimize twitching motility by wild-type *R. solanacearum* in culture, we tested different conditions using AW1, K60 and GMI1000. The degree of twitching was evaluated qualitatively by observing the size and micro-morphology of colonies in low-density areas of plates 20–24 h after streaking. As previously reported (Bradley, 1980; Henrichsen, 1975b), correct preparation of the agar plates was important, with freshly poured and briefly dried plates (see Methods) working better than those ≥ 1 d old. However, all three strains exhibited some twitching motility on 1-d-old plates prepared using standard methods. Incubating inoculated plates in a loosely closed plastic bag along with several moist paper towels to ensure high humidity enhanced the degree of twitching motility and increased colony size (compare Figs 4a and 4b). Twitching was observed on plates solidified with agarose (Genetic Technology Grade; ICN), but colonies consisted mostly of sinuous tendrills with smooth margins (Fig. 4c). Motility was much better on BG agar plates than on MM agar plates (Fig. 4d), but agar from different companies (Difco or Sigma) did not affect twitching. Agar concentrations ranging from 0.8 to 2.0% were tested, and plates with 1.2–1.6% agar were the best for observing twitching. Results were the same whether cells were applied to plates by streaking, spreading or spotting micro-droplets. When plates were stab-inoculated with *R. solanacearum*, twitching motility was observed in the interstitial space between the agar and the polystyrene surface of the Petri dish, but in comparison to colonies on the agar surface, the spearheads were less pronounced and movement away from the point of inoculation was slower. Twitching motility by *P. aeruginosa* responded similarly to most conditions tested, but *P. aeruginosa*

Fig. 4. Micromorphology of *R. solanacearum* wild-type K60 (a–d) and K60-Q (e) colonies cultured using standard or nonstandard conditions. (a) Standard conditions on BG agar, showing typical morphology. (b) High humidity during incubation on BG agar, showing large colony size. (c) Standard conditions on BG agarose (1.6%), showing more compact and distinct tendrills. (d) Standard conditions on MM agar, showing reduced twitching activity. (e) Standard conditions on BG agar, showing a *pilQ* mutant, which does not twitch. Bar, 0.2 mm.

Fig. 5. Predicted amino acid sequence of *R. solanacearum* AW1 PilQ protein and its alignment with selected orthologues. The alignment was generated using CLUSTAL W (<http://www.ch.embnet.org>) with the default parameters. Amino acids that are identical or similar in $\geq 50\%$ of the sequences are shown in white on black or in black on grey, respectively. An 'x' indicates the C-terminus. Homologous PilQ proteins are from *P. aeruginosa* (GenBank accession no. P34750), *N. gonorrhoeae* (S70838) and *M. xanthus* (AF100157). The unaligned N-terminal 150 amino acids of *M. xanthus* PilQ were omitted for simplicity. The C-terminal region (starting with residue 434 of *R. solanacearum* PilQ) that is most conserved among the secretin superfamily is enclosed by a box. The three highly conserved motif sequences are underlined (motifs 1, 2 and 4; see PRINTS accession PR00811).

was much better than *R. solanacearum* in moving between the agar and the Petri dish surface. In summary, only simple procedures and correct timing were necessary to observe twitching motility by *R. solanacearum*.

We investigated the effect that inactivating a variety of regulatory genes had on twitching motility of *R. solanacearum*. Mutants of AW1 that were tested carried single insertions that inactivated either components of the Phc confinement-sensing system (*phcA*, *phcB*, *phcBSR*) (Clough *et al.*, 1997b), two two-component systems that regulate EPS production and other virulence factors (*vsrAD*, *vsrBC*) (Huang *et al.*, 1995), a unique signal-integrating, *trans*-acting protein in *eps* regulation (*xpsR*) (Huang *et al.*, 1995), the stationary-phase sigma factor (*rpoS*) (Flavier *et al.*, 1998), or the acylhomoserine-lactone-responsive quorum-sensing system (*solR*, *soll*) (Flavier *et al.*, 1997). Mutants of GMI1000 carried single insertions that inactivated components regulating *hrp* genes (*prhA*, *prhI*, *prhJ*, *hrpG* and *hrpB*) (Brito *et al.*, 1999; Marena *et al.*,

1998; Schell, 2000) or *phcA*. The mutants all exhibited twitching motility that was comparable to that of the parental strains cultured at the same time, except for the *phcA* mutants, which continued to exhibit twitching motility for days longer than the others. Prolonged motility of bacteria was particularly evident when there were only three or four colonies per plate or if the plate was spot-inoculated at its centre. For example, isolated colonies of the AW1 *phcA* mutant became about three times the size of EPS1⁻ colonies (e.g. 17.2 mm diameter vs 5.5 mm after 3 d incubation). This observation suggests that, like a few other traits, twitching motility is negatively regulated by the increase in PhcA function that occurs when cell density increases (Clough *et al.*, 1997a; Schell, 2000).

Many of the proteins essential for type IV piliation are similar to those required for type II protein secretion across the outer membrane (Nunn, 1999). In *P. aeruginosa* these similarities are so strong that some proteins in both systems are processed by PilD, a specialized

Fig. 6. Virulence of *R. solanacearum* wild-type and a *pilQ* mutant. Unwounded roots of tomato plants were inoculated by drenching the soil with suspensions of wild-type AW1 (filled symbols) or the *pilQ* mutant AW1-Q (open symbols) at concentrations of 2×10^8 c.f.u. ml⁻¹ (circles), 2×10^7 c.f.u. ml⁻¹ (squares), or 2×10^6 c.f.u. ml⁻¹ (triangles). Each treatment was applied to eight plants, and the mean percentage of leaves wilted was determined daily. Results are representative of two or more experiments.

leader peptidase (Strom & Lory, 1993). However, *R. solanacearum* strain AD4, which has its type II protein secretion system inactivated (Kang *et al.*, 1994), exhibited wild-type twitching activity.

R. solanacearum* has at least three orthologues of genes essential for production of functional Tfp in *P. aeruginosa

Movement of *R. solanacearum* cells over the surface of agar plates strongly suggests twitching motility, but proof requires documenting that cells become nonmotile when production of functional Tfp is inactivated. Careful examination of the preliminary GMI1000 genomic sequence revealed six ORFs that strongly resemble genes encoding pilin proteins in *P. aeruginosa* and other bacteria, so it will require substantial effort to determine which ORF encodes the pilin protein, as opposed to a pilin-like protein that is involved in biogenesis of Tfp. Therefore, to test more immediately the connection between twitching motility and Tfp, we targeted conserved genes likely to be essential for Tfp production and function.

One of the more conserved *R. solanacearum* GMI1000 orthologues of interest encodes the putative PilD, which is 41–52% identical to PilD from *P. aeruginosa* (GenBank accession no. M32066), *N. gonorrhoeae* (U32588) and *M. xanthus* (AF003632). In *P. aeruginosa*, *pilD* encodes a leader peptidase that processes the Tfp prepilin protein and N-methylates the pilin N-terminal amino acid (Lory & Strom, 1997; Strom & Lory, 1993). PilD peptidase also processes at least six other proteins involved in pilus biogenesis (FimTU, PilE, PilVWX), and five pseudopilins (XcpTUVWX) in the type II

secretion system (Alm & Mattick, 1997; Nunn, 1999). Therefore, *P. aeruginosa pilD* mutants lack twitching motility and do not secrete a lipase and several other extracellular enzymes. We found the *R. solanacearum pilD* orthologue in a cosmid library of AW1 genomic DNA by its ability to restore lipase activity and twitching motility to a *P. aeruginosa pilD* mutant. After an en masse mating, two transconjugant colonies were positive for lipase activity on plates with olive oil as the sole carbon source. Cosmid DNA (pLAD1 and pLAD2) was extracted from each transconjugant, transformed into *E. coli* DH5 α , and recombined into the *P. aeruginosa pilD* mutant. Both cosmids, which contained overlapping regions of the genome, again restored lipase activity and also restored twitching motility, indicating that *R. solanacearum* AW1 has a functional PilD orthologue. Unfortunately, because *pilD* mutants are pleiotropic, this gene was not a good target to test genetically the connection between Tfp and twitching motility.

A second orthologue of interest was that for *pilQ*. In *M. xanthus*, *N. gonorrhoeae* and *P. aeruginosa* PilQ is a secretin located in the outer membrane, where it is thought to form a gated channel through which the pilin subunits travel (Alm & Mattick, 1997; Fernandez & Berenguer, 2000; Wall *et al.*, 1999; Wolfgang *et al.*, 2000). Inactivation of *pilQ* prevents biogenesis of Tfp, but should have little effect on other secretion systems. In GMI1000, the putative *pilQ* orthologue is the last gene in what appears to be the *pilMNOPQ* operon, an organization like that in *P. aeruginosa* and *M. xanthus* (Alm & Mattick, 1997; Wall & Kaiser, 1999). We PCR-amplified *pilQ* from the genome of AW1 on a 2.2 kb fragment, and DNA sequencing showed it to be 89.9% identical to *pilQ* in GMI1000. The predicted amino acid sequence of AW1 PilQ has 714 residues and is 91% identical to the predicted GMI1000 PilQ, and 30–38% identical to PilQ from *M. xanthus*, *N. gonorrhoeae* and *P. aeruginosa* over its entire length (Fig. 5). Like other members of the secretin superfamily (Genin & Boucher, 1994; Wall *et al.*, 1999), the AW1 PilQ sequence is better conserved in its C-terminal half, with three of the four conserved sequence motifs being present (PRINTS accession PR00811).

The third orthologue we investigated was that for *pilT*, which encodes one of the most highly conserved proteins required for biogenesis of Tfp in *M. xanthus*, *N. gonorrhoeae* and *P. aeruginosa* (Wall & Kaiser, 1999). Evidence is building that PilT, which belongs to a family of presumed ATPases, is required for the retraction of Tfp that powers cell movement (Merz *et al.*, 2000; Wall & Kaiser, 1999; Wolfgang *et al.*, 1998, 2000). Following the same approach as above, we PCR-amplified and cloned the putative *pilT* from AW1, which DNA sequencing showed to be 96.9% identical to *pilT* in GMI1000. The predicted amino acid sequence for AW1 PilT has 347 residues and is 99.7% identical to the predicted GMI1000 PilT; it is 68 and 71% identical to PilT from *N. gonorrhoeae* and *P. aeruginosa*, respectively. Not surprisingly, AW1 PilT has a conserved Walker box ATP/GTP binding-site [AG]-X₄-G-K-[ST]

Table 2. Twitching motility and presence of *pilQ* in a diverse collection of *R. solanacearum*

Strain*	Twt†	<i>pilQ</i> ‡	Race	Biovar	RFLP§		Host	Origin
					Division	Group		
AW1	++	+	1	1	II	ND	Tomato	USA
K60 (UW25)	++	+	1	1	II	1	Tomato	USA
UW26	++	ND	1	1	II	2	Tomato	USA
UW30	++	ND	1	1	II	1	Tomato	Trinidad
UW134	++	ND	1	1	II	1	Potato	Kenya
UW153	++	ND	1	1	II	1	Potato	Australia
UW154	++	ND	1	1	II	6	Tobacco	Columbia
UW256	++	ND	1	1	II	4	Potato	Costa Rica
UW119	++	ND	1	3	I	14	Potato	Costa Rica
UW130	++	+	1	3	I	9	Tomato	Peru
UW143	++	+	1	3	I	10	Tomato	Australia
GMI1000	++	+	1	4	I	ND	Tomato	French Guyana
UW74	++	ND	1	4	I	15	Potato	Sri Lanka
UW141	++	ND	1	4	I	21	Ginger	Australia
UW151	++	ND	1	4	I	22	Ginger	Australia
UW197	++	ND	1	4	I	11	Ginger	The Philippines
UW360	++	ND	1	4	I	16	Mulberry	China
UW361	++	ND	1	5	I	19	Mulberry	China
UW373	+	ND	1	5	I	20	Mulberry	China
UW9	++	ND	2	1	II	24	<i>Heliconia</i>	Costa Rica
UW20	++	ND	2	1	II	28	Banana	Venezuela
UW28	—	+	2	1	II	24	Potato	Cyprus
UW70	++	+	2	1	II	25	Plantain	Columbia
UW127	++	ND	2	1	II	25	Plantain	Peru
UW135	++	ND	2	1	II	24	Banana	Honduras
UW138	—	ND	2	1	II	25	Plantain	Costa Rica
UW160	++	+	2	1	II	25	Plantain	Peru
UW167	++	ND	2	1	II	24	Banana	Costa Rica
UW19	+	+	3	2	II	27	Potato	Columbia
UW23	++	+	3	2	II	26	Potato	Israel
UW73	+	ND	3	2	II	26	Potato	Unknown
UW80	+	ND	3	2	II	27	Potato	Columbia
UW81	+±	+	3	2	II	27	Potato	Columbia
UW145	+	ND	3	2	II	26	Potato	Australia
UW150	+	ND	3	2	II	26	Potato	Australia

* UW strains were from the collection of L. Sequeira (University of Wisconsin). See Cook *et al.* (1989) for additional information about these strains.

† Degree of twitching motility assessed at 24 h when cultured using standard conditions on BG agar. ++, Very active twitching; +±, intermediate twitching; +, less active twitching; —, not twitching. See Fig. 7 for examples of the different twitching activities.

‡ Southern blots probed with the cloned *pilQ*. +, Hybridizing band; ND, not done.

§ RFLP results are from Cook *et al.* (1989).

(ProSite PDOC00017) at the expected location (starting with residue 130).

Inactivation of *pilQ* or *pilT* eliminates twitching motility and reduces virulence of *R. solanacearum*

To inactivate *pilQ* in *R. solanacearum*, we inserted an *nptI* (Km resistance) cartridge into an internal 1.3 kb *pilQ* gene fragment and introduced this into the genome of AW1 and K60 by allelic replacement to create strains

AW1-Q and K60-Q, respectively. As expected, the *R. solanacearum pilQ* mutants did not exhibit twitching motility on BG agar plates, since colonies less than 24 h old had entire margins, and were smaller and more domed than those of the wild-type parents (Fig. 4e). Nevertheless, 2-d-old colonies of the *pilQ* mutants on BG agar plates were very similar to those of the wild-types. Introduction of wild-type *pilQ* on pRQ2+, which has the gene on a 2.2 kb fragment aligned with the vector's *lac* promoter, into the *pilQ* mutants restored

Fig. 7. *R. solanacearum* strains naturally exhibiting different degrees of twitching motility. Each row of images (a–c) illustrates one of the three twitching categories in Table 2. Images in each column were recorded at the indicated time and objective power. (a) Strain UW153 had Twt++ activity. (b) Strain UW145 had Twt+ activity. (c) Strain UW138 had Twt– activity. Bars, 0.2 mm (first and third columns); 50 nm (middle column).

twitching motility (not shown), so the *nptI* insertion in the genome did not have any obvious effects on downstream genes. pRQ2–, in which *pilQ* is aligned opposite to the vector's *lac* promoter, did not restore motility, so the cloned fragment lacks its native promoter as one would expect for a gene located at the end of a large operon.

pilT was inactivated in the genome of AW1 and K60 by allelic replacement, creating strains AW1-T and K60-T. Compared to their wild-type parents, PilT mutants formed a more substantial pellicle at the surface of unshaken cultures or developed as macroscopic clumps of cells that settled to the bottom of the culture tube,

suggesting that they are hyperpiliated. Neither AW1-T nor K60-T exhibited twitching motility on BG agar plates, since 24-h-old colonies looked the same as *pilQ* mutants (not shown). However, 2-d-old cultures of the *pilT* mutants on BG agar plates had smaller colonies than wild-type, less EPS1 slime, and the slime produced was abnormally transparent due to there being fewer suspended cells.

pilQ and *pilT* mutants of AW1 and K60 multiplied normally in BG broth cultures, and AW1-Q survived as well as AW1 during the first 7 d after application to the potting mixture used for growing tomato plants (results not shown). To test whether inactivation of *pilQ* or *pilT*

affected important extracellular products that contribute to virulence of *R. solanacearum*, we quantified EPS1 production, and the activities of two secreted enzymes (endoglucanase and endo-polygalacturonase). The mutants were comparable to the wild-type in all three traits, so the membrane-localized functions for EPS1 biosynthesis and type II secretion were not affected. Like AW1, both AW1-Q and AW1-T elicited a normal HR defence response when infiltrated into nonhost tobacco leaves, so Hrp pili and the rest of the type III protein secretion system also were not affected. However, AW1-Q induced wilt symptoms less quickly than did AW1 when unwounded tomato roots were inoculated by a soil drench (Fig. 6). Although tested less extensively, K60-Q was comparable to AW1-Q in virulence (results not shown). The difference between AW1 and AW1-Q was clearly affected by the inoculum concentration, with higher concentrations of bacteria (e.g. 2×10^8 c.f.u. ml⁻¹) markedly reducing the differential between the wild-type and the mutant in some experiments. In three experiments, K60-T wilted plants less quickly than K60 when inoculated via wounded petioles (results not shown). These results suggest that *R. solanacearum* Tfp, or functions that require Tfp, are important during both invasion and colonization of young tomato plants.

Twitching motility is widespread in *R. solanacearum*

To learn whether twitching motility is a common trait in *R. solanacearum*, we examined the colony morphology of a diverse set of 35 strains under standard conditions (Table 2). Thirty-three strains exhibited twitching motility, with the race 1 and 2 strains generally twitching more actively than the race 3 strains. Two strains were nonmotile, because their colonies resembled those of nontwitching *R. solanacearum pilQ* mutants. The differences between the different twitching categories are illustrated in Fig. 7. Except for the less active twitching in most race 3 strains, there was no correlation between the twitching motility and biovar, restriction fragment length polymorphism (RFLP) division, RFLP group, or the host or country from which the strain originated. Southern-blot analysis of 11 *R. solanacearum* strains using pPILQ1 as the probe revealed that all of them, including the nonmotile strain UW28, had a single fragment that hybridized (Table 2 and results not shown). The four different bands that hybridized to the *pilQ* probe ranged in size from 7 to 10 kb, but the RFLP polymorphisms did not correlate with any of the other characteristics listed in Table 2.

DISCUSSION

Our results revealed that during wild-type colony development *R. solanacearum* has the ability to migrate over an agar surface and that migration of the two strains examined in detail apparently required production of functional Tfp. We referred to this movement as twitching motility, because it closely resembles the Tfp-dependent phenomenon in well-studied strains of *P. aeruginosa* (Bradley, 1980; Darzins, 1993; Semmler

et al., 1999). As previously reported for *P. aeruginosa* (Bradley, 1980; Semmler *et al.*, 1999), we saw smooth movement of *R. solanacearum* in cellular aggregates rather than intermittent and jerky movement of single cells characteristic of the twitching motility reported by Henrichsen (1972). However, the movements Henrichsen used to differentiate twitching and gliding motility (Henrichsen, 1972) seem subtle and subjective, and even he sometimes had trouble making clear distinctions between these motions (Henrichsen, 1975a). We agree with the conclusion of Semmler *et al.* (1999) that, according to Henrichsen's definitions, because the movement of *P. aeruginosa* and *R. solanacearum* was 'continuous and regularly follows the long axis of the cells which are predominantly aggregated in bundles', it could alternatively be called gliding motility.

We were surprised to find, when researching the older literature on twitching motility, that Henrichsen (1975a, 1983) reported many years ago that five strains of *R. solanacearum* exhibited twitching motility. These results were largely forgotten by the scientific community studying this plant pathogen for several reasons. First, until recently twitching motility was not a commonly studied phenomenon so most plant pathologists were (and are) unfamiliar with it. Second, because Henrichsen surveyed many bacteria in addition to *R. solanacearum*, it was not mentioned by name in the abstract, so only the most thorough or directed literature search would have found these reports.

In our study, motility by *R. solanacearum* clearly was much better on a normal agar surface than in the interstitial space between the agar and the plastic Petri dish, in contrast with *P. aeruginosa*, which migrated quickly in both locations. Unlike Semmler *et al.* (1999), we observed that *P. aeruginosa* (i) readily developed rafts when growing on the agar-air interface of BG plates and (ii) did not twitch well on the basal agar surface that was exposed by inverting the agar layer. In addition, in contrast to the results of Henrichsen (1975b) with *Acinetobacter calcoaceticus*, motility of *R. solanacearum* was observed on media solidified with agarose. Therefore, neither BG agarose nor a BG agar surface that solidified while exposed to air was inherently suboptimal for observing twitching motility *in vitro*. At least for *R. solanacearum*, we found that rather than special conditions being required to observe twitching motility, it is more important that one observe the appropriate strains at the correct time.

Twitching motility has not been reported for other plant-pathogenic bacteria, but several xanthomonads and one pseudomonad make typical Tfp. Pili of *Xanthomonas campestris* pv. *hyacinthi* were purified, and the N-terminal amino acid sequence strongly resembled the conserved portion of Tfp (van Doorn *et al.*, 1994). Cells and pili bound to the stomata of hyacinth leaves, but a nonpiliated mutant was not tested so the role of Tfp in pathogenesis on hyacinth remains unclear. For *Xanthomonas campestris* pv. *vesicatoria*, the pilin structural gene was found by reverse genetics and

subsequently inactivated. The nonpiliated mutant retained full virulence on tomato plants when tested under controlled conditions, but because Tfp promoted aggregation on leaf surfaces and increased resistance to killing by UV light the authors speculated that piliation might increase survival on leaf surfaces (Ojanen-Reuhs *et al.*, 1997). Similarly, the Tfp of *Xanthomonas campestris* pv. citri were reportedly not important for virulence when a nonpiliated mutant was sprayed on citrus leaves in a humid environment (Su *et al.*, 1999). It would have been more interesting and informative if these pathogens had been tested on plants in environmental conditions that resemble natural settings. One example where a mutant strain lacking Tfp was tested in the field was for *Pseudomonas syringae* pv. tomato (Roine *et al.*, 1998). Even here, however, Tfp did not contribute measurably to virulence, although piliation was again associated with UV tolerance and survival on the leaf surface. *Xylella fastidiosa* probably makes Tfp, because 26 orthologous genes for pili synthesis and function were found in its genomic sequence (Simpson *et al.*, 2000), but this remains to be proven.

We did not demonstrate the physical presence of Tfp on *R. solanacearum*, but the literature suggests that this organism can make two or more types of pili. In the only contemporary research, van Gijsegem *et al.* (2000) observed two types of polar pili on GMI1000 during electron microscopy of negatively stained cells. One type of pilus was 3–5 nm wide and was produced preferentially when cells were cultured on rich medium, but was not further characterized. The second type of pilus had a constant diameter of 6.6 ± 1 nm and it is composed of 7 kDa pilin monomers encoded by *hrpY*. The HrpY pilin has a much lower molecular mass than a typical type IV pilin (16–17 kDa), and its amino acid sequence is completely unrelated. It was clear from our results that neither Hrp pilus nor any of the Hrp regulatory system tested were necessary for twitching motility.

In the older literature on *R. solanacearum*, it is not clear what type of pili were detected. The first report was by Fuerst & Hayward (1969), who observed sparse, polar pili on wild-type biovar 2, 3 and 4 strains. Interestingly, the biovar 4 strain 003A they reported using is probably similar to UW141 (originally designated as 007A), which we found to exhibit active twitching motility, because both were isolated by Hayward from ginger plants in Australia during April, 1965. Henrichsen & Blom (1975) observed polar pili on what appears to be the same biovar 2 strain as used by Fuerst & Hayward (1969). More recently, a pilin protein was purified two separate times from K60 (Stemmer & Sequeira, 1987; Young *et al.*, 1985), and neither the molecular mass (9.5 kDa) nor the amino acid compositions matched either *R. solanacearum* HrpY pilin or typical Tfp. Because these authors used very different culture conditions and methods to observe or isolate pili, the discrepancies in their results do not necessarily indicate production of different pili.

In contrast to the results with other Tfp[−] phytopathogenic bacteria, eliminating the PilQ secretin in *R.*

solanacearum (and presumably Tfp, since the mutants were nonmotile), markedly reduced virulence on tomato plants, indicating that motility and/or Tfp play a significant role during pathogenesis. Although there was no change in type II or type III protein secretion or in EPS1 production when *pilQ* was inactivated, we cannot exclude the possibility that loss of this outer-membrane protein has unknown secondary effects. Addressing this concern will require additional work to make a mutant that is defective only in synthesis of the pilin subunit. Nevertheless, that twitching motility appears to be negatively regulated by PhcA, the global virulence regulator, supports the likelihood that motility and/or Tfp are important factors in pathogenesis. Regulation of twitching appears to be on a separate circuit from most other known virulence and pathogenicity factors, since none of the other 12 regulatory genes or two-component systems tested seemed to have any effect. However, we did not test the *R. solanacearum* *pehSR* two-component system, which positively regulates both transcription of *pehA*, the endo-polygalacturonase structural gene, and flagellar motility (Allen *et al.*, 1997; Tans-Kersten *et al.*, 2001). Based on the similarity of PehS and PehR to the two-component regulatory system that regulates pilin synthesis in *P. aeruginosa*, Allen *et al.* (1997) speculated that this system might also positively regulate Tfp production in *R. solanacearum*. Because expression of *pehSR* is negatively controlled by PhcA (Allen *et al.*, 1997), our observation of prolonged twitching motility by *phcA* mutants supports this proposed regulatory pathway.

It is interesting to consider why type IV piliation is more important for virulence of *R. solanacearum* than it appears to be for *X. campestris* or *P. syringae*. One explanation might be that, because *R. solanacearum* must systemically colonize tomato plants to cause disease symptoms (Saile *et al.*, 1997; T. P. Denny, unpublished observations), it has different requirements for Tfp *in planta* than do the other pathogens tested, which cause only localized lesions. It would be premature to suggest what these requirements might be, because the process of systemic colonization of a host by *R. solanacearum* is not well understood. Another possibility is that contact with and invasion of unwounded tomato roots by *R. solanacearum* when poured into the soil is a more demanding process than that when the foliar pathogens are sprayed directly onto leaf surfaces. It also is fair to wonder what role twitching motility might have for *R. solanacearum* in natural settings. Here, the best possibility is that, as proposed for *M. xanthus* and *P. aeruginosa* (Wall & Kaiser, 1999), which are common soil microbes, Tfp-mediated migration over surfaces could promote acquisition of nutrients. Regardless of whether these explanations are correct, our results with *R. solanacearum* reinforce the conclusion by Semmler *et al.* (1999) that twitching motility is a highly organized process requiring cell-cell interactions similar to the social gliding motility in *M. xanthus*, rather than the slow, disorganized mode of movement by individual cells originally proposed by

Henrichsen (1972). Consequently, the role of twitching motility for pathogenesis and survival of *R. solanacearum* will be all the more interesting and important to understand.

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