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Ralstonia solanacearum Needs Motility for Invasive Virulence on Tomato

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***Ralstonia solanacearum*, a widely distributed and economically important plant pathogen, invades the roots of diverse plant hosts from the soil and aggressively colonizes the xylem vessels, causing a lethal wilting known as bacterial wilt disease. By examining bacteria from the xylem vessels of infected plants, we found that *R. solanacearum* is essentially nonmotile in planta, although it can be highly motile in culture. To determine the role of pathogen motility in this disease, we cloned, characterized, and mutated two genes in the *R. solanacearum* flagellar biosynthetic pathway. The genes for flagellin, the subunit of the flagellar filament (*fliC*), and for the flagellar motor switch protein (*fliM*) were isolated based on their resemblance to these proteins in other bacteria. As is typical for flagellins, the predicted FliC protein had well-conserved N- and C-terminal regions, separated by a divergent central domain. The predicted *R. solanacearum* FliM closely resembled motor switch proteins from other proteobacteria. Chromosomal mutants lacking *fliC* or *fliM* were created by replacing the genes with marked interrupted constructs. Since *fliM* is embedded in the *fliLMNOPQR* operon, the *aphA* cassette was used to make a nonpolar *fliM* mutation. Both mutants were completely nonmotile on soft agar plates, in minimal broth, and in tomato plants. The *fliC* mutant lacked flagella altogether; moreover, sheared-cell protein preparations from the *fliC* mutant lacked a 30-kDa band corresponding to flagellin. The *fliM* mutant was usually aflagellate, but about 10% of cells had abnormal truncated flagella. In a biologically representative soil-soak inoculation virulence assay, both nonmotile mutants were significantly reduced in the ability to cause disease on tomato plants. However, the *fliC* mutant had wild-type virulence when it was inoculated directly onto cut tomato petioles, an inoculation method that did not require bacteria to enter the intact host from the soil. These results suggest that swimming motility makes its most important contribution to bacterial wilt virulence in the early stages of host plant invasion and colonization.**

Motility solves many of the problems that confront microbes: it allows them to obtain more or better nutrients, avoid toxic substances or unfavorable environments, find a host, and disperse effectively. Many species of bacteria, including most soil-borne species studied to date, can move by swimming, gliding, twitching, or swarming (37, 58). Swimming motility is mediated by flagella, structures consisting of a long, helical filament anchored in the cell envelope by a flexible hook and basal body complex. The flagellar filament is a hollow tube composed of about 20,000 copies of a single protein called flagellin (FliC) polymerized into a complex helix. Rotation of the flagellum is controlled in the basal body by the flagellar motor switch, which is composed of FliG, FliM, and FliN (37). Many additional proteins make up the flagellar basal body and secretion apparatus, which is evolutionarily related to type III secretion systems (22).

The ability to move toward or within a potential host generally confers a significant selective advantage on bacteria pathogenic to animals (46). However, the role of motility in plant-associated microbes is not as well understood (58). Undefined chemically induced nonmotile mutants of the plant pathogens *Erwinia amylovora, Pseudomonas phaseolicola*, and *P. syringae* pv. glycinea were significantly reduced in virulence in assays that required bacteria to actively enter the plant, though all three mutants were fully virulent when injected directly into host tissue (7, 29, 47). In contrast, nonmotile mutants of the crown gall pathogen *Agrobacterium tumefaciens* were fully virulent (10), although another group found that an *A. tumefaciens* flagellin mutant induced smaller and fewer tumors even when it was inoculated directly onto wounded plant tissue (15). Moreover, a chemically induced nonmotile *A. tumefaciens* mutant could not cause disease when inoculated in soil, though it was still fully virulent when applied directly to wounded roots (30). Among nonpathogenic plant-associated microbes, swimming motility increased epiphytic fitness of leaf-colonizing strains of *P. syringae* (27), while nonmotile strains of the free-living nitrogen-fixing bacterium *Azospirillum brasilense* and of a plant growth-promoting *P. fluorescens* strain were much less able to colonize host roots (19, 57). Conversely, nonmotile *P. fluorescens* biocontrol strains colonized wheat and pea roots as well as wild-type motile strains (9, 33). Some, but not all, data suggest that motility increases competitiveness of *Rhizobium* strains under field conditions (5, 13, 38). These mixed results permit no generalization about the role of motility in plant-microbe interactions, although it is clear that the stringency of the virulence assay and the method of inoculation can dramatically affect the apparent importance of motility.

Ralstonia solanacearum is a soil-borne gram-negative bacterium that causes bacterial wilt disease in over 200 families of plants, including such mainstay crops as potatoes, tomatoes, peanuts, tobacco, bananas, and plantains, as well as many native plant species (31). Losses caused by the disease are known to be enormous but cannot be accurately estimated because of

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TABLE 1. Bacterial strains and plasmids used in this study[a]

Designation	Relevant characteristics	Reference or source
E. coli		
BL21(DE3)	*hsdS gal* (λcI *ts857 ind1 Sam7* UV5-T7 gene-1)	53
DH5α	F$^-$ *endA1 relA* ϕ80 *lacZΔM15 hsdR17 supE44 thi-1 recA1 gyrA96*	28
R. solanacearum		
K60	Wild type, race 1, biovar 1, tomato isolate	35
K71	K60 *pehR*::Tn*3-uidA*, Km^r	3
K701	K60 *fliC*::Gm^r	This study
K724	K60 *fliM*::*aphA*, Km^r	This study
Plasmids		
pBS	Ap^r	Stratagene
pLAFR3	Tc^r	51
pGEM-T Easy	A-T cloning vector, Ap^r	Promega
pUC18K	pUC18 containing *aphA* cassette, Km^r	41
pUCGM	Contains Gm^r cassette, Gm^r	Novagen
pGp5Gm	Plasmid containing Tn*5*, Gm^r	E. Stabb, personal communication
pHUHFli	7.0-kb *Bam*HI fragment containing *fliC* and *fliD* in pBS SK+, Ap^r	This study
pFliC1	0.85-kb fragment containing *fliC* in pGEM-T Easy, Ap^r	This study
pFliC2	pFLIC1 with the 0.85-kb Gm^r cassette in *Bal*I site of *fliC*, Gm^r Ap^r	This study
pFliC9	1.1-kb *Ava*I fragment containing *fliC* in pET29b	This study
pFliM1	7.5-kb *Hin*dIII fragment containing *fliM* in pBS KS+, Ap^r	This study
pFliM2	1.1-kb fragment containing *fliM* in pGEM-T Easy, Ap^r	This study
pFliM3	pFLIM2 with the 0.85-kb *aphA* cassette in *Bsr*BRI site of *fliM*, Ap^r Km^r	This study

[a] pBS, pBluescript; Ap, ampicillin; Tc, tetracycline; Gm, gentamicin; Km, kanamycin.

its large but undocumentable impact on subsistence agriculture and because of the abandonment of wilt-susceptible crops in many parts of the world (31). The pathogen invades plant roots through wounds, notably those created by lateral root emergence. Once inside a susceptible host, the bacteria multiply in the cortical tissue before invading the xylem elements. In a matter of hours, the bacteria spread into the crown and stem through the plant's own vascular system, presumably carried along by the transpirational flow (59). Symptoms of bacterial wilt disease include yellowing and wilting, followed by generalized necrosis and death.

R. solanacearum possesses swimming motility mediated by one to four polar flagella (12). In this species, motility is a quantitative trait; at minimal motility, up to 1% of cells are still moving, while only around 60% of cells are moving at any given time in a culture at maximum motility (17). Motility is coregulated with several known virulence factors in a regulatory cascade ultimately controlled by a cell density-responsive LysR-type global regulator called PhcA. In culture, expression of PhcA increases when the population reaches about 10^7 CFU/ml (23). PhcA then induces expression of the virulence factors extracellular polysaccharide (EPS), endoglucanase, and possibly others via a complex cascade involving additional regulatory elements (25). At the same time that it induces some virulence factor genes, PhcA represses expression of others by reducing the transcription of a two-component regulator called PehSR. PehSR is a positive regulator of plant cell wall-degrading polygalacturonases, which are also virulence factors. In addition, PehSR positively regulates bacterial motility (3). It is hypothesized that during saprophytic life and early in wilt disease development, when bacterial cell density is low and *phcA* is not expressed, *R. solanacearum* cells are motile and highly pectolytic. As bacterial populations increase in the host xylem elements, *phcA* is expressed, inducing production of EPS and other late-stage virulence factors and reducing motility by repressing *pehSR* expression (49). However, bacterial motility has not been directly measured in the plant during pathogenesis.

The fact that motility is coregulated with known virulence factors suggests that motility may also contribute to virulence. Although *phcA* mutants are hypermotile and *pehR* mutants are completely nonmotile, both strains are strikingly affected in virulence; indeed, *phcA* mutants are avirulent (11). However, since both are pleiotropic regulatory mutants, it is impossible to deduce the importance of motility in bacterial wilt disease from their behavior. Results from other systems suggest that bacterial motility can play an important ecological role in plant-microbe interactions. However, inconsistent and artificial virulence assays make it difficult to compare results from different groups. Further, much of the research in this area is inconclusive because it was conducted with uncharacterized chemical or spontaneous nonmotile mutants that may have been affected in other traits. To more conclusively determine the role of motility in bacterial wilt disease, we constructed two defined, nonpleiotropic nonmotile mutants of *R. solanacearum* and measured their virulence on tomato plants using two different assays. We also compared the motility of bacteria in host plant xylem vessels with that of bacteria growing in culture.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *R. solanacearum* strains were grown at 28°C in CPG broth (32) or on TZC plates containing CPG plus 1.8% agar and 0.05% 2,3,5-triphenyltetrazolium chloride (35). *Escherichia coli* strains were cultured at 37°C in Luria-Bertani medium (42). Antibiotics were added as required in the following concentrations (milligram per liter): ampicillin, 50; kanamycin, 25; tetracycline, 15; and gentamicin, 25.

Growth levels of wild-type and mutant strains were compared in Boucher's minimal medium (BMM) broth supplemented with 0.2% glucose (8). Cultures were inoculated in triplicate to an initial optical density at 600 nm of 0.02, and bacterial growth was measured over time as turbidity using a spectrophotometer. To measure growth of wild-type and mutant strains in planta, we infused leaves of 3-month-old tobacco (cv. Bottom Special) with a 10^8-CFU/ml suspension of bacteria. At various time points, 1-cm^2 leaf punches were ground and dilution plated on TZC plates to quantify bacteria; infusions and platings were triplicated. The ability of bacterial strains to elicit a hypersensitive response (HR) in nonhost plants was tested in 2-week-old cucumber cotyledons as previously described (60).

DNA manipulations. Isolation of plasmid and chromosomal DNA, restriction mapping, cloning, subcloning, Southern blot hybridization, and PCR were performed by standard procedures (6). *R. solanacearum* and *E. coli* were transformed by electroporation as described previously (4). DNA sequencing was performed at the University of Wisconsin—Madison Biotechnology Center. Genomic DNA sequence from the closely related *R. solanacearum* GMI1000 strain was generously provided by Christian Boucher (INRA-Toulouse, Toulouse, France). DNA sequence was analyzed with software from DNASTAR (Madison, Wis.).

Motility assays. Motility of wild-type and mutant strains was determined by transferring individual colonies to semisolid motility medium plates (36). After 4 to 6 days at 28°C, motile colonies were surrounded by an even white halo or, occasionally by radiating streaks. We also directly observed motility of bacteria by microscopy after growth in BMM broth or in xylem fluid collected from symptomatic plants. Xylem fluid from infected tomato plants was obtained by cutting plant stems with a razor blade above the cotyledons and collecting the extruding xylem fluid with a large-bore pipetter. Motility was determined microscopically within 10 mins of extraction from xylem vessels by counting motile and nonmotile cells in a known volume of fluid on a hemocytometer slide; bacterial densities were confirmed by dilution plating in triplicate.

Cloning and mutagenesis of the *fliM* and *fliC* genes. A nonmotile *R. solanacearum* transposon Tn*5* mutant was created by standard methods using suicide plasmid pGp5Gm; sequencing of the DNA flanking the Tn*5* insertion suggested that the *fliM* gene had been interrupted. Using this sequence and a putative *fliM* sequence from the GMI1000 genome, we designed primers to amplify an internal 200-bp fragment of *fliM* from chromosomal DNA of wild-type *R. solanacearum* strain K60 (5′GGAAAGGTTCCGTTCAGG3′ and 5′TTCGCTGTACTTCTG GAC3′). The resulting amplified fragment was used to probe a K60 genomic library (54). A 7.5-kb *Hin*dIII *fliM*-containing fragment was subcloned from a hybridizing cosmid into pBluescript, creating pFliM1, which was used to sequence *fliM* (GenBank accession number [hereafter simply GenBank] AF283286). A 1.1-kb subclone was amplified from pFliM1 using primers 5′AT GCTGCTGTCGTCCAAG3′ and 5′TTCAGCATGCGGTTGACG3′ and cloned into pGEM-T Easy to create pFliM2. To make a nonpolar mutation in *fliM*, the *aphA* cassette was inserted into the *Bsr*BRI site of pFliM2, creating pFliM3. The resulting construct was sequenced to confirm in-frame insertion of the *aphA* cassette.

We designed oligonucleotide primers based on a putative *fliC* sequence from *R. solanacearum* GMI1000 to amplify an internal fragment of the *fliC* gene from *R. solanacearum* strain K60 genomic DNA (5′GTCCCTCAGCCTCAATACCA A3′ and 5′GCCCTTCAGCAGGTTCAGAAT3′). The resulting 0.8-kb amplified fragment was A-T cloned into the pGEM-T Easy vector to create pFliC1; its identity was confirmed by sequencing. A 7.0-kb *Bam*HI fragment containing *fliC* was subcloned from the K60 genomic library into pBluescript to create pHUHFli, and the region containing *fliC* was sequenced (GenBank AF283285). To interrupt the *fliC* gene, we inserted a 0.85-kb *Sma*I fragment of the pUCGM gentamicin resistance (Gm^r) cassette into the *Bal*I site of *fliC* to create pFliC2. Both the *fliM*::*aphA* and *fliC*::Gm^r interrupted gene constructs were introduced into wild-type *R. solanacearum* strain K60 by homologous recombination as previously described (4). Transformants with appropriate antibiotic resistance phenotypes were screened for loss of motility on semisolid motility medium plates.

Electron microscopy. Cultures were grown to a density of 10^7 CFU/ml in BMM broth supplemented with 0.2% glucose. Cells were placed on copper grids, stained with 1% uranyl acetate, and examined with a Philips model EM-120 electron microscope. For each strain, a minimum of 1,000 cells were examined for presence or absence of flagella and, where applicable, for flagellar morphology.

Overexpression and isolation of flagellin. A 1.1-kb *Ava*I fragment containing the *fliC* open reading frame (ORF) and flanking regions was subcloned from pHUHFli into the T7 expression vector pET29b (Novagen) to create pFliC9. This construct was transformed into the *E. coli* overexpression strain BL21(DE3), and protein expression was induced with 2 mM isopropylthiogalactopyranoside (IPTG) according to Novagen's instructions. Crude flagellin was isolated as sheared bacterial cell extracts as previously described (56). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels and stained with Coomassie brilliant blue (6).

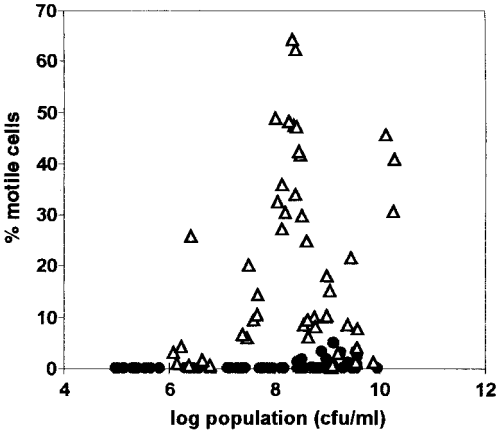

FIG. 1. *R. solanacearum* motility in culture and in planta. Wild-type strain K60 cells were grown in BMM broth (open triangles) or extracted from midstem xylem vessels of infected tomato plants (closed circles). The percentage of motile cells was determined microscopically by counting motile and nonmotile cells on a hemocytometer slide. Cell density was confirmed by dilution plating in triplicate.

Virulence assays. We measured the virulence of *R. solanacearum* strains on tomato (highly susceptible cv. Bonnie Best). Virulence of strains on unwounded plants was quantified by soil inoculation as previously described (54). In summary, we poured a 50-ml bacterial suspension onto the soil of 15-day-old plants to achieve a final concentration of about 6.25×10^6 CFU/g of soil. Direct petiole inoculation of tomato plants was as previously described (48). Briefly, we cut the petiole of the first true leaf 0.5 cm from the stem and placed 10^4, 10^3, or 10^2 bacteria in a 2-μl volume onto the freshly cut surface. For both assays, inoculum concentrations were determined turbidimetrically and confirmed by dilution plating. Plants were coded and inspected for wilt symptoms daily for 14 days following inoculation by a rater blind to treatment identity. Plants were rated on a disease index scale of 0 to 4 (0, no wilting; 1, 1 to 25% wilting; 2, 26 to 50% wilting; 3, 51 to 75% wilting; and 4, 76 to 100% wilted or dead). Each assay was repeated in three successive trials. Within each trial we treated 16 plants with each strain, yielding a total of 48 plants for each strain.

Analysis. We analyzed the data together by combining results from all 48 plants treated with a given strain across the three trials. We analyzed the daily disease indices in a combined analysis of variance (ANOVA) using repeated-measure analysis (50). This analysis accounts for the autocorrelation of disease index scored on successive days. Trial and pathogen strain were treated as fixed effects in the factorial design. All analyses were done using Systat version 5.2 (Systat Inc., Evanston, Ill.).

RESULTS

***R. solanacearum* is virtually nonmotile in planta.** To determine if motility is expressed differentially in plants and in culture, we extracted and quantified bacteria in xylem fluid from infected tomato plants and in BMM broth and viewed them under the microscope. In minimal medium, wild-type strain K60 behaved as previously observed in related race 1 strain AW-1 (17): motility increased with cell density and was greatest (about 60% motile) around 10^8 CFU/ml before declining with higher cell densities (Fig. 1). Surprisingly, we found that bacteria behaved very differently in host plant xylem vessels. In the plant, *R. solanacearum* was almost never motile below 5×10^8 CFU/ml and reached maximum motility of only 5% at about 10^9 CFU/ml (Fig. 1).

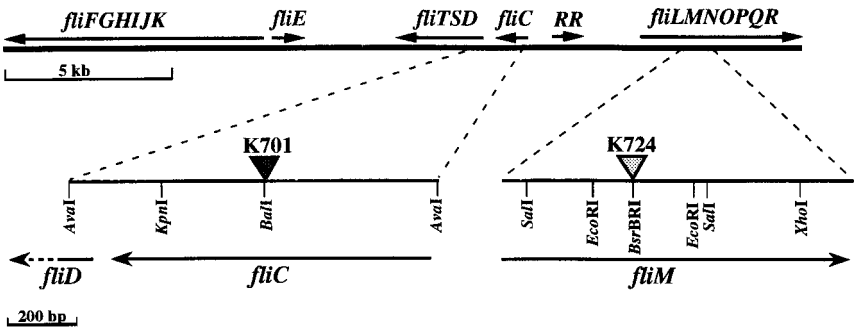

FIG. 2. Large-scale physical map of *R. solanacearum* flagellar gene cluster, assembled from related strain GMI1000 genome sequence and wild-type strain K60 genomic library cosmids 19-84 and 18-73. Filled triangle, Gm^r gene cassette; stippled triangle, nonpolar *aphA* mutagenesis cassette; *RR*, putative response regulator gene of unknown function.

Cloning and analysis of the *fliC* gene. An 850-bp amplified *fliC* gene fragment hybridized to K60 genomic library cosmid 18-73, which contained several flagellar genes (Fig. 2). The 822-bp *fliC* ORF encodes a putative protein with a calculated molecular mass of 28,283 Da. The strain K60 FliC is 94% identical at the predicted amino acid level to FliC from the closely related *R. solanacearum* strain GMI1000 and has at least 35% overall identity with several known flagellins, notably those of *Aeromonas caviae* (40%; GenBank AF198617), *P. fluorescens* (40%; GenBank AF034765), and *Legionella pneumophila* (38%; GenBank X83232). At the N terminus, which is conserved among flagellins from gram-negative bacteria, the first 120 amino acids are over 50% identical to flagellins from *Burkholderia pseudomallei* (GenBank AF078151) and *B. cepacia* (GenBank AF080259). The N-terminal region also contains a small, highly conserved 15-amino-acid domain that is over 60% identical to other flagellins (21).

Mutagenesis of the *fliC* gene. A construct containing *fliC* interrupted by a Gm^r cassette was recombined into the *R. solanacearum* strain K60 genome to create K701 (Fig. 2). Southern blot analysis confirmed that K701 contains a single interrupted copy of *fliC* (data not shown). It is unlikely that other genes are cotranscribed with *fliC* since there is a stem-loop structure that may function as a factor-independent terminator located 20 bp 3′ of the *fliC* stop codon and the closest 3′ gene, *fliD* (encoding HAP2, the flagellar cap/hook-associated protein), is 179 bp away.

Cloning, analysis, and mutagenesis of the *fliM* gene. The *R. solanacearum* K60 *fliM* gene was identified by sequence flanking the transposon insertion in a nonmotile mutant and by the putative *fliM* sequence from the GMI1000 genome. A 200-bp PCR fragment internal to *fliM* hybridized to genomic library cosmid 19-84, which contains *fliM* and apparently several other genes in the flagellar regulon. The 1,008-bp *fliM* ORF encodes a putative protein of 335 amino acids with a predicted molecular mass of 38,000 Da. 5′ to *fliM* lies *fliL*, with only one base separating the two ORFs. The *fliN* locus is 20 bp downstream (3′) of *fliM*; this arrangement suggests that these genes are transcribed in one unit, as in other bacteria, with the promoter located 3′ to the *fliL* ORF (37). The *fliM* protein is similar to flagellar motor switch proteins from *Pseudomonas putida* (43.2% identity; GenBank AF031418) and *P. aeruginosa* (42.7% identity; GenBank AE004574).

Because *fliM* is embedded in the *fliLMNOPQR* operon, we used the *aphA* cassette to create a nonpolar *fliM* mutant. This cassette contains a kanamycin resistance marker which is preceded by translation stop codons in all three reading frames and is followed by a consensus ribosome binding site and start codon to ensure continuing translation 3′ of the insertion (41). We cloned the *aphA* cassette into the *Bsr*BRI site of *fliM* and recombined this construct into the K60 genome to create K724. Southern blot analysis confirmed that a single copy of *fliM* was interrupted by the *aphA* cassette in this strain, and sequencing confirmed that the cassette was inserted properly for continued translation of downstream genes (data not shown).

Phenotypes of nonmotile mutants. Mutants K701 (*fliC*) and K724 (*fliM*) had normal mucoid colony morphology on solid media and grew as well as wild-type strain K60 in minimal media supplemented with 0.2% glucose and in tobacco plant leaves (data not shown). K701 (*fliC*) caused a normal, wild-type HR when infused into cucumber cotyledons. To directly visualize bacterial motility, cultures of the nonmotile mutants grown in BMM-glucose were compared to wild-type cells under a light microscope. At a cell density of about 10^8 CFU/ml, when *R. solanacearum* would normally be expressing greatest motility, up to 60% of the wild-type cells were motile. However, no motile bacteria were ever seen in cultures of strains K701 (*fliC*) and K724 (*fliM*) under these conditions. We also examined the motility of bacteria in xylem fluid from infected tomato plants; about 5% of wild-type K60 cells were motile in the xylem fluid (which contained about 10^9 CFU/ml), but motile K701 or K724 cells were never observed in infected plants. The instability of plasmid vectors in *R. solanacearum* made it impossible to examine the in planta virulence or motility of K701 or K724 cells complemented with wild-type *fliC* or *fliM* genes.

We examined the degree of flagellation and the flagellar morphology of various strains directly by electron microscopy. Approximately 50% of wild-type strain K60 cells had one to five helical polar flagella (Fig. 3A), while the rest were aflagellate, with many broken flagella present. None of the K701 (*fliC*) mutant cells had flagella, and no broken flagella were present (Fig. 3B). Most of the K724 (*fliM*) cells were aflagellate as well (Fig. 3C), although abnormal truncated or branched flagella were present on fewer than 10% of the K724 cells, and

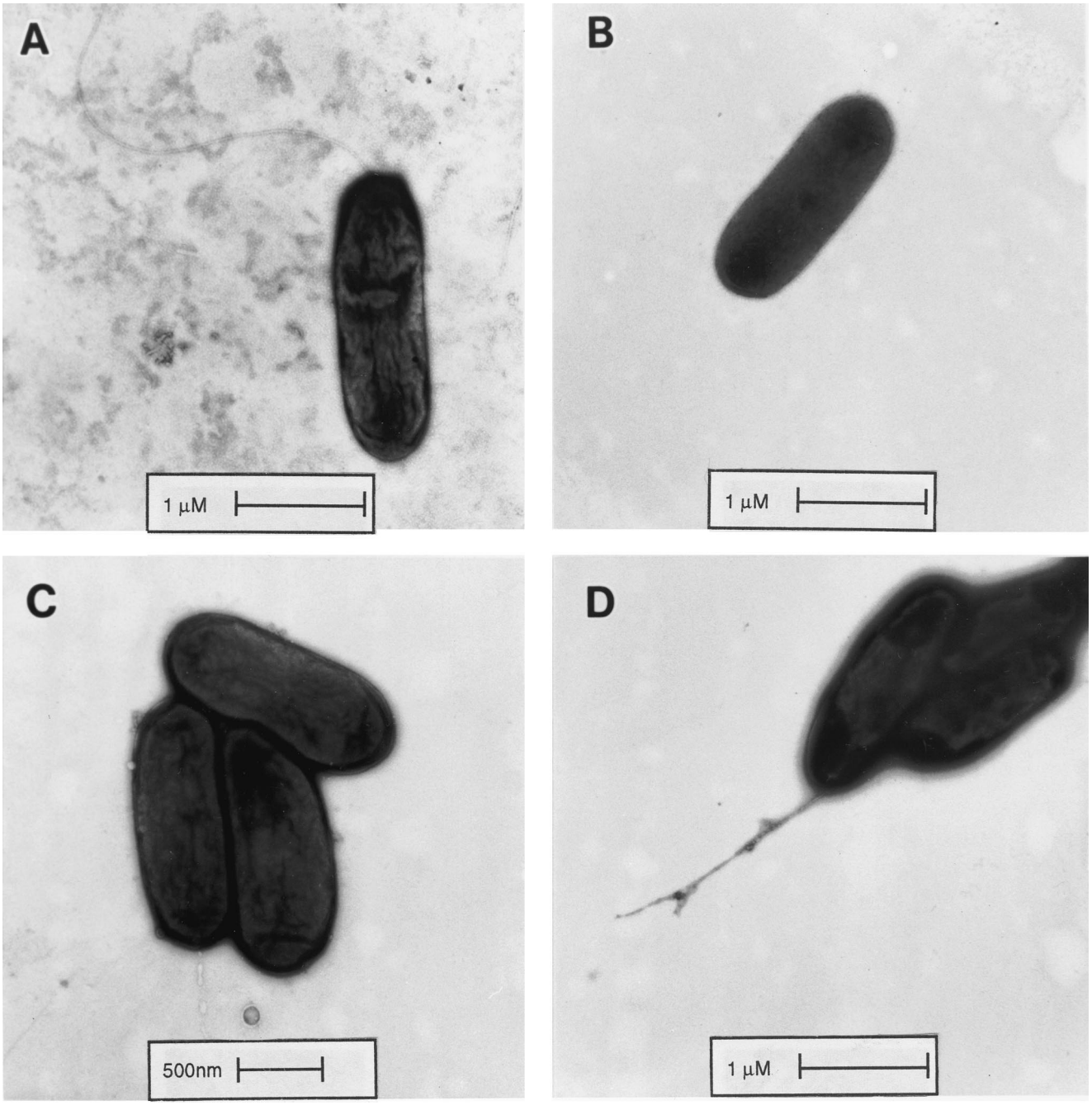

FIG. 3. Morphology of wild-type and nonmotile *R. solanacearum* cells. Cells were grown to 10^7 CFU/ml, stained with 1% uranyl acetate, and examined by electron microscopy. (A) Wild-type strain K60; (B) K701 (*fliC*) aflagellate cells; (C) K724 (*fliM*) aflagellate cells; (D) K724 cells showing abnormal truncated or branched flagella.

a few broken flagella were seen in the preparations (Fig. 3D). No flagella on K724 had the long, curved appearance of the wild-type K60 flagella. Nonmotile strain K71 (*pehR*::*gus*), which lacks the *pehR* response regulator that positively controls motility, had no flagellate cells (data not shown).

Expression and isolation of flagellin. Flagellin is usually present in sheared-cell extracts, the fraction of outer cell-associated protein that can be removed only by high-speed vortexing or blending. Wild-type strain K60 sheared-cell extracts contained an abundant band that closely corresponds to the predicted molecular mass of *R. solanacearum* flagellin (30 kDa); however, this band was completely absent from sheared-cell extracts of the *fliC* mutant K701 (Fig. 4, lanes 5 and 6). When the cloned *fliC* gene was overexpressed in *E. coli*, a 30-kDa protein band was induced which comigrated with the apparent flagellin from the *R. solanacearum* sheared-cell extracts (Fig. 4, lane 4).

Virulence of nonmotile mutants. To determine whether the ability to move contributes to invasive bacterial wilt virulence on intact host plants, we inoculated tomatoes with the nonmo-

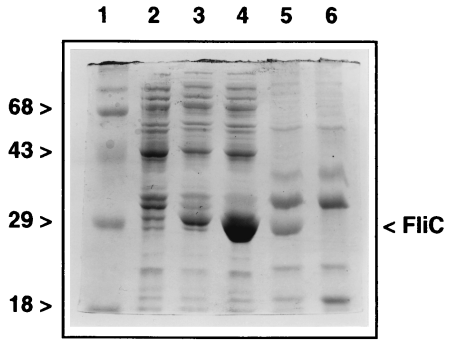

FIG. 4. Visualization of flagellin from *R. solanacearum* and its overexpression in *E. coli*. Crude flagellin was isolated as sheared-cell extracts. Protein expression was induced in mid-log-phase *E. coli* BL21(DE3) with 2 mM IPTG. Proteins were separated by SDS-PAGE on 12% gels and stained with Coomassie brilliant blue. Lane 1, molecular weight markers (positions are indicated in kilodaltons); lane 2, *E. coli* BL21(DE3)(pET29b) induced for 1 h; lane 3, *E. coli* BL21(DE3)(pFliC9) uninduced; lane 4, *E. coli* BL21(DE3)(pFliC9) induced for 1 h; lane 5, wild-type strain K60 sheared cell extract; lane 6, K701 (*fliC*) sheared-cell extract.

tile mutants using a natural soil-soak inoculation. Under these conditions, wild-type strain K60 completely wilted nearly all tomato plants by about 8 days after inoculation, with an average disease index of 3.8 (Fig. 5A). Both K701 (*fliC*) and K724 (*fliM*) were significantly reduced in virulence, with an average disease index of around 2.4 by the end of the assay. Consistent with previous observations, K71 (*pehR*), a nonmotile pleiotropic regulatory mutant, had the lowest virulence, with an average disease index of only 1.2 by day 14 postinoculation. Repeated-measures ANOVA demonstrated that the virulence of nonmotile strains K701 (*fliC*) and K724 (*fliM*) was significantly different from that of wild-type strain K60 and from that of K71 (*pehR*) ($P < 0.001$), but K701 and K724 did not differ significantly from each other by this test.

To determine whether a nonmotile mutant could cause disease normally if it did not have to invade and colonize from the soil, we inoculated bacteria directly into the host vascular system via the cut leaf petioles of tomato plants. When 10,000 bacteria were applied to each plant, wild-type strain K60 and K701 (*fliC*) both wilted all tomatoes by day 10 postinoculation, and K701 was statistically indistinguishable from the wild type. However, K71 (*pehR*) differed significantly from either K60 or K701 ($P < 0.001$); K71 wilted only a few of the plants, reaching a final disease index of below 2.0 (Fig. 5B). At lower inoculum levels (1,000 and 100 cells), K60 (wild type) and K701 (*fliC*) remained indistinguishable from each other in virulence. Maximum disease indices for both strains were 3.8 at 10^3 CFU/plant and 2.5 at 10^2 CFU/plant, where about 35% of the inoculated plants remained asymptomatic. *pehR* regulatory mutant K71 did not cause any detectable disease symptoms at an inoculation level of either 10^3 or 10^2 CFU/plant (data not shown).

DISCUSSION

The expression pattern of *R. solanacearum* virulence genes in culture is well understood, but less is known about how these genes are regulated in the host plant. As bacterial population density increases during growth in broth, the global virulence regulator PhcA induces expression of EPS and endoglucanase while concomitantly reducing polygalacturonase expression

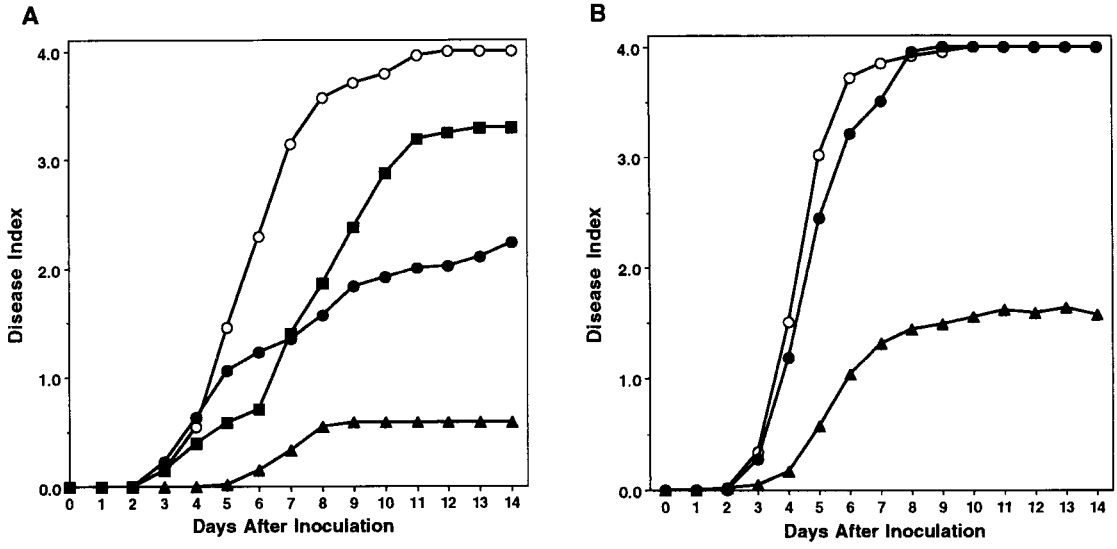

FIG. 5. Virulence of *R. solanacearum* nonmotile mutants on tomato plants by different inoculation methods. Fifteen-day-old tomato plants were inoculated either by soaking the soil to a final bacterial population of about 6.25×10^6 CFU/g (A) or by applying 10^4 bacteria directly to the cut surface of the first true leaf petiole (B). Plants were rated daily on a disease index scale of 0 to 4; each point represents the mean disease index of 48 plants combined from three separate experiments. (A) Closed triangles, K71 (*pehR*); closed circles, K701 (*fliC*); open circles, K60 (wild type); closed squares, K724 (*fliM*); (B) closed triangles, K71; closed circles, K701; open circles, K60. Repeated-measure ANOVA found that for panel A, the virulence of nonmotile mutants K701 and K724 was significantly different from that of wild-type strain K60 and from that of regulatory mutant K71 ($P < 0.001$), but the virulence of K701 and K724 was not significantly different. In panel B, the virulence of wild-type and nonmotile strains was not significantly different, though both differed from that of K71 ($P < 0.001$).

and motility by repressing *pehSR*. In planta studies found that EPS is regulated similarly in tomato plants and in culture (34, 40). However, when we quantified motility of bacteria from the xylem vessels of infected tomato plants, we found that this bacterium was completely nonmotile at low to moderate cell densities in the plant host, becoming only rarely motile (fewer than 5% of cells) around 10^9 CFU/ml. We note that free bacteria floating in the xylem fluid represent only part of the population in planta; bacteria aggregated in biofilms on vessel walls would not be measured in our assay. However, free bacteria are more likely to be motile than those in biofilms, suggesting that our numbers may actually overrepresent the overall motility of *R. solanacearum* living in xylem vessels (18).

This result highlights the importance of in planta studies and significantly alters the existing regulatory model. Because *pehSR* expression is similar in culture and in planta (D. Brown and C. Allen, unpublished results), we suspect the existence of additional regulatory elements intervening between *pehSR* and the expression of motility. Such elements may respond to conditions inside the plant or to a specific plant signal (which may trigger phosphorylation of the PehR response regulator by the PehS sensor-kinase). Given the complex networks that regulate motility in other systems, additional levels of regulation would not be surprising. Many mammalian pathogens are motile in the external environment or in culture but quickly shed their flagella and become nonmotile once they are inside a host. For example, *Bordetella pertussis* and *B. brochiseptica* are motile at temperatures below 37°C, corresponding to conditions outside the host, but at 37°C or above, they become nonmotile and simultaneously produce an array of toxins and other virulence factors (2, 39). Indeed, constitutively motile *B. bronchiseptica* mutants were avirulent (1), suggesting that motility within the host can be actively disadvantageous to the pathogen.

To separate the effects of the flagellum itself from those of bacterial movement, we attempted to create a paralyzed strain of *R. solanacearum* (with intact but nonfunctional flagella) by mutating the flagellar motor switch protein gene (*fliM*). However, most cells of the nonmotile *fliM*::*aphA* mutant K724 were aflagellate rather than paralyzed, though a few cells had truncated or branched flagella. The presence of these occasional abnormal flagella in K724 suggests that the *aphA* cassette did not have a polar effect on downstream genes, since loss of the entire *fliL* operon generally creates a completely aflagellate phenotype (37).

To determine the importance of pathogen motility in bacterial wilt disease development, we assayed the virulence of nonmotile mutants K701 (*fliC*) and K724 (*fliM*) on tomato plants, using a biologically representative soil-soak inoculation that required bacteria to actively find and invade host plant roots from the soil. Under these conditions, the nonmotile mutants caused significantly less disease on tomato plants than the wild-type strain, demonstrating that pathogen motility is necessary for full virulence in *R. solanacearum*.

Interestingly, the low-virulence phenotype of nonmotile mutant K701 could be rescued by inoculating the strain directly into the tomato stem vascular system. When bacteria were applied to cut leaf petioles, the nonmotile strain was indistinguishable in virulence from the wild-type strain. Indeed, in this assay the nonmotile strain had wild-type virulence even at very low inoculum densities (100 cells per plant). The finding that motility does not appear to be required once the pathogen reaches the stem vasculature is consistent with our observation that wild-type bacteria do not express motility in xylem fluid from the stem. On a biological level, this result demonstrates that flagella make their contribution to virulence before bacteria reach the stem, in the early stages of disease development. Several animal pathogens also depend on motility primarily to invade and colonize hosts. Nonmotile *P. aeruginosa* cells are much less able to invade mice from burn wounds, and a nonmotile mutant of the fish pathogen *Vibrio anguillarum* is virtually avirulent in an immersion assay; however, both strains are fully virulent when they are injected directly into the host peritoneal cavity (20, 43).

How might motility contribute to *R. solanacearum* virulence early in pathogenesis? Swimming motility is obviously necessary for chemotaxis through soil to optimal infection sites on host roots, and from there through the cortex to developing protoxylem tissue. As disease progresses, motility can help the pathogen spread out of infected xylem vessels into adjacent uninfected vessels and xylem parenchyma cells. Flagella may also help bacteria attach to host cells (44, 46); if this is the case, there should be a difference in the ability of wild-type, paralyzed, and aflagellate cells to attach to tomato root surfaces. Finally, swimming motility is usually required for biofilm formation (45, 55). Microscopic observations suggest that *R. solanacearum* forms biofilms on host xylem vessel walls; these specialized aggregates likely protect bacteria from host defenses and may also contribute to bacterial survival during latent infections and saprophytic life.

The flagellar complex may also play a more direct role in bacterial wilt virulence. Several proteins that assemble flagella are evolutionarily related to type III secretion systems, which inject bacterial virulence factors into plant or animal cells; in *Yersinia enterocolitica*, the flagellar assembly apparatus itself secretes a key virulence factor, phospholipase (24, 61). At this point we cannot rule out the possibility that the *R. solanacearum* flagellar apparatus also transports virulence factors.

The flagellum itself can be highly antigenic in mammals, since it is abundant, exposed on the bacterial cell surface, and capable of eliciting a strong host immune response (16, 52). Although plants lack immune systems, there is some evidence that they can recognize and respond to flagellins. Flagellin from an incompatible strain (but not from a compatible strain) of *Pseudomonas avenae* induces a resistance response in cultured rice cells (14). Synthetic 15-mer oligopeptides derived from the conserved N terminus of *P. aeruginosa* flagellin elicit defense responses in tomato cell lines and *Arabidopsis* (21). Interestingly, the corresponding 15 amino acids are less conserved (50% identity) in the plant-associated species *A. tumefaciens* and *Rhizobium meliloti*, possibly so that these bacteria do not trigger host defenses (21). We note that *R. solanacearum* flagellin has only 60% identity to this conserved region, suggesting that this protein may have evolved to avoid host plant recognition. Alternatively, if host plants can recognize *R. solanacearum* flagellin, avoiding host recognition of flagellin may be one reason why this species loses motility once it is inside a plant host. To distinguish between these two possibilities, we need to determine if purified wild-type flagellin or the isolated 15-mer flagellin oligopeptide can elicit defense re-

sponses in tomato and other plant species. Arguing against this possibility is our observation that aflagellate strain K701 bacteria still induced a wild-type HR on the nonhost plant cucumber. However, this result is not conclusive because many factors contribute to HR induction by *R. solanacearum*, including several proteins injected by the *hrp* secretion system (26). Thus, even if flagellin is recognized by the host plant, it is not likely to act alone.

We have shown that *R. solanacearum* needs motility to effectively invade and colonize host plants. Many questions still remain about the process and function of bacterial movement in bacterial wilt disease development. A paralyzed mutant should allow us to separate the effects of bacterial motility from those of the flagellum itself, such as attachment and stimulation of host defenses. We found that *R. solanacearum* is effectively nonmotile in host xylem vessels, suggesting the existence of a regulatory structure that responds to a plant signal. Reporter gene analyses should help define the regulation of motility in this species.

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