Swimming Motility, a Virulence Trait of *Ralstonia solanacearum*, Is Regulated by FlhDC and the Plant Host Environment

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Swimming motility allows the bacterial wilt pathogen *Ralstonia solanacearum* to efficiently invade and colonize host plants. However, the bacteria are essentially nonmotile once inside plant xylem vessels. To determine how and when motility genes are expressed, we cloned and mutated *flhDC*, which encodes a major regulator of flagellar biosynthesis and bacterial motility. An *flhDC* mutant was nonmotile and less virulent than its wild-type parent on both tomato and *Arabidopsis*; on *Arabidopsis*, the *flhDC* mutant also was less virulent than a nonmotile *fliC* flagellin mutant. Genes in the *R. solanacearum* motility regulon had strikingly different expression patterns in culture and in the plant. In culture, as expected, *flhDC* expression depended on PehSR, a regulator of early virulence factors; and, in turn, FlhDC was required for *fliC* (flagellin) expression. However, when bacteria grew in tomato plants, *flhDC* was expressed in both wild-type and *pehR* mutant backgrounds, although PehSR is necessary for motility both in culture and in planta. Both *flhDC* and *pehSR* were significantly induced in planta relative to expression levels in culture. Unexpectedly, the *fliC* gene was expressed in planta at cell densities where motile bacteria were not observed, as well as in a nonmotile *flhDC* mutant. Thus, expression of *flhDC* and flagellin itself are uncoupled from bacterial motility in the host environment, indicating that additional signals and regulatory circuits repress motility during plant pathogenesis.

The soilborne bacterium *Ralstonia solanacearum* causes bacterial wilt, a widespread and economically destructive plant disease. Bacterial wilt disease is responsible for serious losses, attacking over 50 plant families, including such major crops as tomato, potato, and banana (Hayward 1991). The bacterium generally invades its host through wounds in the roots, colonizes the xylem vessels, and spreads rapidly up the stem and through the plant, ultimately reaching cell densities as high as 10^9 CFU/g of stem. Once wilt symptoms develop, plants usually die quickly and the bacteria are released into the soil. The pathogen is common in tropical and subtropical regions worldwide; however, cold-tolerant strains of the bacterium recently became established in Northern Europe, demonstrating the versatile and aggressive nature of this pathogen (Janse 1996). Although *R. solanacearum* has been studied since its discovery over 100 years ago, we still do not fully understand how it causes disease or interacts with its host plants. Recently, the genome of a

Race 1 strain, GMI1000, has been sequenced, opening new avenues for exploring the biology of this complex and deadly bacterium (Salanoubat et al. 2002).

Both twitching motility mediated by type IV pili and swimming motility mediated by flagella are required for full virulence in *R. solanacearum* (Liu et al. 2001; Tans-Kersten et al. 2001). However, injecting an aflagellate *fliC* (flagellin) mutant directly into host stems can rescue its wild-type virulence phenotype, suggesting that swimming motility makes its contribution to virulence early in disease development, perhaps enabling the pathogen to move toward the host rhizosphere, attach to host roots, and invade root tissue (Tans-Kersten et al. 2001). Motility also is an important virulence factor in several animal–host systems where it mediates adherence, invasion, or colonization (Giron et al. 2002; Ottemann and Lowenthal 2002; Tomich et al. 2002; Young et al. 2000). Moreover, biofilm formation mediated by motility or the flagella themselves is an integral part of certain infection processes (O’Toole and Kolter 1998; Pratt and Kolter 1998). The flagellar apparatus, with components similar to the type III secretion system, also can export such virulence factors as phospholipase (Hueck 1998; Young et al. 1999). In addition, bacterial flagellins can specifically trigger antimicrobial defense responses in both plant and animal systems (Felix et al. 1999; Gerwitz et al. 2001; Wyant et al. 1999). An oligopeptide derived from the most conserved region of the *Pseudomonas aeruginosa* flagellin protein sequence elicits a strong defense response in tomato cells (Felix et al. 1999; Pfund et al. 2004).

Flagellar biosynthesis and function involve over 40 genes in gram-negative bacteria. Generally, motility genes are regulated transcriptionally by a three-tiered cascade (MacNab 1996). At the apex of the hierarchy is FlhDC, a heterotetrameric regulatory protein that activates expression of class II flagellar genes encoding the hook and basal body complex, the flagellar sigma factor σ^{28} (*fliA*), and an anti-sigma factor (*flgM*). Once the hook and basal body complex are fully assembled, *flgM* is secreted out of the cell by the flagellum-specific export system, allowing class III genes to be expressed. Class III genes are involved in chemotaxis, flagellar rotation, and synthesis of the filament (including *fliC*) (MacNab 1996). FlhDC itself responds to many cell-signaling systems, including the cyclic AMP-catabolite activator protein (CAP) complex and the histone-like nucleoid structuring (H-NS) protein (Soutourina et al. 1999) and is both transcriptionally and postranscriptionally regulated (Claret and Hughes 2000). Beyond its role in motility, FlhDC also is known to affect cell septation, cell division, and anaerobic respiration, and it has alternate functions during stationary phase (Pruß and Matsumura 1996; Pruß et al. 2001, 2003).

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R. solanacearum owes its pathogenic versatility to an array of virulence factors controlled by complex regulatory cascades that respond to environmental conditions, the presence of host cells, and bacterial density. In addition to motility, these virulence factors include production of extracellular polysaccharide (EPS), a set of effector proteins secreted through the type III secretion system, and a consortium of plant cell wall-degrading enzymes (Genin and Boucher 2002). The LysR-type global regulator PhcA controls expression of several virulence factors in response to cell density by a quorum sensing mechanism. At high cell density, accumulation of the autoinducer 3-hydroxy palmitic acid methyl ester activates expression of PhcA, which in turn induces production of late disease virulence factors such as EPS and endoglucanase; PhcA also represses expression of the two-component regulator PehSR (Schell 2000). A *phcA* mutant is nonmucoid and nearly avirulent, but is motile and overproduces cell wall-degrading polygalacturonases (Brumbley and Denny 1990). At low cell densities in culture, presumably corresponding to saprophytic life and early plant colonization, PhcA is not expressed, allowing PehSR to activate expression of the early disease virulence factors, including polygalacturonases (PehA, PehB, and PehC) and both twitching and swimming motility (Schell 2000). *pehSR* mutants are severely attenuated in virulence, produce decreased polygalacturonase activity, and are nonmotile (Allen et al. 1997). In culture, approximately 65% of wild-type *R. solanacearum* cells are swimming at peak motility, which occurs between 10^7 and 10^8 CFU/ml, consistent with a cell density-responsive regulation of motility via PhcA and PehSR. However, bacteria growing in the xylem of infected tomato plants are essentially nonmotile until they reach densities of 10^9 CFU/ml, when approximately 5% of cells are motile (Tans-Kersten et al. 2001). This unexpected result suggests that

motility is regulated differently in culture than during growth in the host.

To better understand the regulation and role of motility in *R. solanacearum*, we created a nonmotile *flhDC* regulatory mutant and compared its virulence with that of an aflagellate *fliC* mutant. Using reporter gene constructs, we tracked the expression of class I (*flhDC*) and class III (*fliC*) flagellar genes over a range of cell densities in culture and in tomato plants, and in various regulatory mutant backgrounds. Because *R. solanacearum* is highly motile at mid-log phase in culture, but is rarely motile in host xylem tissue, we were particularly interested in profiling expression of motility genes (*flhDC* and *fliC*) in the plant and the possible effects of host signals on this regulon. PehSR positively regulates motility in *R. solanacearum*; therefore, we also monitored expression of this regulator at different cell densities in culture and in the plant.

RESULTS

Cloning, mutation, and analysis of the *flhDC* locus.

A polymerase chain reaction (PCR) product containing *flhDC* from *R. solanacearum* strain GMI1000 hybridized to six cosmids from an *R. solanacearum* strain K60 chromosomal library. Cosmids 15-2 and 2-89 both contained *flhD*, but only cosmid 2-89 contained the entire *flhDC* locus and upstream (5') region. Two fragments containing the *flhDC* locus were subcloned and named pflhDC1 and pflhDC4 (Table 1).

Sequence analysis of the K60 *flhD* locus revealed that it is 98% identical at the DNA and protein level to its homologue in the GMI1000 genome, and that gene order is conserved between the strains. In strain K60, *flhD* and *flhC* are small genes (314 bp and 105 amino acids [aa] and 561 bp and 186 aa, respectively) transcribed in the same direction, with 78 bp

Table 1. Bacterial strains and plasmids

Designation	Relevant characteristics ^a	Reference or source
<i>Escherichia coli</i>		
DH5 α	F- <i>endA1 relA</i> ϕ 80 <i>lacZAM15 hsdR17 supE44 thi-1 recA1 gyrA96</i>	Hanahan 1983
<i>Ralstonia solanacearum</i>		
K60	Wild-type, race 1 biovar 1 tomato isolate	Kelman 1954
GMI1000	Wild-type race 1 biovar 4 tomato isolate	Boucher et al. 1985
K71	K60 <i>pehR::Tn3-uidA</i> , Km ^r	Allen et al. 1997
K701	K60 <i>fliC::Gm</i> ^r	Tans-Kersten et al. 2001
K702	K60 <i>flhDC::Gm</i> ^r	This study
K60- <i>phcA</i>	K60 <i>phcA::Ω</i> , Sm ^r	Allen et al. 1991
KB5	K60 <i>pehR::Tn5</i> Km ^r	Allen et al. 1997
K60 <i>fliC::gus</i>	pfliCGUS1 integrated into K60 chromosome; cis-merodiploid	This study
K702 <i>fliC::gus</i>	pfliCGUS1 integrated into K702 chromosome; cis-merodiploid	This study
Plasmids		
pBluescript (pBS SK+)	Ap ^r	Stratagene
pLAFR3	Tc ^r replicates in <i>R. solanacearum</i>	Staskawicz et al. 1986
pSTBlue-1	A-T cloning vector, Km ^r , Amp ^r	Novagen, Inc.
pUCGM	Gentamycin resistance cassette (<i>aacCI</i>), Gm ^r	Schweizer 1993
pRTL2-NIa/GUS	Plasmid containing 1,900-bp promoterless <i>gus</i> open reading frame, Ap ^r	Jefferson 1987
15-2	pLAFR3 cosmid containing <i>flhD</i> , Tc ^r	This study
2-89	pLAFR3 cosmid containing <i>flhDC</i> , Tc ^r	This study
pflhDC1	4.0-kb <i>EcoRI</i> fragment containing <i>flhDC</i> in pBS	This study
pflhDC2	1.1-kb <i>ClaI-EcoRI</i> fragment containing <i>flhDC</i> in pBS	This study
pflhDC3	850-bp Gm ^r cassette in 96-bp <i>BalI</i> deletion of <i>flhD</i> in pflhDC2	This study
pflhDC4	4.7-kb <i>ClaI</i> fragment containing <i>flhDC</i> in pBS	This study
pflhDC5	1,300-bp PCR fragment containing <i>flhDC</i> and promoter in pSTBlue-1	This study
pflhDC6	1,300-bp <i>BamHI-HindIII</i> fragment from pflhDC5 in pLAFR3	This study
pflhDCpro	372-bp <i>flhDC</i> promoter in <i>EcoRV</i> site of pSTBlue-1	This study
pflhDCGUS1	1,900-bp <i>uidA (gus)</i> fragment in <i>BamHI</i> site of pflhDCpro	This study
pflhDCGUS2	2,300-bp <i>BamHI-HindIII</i> fragment from pflhDCGUS1 in pLAFR3	This study
pLAFR3GUS	1,900-bp Promoterless <i>gus</i> fragment in <i>BamHI</i> site of pLARF3	This study
pfliCpro	400-bp <i>fliC</i> promoter in <i>EcoRV</i> site of pSTBlue-1	This study
pfliCGUS1	1,900-bp <i>uidA (gus)</i> fragment in <i>BamHI</i> site of pfliCpro	This study
pfliCGUS2	2,400-bp <i>BamHI-HindIII</i> fragment from pfliCGUS1 in pLAFR3	This study

^a Ap = ampicillin, Tc = tetracycline, Nal = naladixic acid, Gm = gentamycin, Km = kanamycin, Sm = streptomycin.

between the two open reading frames (ORFs). In strain GMI1000, the *flhDC* locus is located on the megaplasmid in a region containing chemotaxis and other motility-related genes. *motA* is located 456 bp downstream (3') of *flhC*, followed by *motB*, *cheY*, *cheA*, and *cheW* (Salanoubat et al. 2002). There are no significant ORFs within 755 bp upstream (5') of *flhDC* (Fig. 1). In strain GMI1000, *flhDC* is located over 1 Mb from a cluster containing *fliC* and other class II and class III genes in the motility regulon.

To create an *flhD* mutant, we inserted a gentamycin resistance (*aacCI*) gene cassette into a 96-bp *BalI* deletion of the subcloned *flhD* gene and homologously recombined this mutagenesis construct into the wild-type strain K60 chromosome. Because FlhD and FlhC form a heterotetrameric protein, mutations in *flhD* would likely affect the entire protein structure and function; thus, an *flhD* mutant is essentially an *flhDC* double mutant. The resulting mutant was named K702 (Fig. 1).

flhDC mutant K702 is nonmotile.

flhDC mutant cells were completely nonmotile on semisolid agar motility plates. Strain K702 cells also were nonmotile when examined under the light microscope after growth in minimal broth or harvested from the xylem of infected tomato plants. Growth of K702 was indistinguishable from that of its wild-type parent in minimal medium and in tobacco leaves (data not shown). K702 had normal colony morphology and normal cellular morphology when viewed under a light microscope, and its cells did not form chains or clumps, indicating there was no obvious defect in the cell division process.

The cloned *flhDC* locus complemented the motility phenotype of K702.

Both cosmid 2-89 and cosmid pflhDC6, which contains only the *flhDC* locus and promoter, restored motility to nonmotile *flhDC* strain K702 when present in trans, indicating that the motility defect of K702 resulted from disruption of the *flhDC* locus. However, for reasons we do not understand, pflhDC6 significantly reduced the fitness of any strain that carried it. Wild-type and *flhDC* mutant strains carrying pflhDC6 did not grow as well as strains carrying the empty pLAFR3 vector (data not shown). Similarly, on semisolid mo-

tility plates, the motility halo of K702(pflhDC6) and K60(pflhDC6) was not as large as that of wild-type strain K60 carrying pLAFR3 (data not shown). Additionally, though pflhDC6 could not restore wild-type virulence to strain K702 in tomato soil soak assays, wild-type strain K60 carrying pflhDC6 also was reduced similarly in virulence compared with K60(pLAFR3) (data not shown). This growth and virulence defect may reflect deleterious effects and fitness costs of carrying multiple copies of a pleiotropic regulator.

Mutation of *flhDC* reduced *R. solanacearum* virulence.

We used two different assays to measure the virulence of the *flhDC* mutant in tomato plants. First, bacterial strains were assayed by a soil soak inoculation method that mimics natural conditions, where bacteria in the soil must find roots, attach, infect, and colonize (Tans-Kersten et al. 2001). In this assay, by 14 days post inoculation, wild-type K60 induced a disease index of nearly 4.0 (100% wilted or dead), whereas strains K701(*fliC*) and K702(*flhDC*) caused a significantly lower disease index of only 2.5 ($P < 0.001$) (Fig. 2A). Strains K701 and K702 were not significantly different from each other. A petiole inoculation, which bypasses the normal infection court and introduces bacteria directly into the vascular system, rescued the virulence phenotypes of both motility mutants, so neither K701 nor K702 were different from their wild-type parent (Fig. 2B). As previously observed (Allen et al. 1997), a *pehR* pleiotropic regulatory mutant K71(*pehR*) was severely reduced in virulence in both assays, with a disease index of less than 1.0.

To measure the virulence of nonmotile mutants on wounded *Arabidopsis* Col-0 plants, we also soil-soak inoculated this very different host. Both wild-type strain K60 and K701(*fliC*) reached an average disease index greater than 2.7 and were not significantly different from each other (Fig. 2C). In contrast to results in the tomato assay, K702(*flhDC*) was slightly but significantly less virulent than both wild-type and K701 on *Arabidopsis*, with a final disease index of 2.2 ($P < 0.001$).

pehR expression decreases as disease develops.

To better understand the expression of virulence regulator *pehR* under various conditions, we measured β -glucuronidase (GUS) activity produced during bacterial growth in minimal medium and in tomato plants by strain K71, which carries a chromosomal *pehR::Tn3-gus* insertion. In culture, *pehR::gus* expression ranged from 1×10^{-8} to 4×10^{-7} pmoles of 4-methylumbelliferone (4MU) produced per minute per cell (PMPC). Consistent with the negative regulation of *pehR* in culture by the cell density-responsive global regulator PhcA, *pehR::gus* expression in minimal medium showed a slight downward trend when graphed versus cell density (CFU/ml) (Fig. 3). In contrast, when K71 cells were harvested from stem tissue samples, expression levels were up to 1,000 times higher than expression in culture, peaking at an estimated bacterial cell density in xylem fluid of less than 1×10^6 CFU/ml. In planta *pehR::gus* expression data showed a strong downward linear trend as disease progressed, decreasing 10,000-fold on a per-cell basis as cell density increased. This suggests that, in the plant, *pehR* expression is even more responsive to cell numbers or to changes in the host environment associated with disease development.

flhDC expression in minimal medium peaks in early log and in stationary phase.

We measured *flhDC* expression under various conditions using pflhDCGUS2, which contains the *flhDC* promoter driving expression of GUS on pLAFR3, a plasmid that is maintained in *R. solanacearum* at three to five copies per cell (Staskawicz et al. 1986). Wild-type K60 harboring either empty pLAFR3

Fig. 1. Cloning and mutagenesis of the *flhDC* region. The *flhDC* region in *Ralstonia solanacearum* strain GMI1000 is shown, together with downstream (3') chemotaxis genes. The 850-bp gentamycin resistance cassette was cloned into a *BalI* deletion within *flhD* and inserted into the chromosome of wild-type K60 by homologous recombination to create K702. Arrows represent putative operons.

Fig. 2. Virulence of nonmotile strains on tomato and *Arabidopsis* spp. **A**, Soil-soak virulence assay in tomato cv. Bonny Best. Unwounded 15-day-old tomato plants were inoculated with a bacterial suspension to an inoculum density between 5.38×10^6 and 2.3×10^7 CFU/g of soil. Each point represents the mean of four assays containing 96 plants total. Open circles, K60 (wild-type); closed circles, K701 (*fliC*); open triangles, K702 (*flhDC*); closed triangles, K71 (*pehR*). **B**, Petiole inoculation virulence assay in tomato: 21-day-old tomato plants were inoculated by introducing between 418 and 1,732 cells directly into the cut petiole of the first true leaf. Each point represents the mean of three experiments containing 56 plants per treatment in total. Open circles, K60 (wild-type); closed circles, K701 (*fliC*); open triangles, K702 (*flhDC*); closed triangles, K71 (*pehR*). **C**, Soil-soak virulence assay in *Arabidopsis* ecotype Col-0. After their roots were wounded, 3- to 4-week-old *Arabidopsis* plants were inoculated with a bacterial suspension containing 1.8 to 9.6×10^9 CFU/plant. Each point represents the average of five assays, with a total of 72 to 84 plants per treatment. Open circles, K60 (wild-type); closed circles, K701 (*fliC*); open triangles, K702 (*flhDC*). Bars indicate standard error.

vector or pLAFR3GUS (containing a promoterless *gus* gene) produced no detectable GUS activity under any of the conditions we tested ($<1 \times 10^{-8}$ pmoles of 4MU produced PMPC). In the wild-type K60 background, *flhD::gus* activity varied with cell density, ranging from 1.33×10^{-6} to 3.94×10^{-5} pmoles of 4MU produced PMPC in minimal medium. Two expression peaks were seen, one at 5×10^6 CFU/ml and one in stationary phase at 1×10^9 CFU/ml (Fig. 4A, open circles).

PehR and PhcA regulators affect *flhDC* expression in culture.

In the *phcA* mutant background, *flhD::gus* activity ranged from 4.41×10^{-7} to 3.9×10^{-5} pmoles of 4MU produced PMPC, similar to or slightly lower than in the wild-type genetic background (Fig. 4A, crosses). *flhD::gus* had only one peak of activity in the *phcA* background at 5×10^8 CFU/ml; an expression peak seen at 5×10^6 CFU/ml in the wild-type profile was completely absent. There was no detectable *flhD::gus* activity ($<1 \times 10^{-8}$ pmoles of 4MU produced PMPC) in the *pehR* mutant background, indicating that, in culture, *pehR* is an essential positive regulator of *flhDC*, functioning upstream of *flhDC* in the motility regulatory cascade (Fig. 4A, solid squares).

***flhDC* expression increases in planta.**

To study *flhDC* expression in the plant, we harvested bacteria from stems of tomato plants inoculated with *R. solanacearum* carrying pflhDCGUS2. Wild-type *flhD::gus* expression in planta ranged from 2.59×10^{-5} to 4.15×10^{-3} pmoles of 4MU produced PMPC, up to 100-fold higher than wild-type expression levels in minimal media at corresponding cell densities. *flhD::gus* expression decreased linearly as cell density increased and then leveled off at stationary phase in the wild-type background (Fig. 4B, crosses). In the *phcA* mutant background, *flhD::gus* expression levels were similar to those observed in the wild-type background, decreasing from 3.6×10^{-4} to 2.04×10^{-5} pmoles of 4MU produced PMPC as cell densities increased. Because the PhcA mutant strain does not infect and

Fig. 3. *pehR::gus* reporter gene expression in planta and in culture. β -Glucuronidase (GUS) activity produced by the *pehR* mutant K71 (*pehR::Tn3-gus*) is expressed as pmoles of 4-methylumbelliferone (MU) produced per minute per cell (PMPC). Bacterial populations from plant samples were converted from CFU/g to estimated cell density in xylem fluid (CFU/ml) to allow direct comparison of activity levels. Solid squares, *pehR::gus* expression in minimal medium; open circles, in planta *pehR::gus* expression versus estimated xylem fluid cell density (CFU/ml).

colonize tomato plants well, we were unable to gather *flhD::gus* data at high cell densities ($>1 \times 10^9$ CFU/ml estimated cell density in xylem fluid) in this mutant background.

Unexpectedly, there was detectable *flhD::gus* expression in the *pehR* mutant background in planta, where expression levels decreased in a strictly linear fashion from 4.6×10^{-5} to 4.7×10^{-8} pmoles of 4MU produced PMPC as cell density increased. This result sharply contrasts with the expression pattern in minimal medium, where PehR was absolutely necessary for *flhDC* expression. In all genetic backgrounds studied, *flhDC* expression peaked at low bacterial numbers, approximately 1×10^7 CFU/ml estimated xylem fluid cell density (Fig. 4B).

To explore the correlation between *flhDC* expression and severity of disease in tomato plants, we graphed wild-type *flhD::gus* expression in planta versus disease index (Fig. 5). The highest *flhD::gus* expression level and the greatest vari-

ability in expression occurred just before tomato plants showed disease symptoms, when internal bacterial populations were high but the disease index was 0. Once early disease symptoms appeared (disease index = 1), *flhDC* expression levels decreased substantially, up to 40-fold, and remained lower and less variable until plant death.

FlhDC is a positive regulator of *flhC* expression in culture.

To discover how and when the *flhC* flagellin gene is expressed in *R. solanacearum*, we used the *pfliCGUS2* reporter construct in K60 (wild-type) and K702 (*flhDC*) genetic backgrounds. In culture, *flhC::gus* expression levels ranged from 9.45×10^{-8} to 7.08×10^{-6} pmoles of 4MU produced PMPC, with expression levels peaking at approximately 8×10^8 CFU/ml (Fig. 6A, open circles); this is consistent with previous results showing that bacterial motility peaks in culture at approximately 10^8 CFU/ml (Clough et al. 1997; Tans-Kersten et al. 2001). As expected, when the transcriptional activator of motility, FlhDC, was interrupted in strain K702, *flhC::gus* expression levels were at or below background levels ($<1 \times 10^{-8}$ pmoles of 4MU produced PMPC) in all samples.

flhC is expressed during growth in the host plant.

Interestingly, *flhC::gus* expression was detectable in planta, peaking at low cell densities ($<1 \times 10^7$ CFU/ml) and decreasing linearly from 4.38×10^{-6} to 1.23×10^{-7} pmoles of 4MU produced PMPC as cell density increased (Fig. 6B). This result was surprising because *R. solanacearum* cells are almost completely nonmotile in the plant, with less than 5% of cells motile at cell densities above 10^9 CFU/ml of xylem fluid (Tans-Kersten et al. 2001). An even more unexpected result was the finding that *flhC* is still expressed at wild-type levels in the K702 (*flhDC*) background in planta. Thus, the expression profile for *flhC* was completely different in planta than in culture. Further, *flhC* was expressed in planta when the majority of bacterial cells was nonmotile, and even when the master regulator of flagellar biosynthesis, FlhDC, was not functional.

Fig. 4. *flhDC::gus* expression in plants and in culture in various mutant backgrounds. β -Glucuronidase (GUS) activity is expressed as pmoles of 4-methylumbelliferone (MU) produced per minute per cell (PMPC). **A**, *flhD::gus* expression in minimal medium in wild-type and mutant backgrounds. Open circles, K60 (wild-type); crosses, K60-phcA (*phcA*); and solid square, KB5 (*pehR*). **B**, *flhD::gus* expression in planta; bacterial populations from plant samples were converted from CFU/g to estimated cell density in xylem fluid (CFU/ml) to allow direct comparison of activity levels. Open circles, K60 (wild-type); crosses, K60-phcA (*phcA*); and solid squares, KB5 (*pehR*).

Fig. 5. In planta *flhD::gus* expression in wild-type K60 background as a function of disease index. Inoculated tomato plants were rated on a 0-to-4 disease scale before plant tissue samples were harvested (2 to 7 days post inoculation) to measure *flhD::gus* activity. β -Glucuronidase (GUS) activity is expressed as pmoles of 4-methylumbelliferone (MU) produced per minute per cell (PMPC).

Confirmation of *fliC::gus* expression results using *cis*-merodiploid strains.

To confirm the accuracy of our *in trans* GUS reporter activity data, we constructed *cis*-merodiploid strains in which the *fliC* promoter::*gus* fusion was integrated into the chromosome of wild-type strain K60 (K60*fliC::gus*) or *flhDC* mutant K702 (K702*fliC::gus*). In these *cis*-merodiploids, the intact native *fliC* promoter drives expression of a single copy of the *gus* gene. Both in planta and in culture *fliC* expression profiles from the *cis*-merodiploids were indistinguishable from *in trans* reporter expression profiles deriving from expression of the p*fliC*GUS2 reporter plasmid construct (data not shown).

DISCUSSION

We describe cloning of the *flhDC* motility regulatory locus and analysis of an *R. solanacearum flhDC* mutant. This mutant, K702, grew like the wild-type in culture and in tobacco leaves but was completely nonmotile under all conditions tested. When tomato plants were inoculated using a soil-soak method, the *flhDC* mutant was significantly attenuated in virulence relative to wild-type strains. However, when K702 was inoculated directly into tomato vasculature through cut petioles, it caused wild-type levels of disease. This result suggests that motility plays a role early in disease development but is no longer needed once the pathogen is inside the host plant xylem, a finding congruent with previous studies using a nonmotile *fliC* mutant (Tans-Kersten et al. 2001).

flhDC mutant K702 was less virulent than flagellin mutant K701 in *Arabidopsis* soil-soak assays. This small but significant virulence difference between K701 and K702 was not detected in tomato plant assays. Why might an *flhDC* mutant be less virulent than a flagellin mutant? The *fliC* mutation affects only the flagellin locus, while *flhDC* affects expression of several regulatory pathways in *Escherichia coli* and over 40 genes in the flagellar biosynthesis pathway, including those encoding the flagellar basal body. The flagellar basal body functions as a protein secretion system in other bacteria, including *Yersinia enterocolitica*, where it translocates the virulence factor phospholipase (Young et al. 1999). If *R. solanacearum* uses its flagellar basal body for export of virulence factors, this function should be absent in an *flhDC* mutant. K702 (*flhDC*) had no obvious deficiencies in protein secretion when we used sodium dodecyl sulfate gel electrophoresis to compare culture supernatants and purified crude boiled extracts from nonmotile strains K701 and K702 to those of wild-type strains. While the 30-kDa flagellin band was not present in the nonmotile strains, there were no other visible differences among strains (data not shown), although biologically important protein bands might not be seen with this method. Our finding that K702 is less virulent than K701 suggests that the flagellar pore may secrete proteins that contribute to *R. solanacearum* virulence in *Arabidopsis* plants. It is possible that these factors are only produced or important in *Arabidopsis* plants. The difference between *Arabidopsis* and tomato also may be a consequence of relative wilt resistance in the two hosts. *Arabidopsis* ecotype Columbia is not especially susceptible to bacterial wilt; to obtain a moderate level of disease, plants must be wounded and treated with inoculum densities of 10^9 CFU/ml. In contrast, *R. solanacearum* kills 100% of the highly susceptible heirloom tomato cv. Bonny Best when inoculated at 10^7 CFU/g of soil with no wounding. The loss of virulence factors that make a subtle contribution may be detectable only in a more resistant tomato host (Chen et al. 2000).

A previously observed discordance between this pathogen's patterns of motility in culture and in planta led us to study the expression of genes in the *R. solanacearum* motility regulon.

Because *R. solanacearum* virulence regulons are known to respond to cell density or confinement through the global regulator PhcA, we graphed all expression data against bacterial cell density. Using a chromosomal *pehR::gus* insertion in strain K71, we studied expression of the two-component motility regulator, PehSR (Fig. 3). In culture, *pehR* was not strongly expressed and was only slightly responsive to cell density, with expression decreasing moderately as cell density increased. In planta, however, *pehR::gus* expression was as much as 1000-fold higher than in minimal media and declined linearly with increasing cell numbers. It is not surprising that *pehR* is highly expressed at low cell densities because it is a known regulator of early virulence genes, itself suppressed by the high-density responsive regulator PhcA (Allen et al. 1997). Because K71 is a *pehR* mutant, our results may not represent PehR expression in a wild-type background; however, our *in culture* expression data was in good agreement with regulatory

Fig. 6. *fliC* (flagellin) expression in wild-type and *flhDC* mutant backgrounds. β -Glucuronidase (GUS) activity is expressed as pmoles of 4-methylumbelliferone (MU) produced per minute per cell (PMPC). **A**, *fliC::gus* expression in minimal media versus cell density (CFU/ml). Open circles, K60 (wild-type); solid square, K702 (*flhDC*). **B**, *fliC::gus* expression in planta; bacterial populations from plant samples were converted from CFU/g to estimated cell density in xylem fluid (CFU/ml) to allow direct comparison of activity levels. Open circle, K60 (wild-type); solid square, K702 (*flhDC*).

model predictions. The significant difference between *pehR* expression in culture and in planta indicates that this regulator responds not only to bacterial density in the plant but also to signals or conditions present in the plant as well. Moreover, the *pehSR* promoter was identified as a plant-induced promoter in an *in vivo* expression technology (IVET) screen for *R. solanacearum* genes up-regulated during growth in tomato plants (Brown and Allen 2004). Because *pehR* is strongly expressed in the plant, it is possible that, in addition to motility, it regulates expression of yet unknown virulence genes that are expressed exclusively in the plant.

We used an *flhD::gus* reporter gene construct to study expression of FlhDC, considered the master regulator of motility, in wild-type and various regulatory mutant backgrounds both in culture and in planta (Fig. 4). In a wild-type background in minimal medium, *flhD::gus* activity peaked twice, one slight peak at 5×10^6 CFU/ml and again in stationary phase at 1×10^9 CFU/ml. This expression profile is not unexpected because *flhDC* controls stationary phase functions as well as motility in other bacteria (Pruß and Matsumura 1996). However, the expression profile for this locus was markedly different when *R. solanacearum* grew in tomato plants. FlhDC expression was 10- to 100-fold higher in the plant than in minimal medium. As bacterial populations and disease increased in the plant, *flhDC* expression decreased, then leveled off in stationary phase. In a *pehR* mutant background, there was no detectable *flhD::gus* expression in culture, indicating that *flhDC* is wholly dependent on *pehSR* for expression, as expected. In contrast, *pehSR* mutant bacteria harvested from the plant did have detectable levels of *flhD::gus* activity, albeit slightly lower than the wild-type background. Expression levels decreased linearly as cell density increased, paralleling the decrease in expression of *pehSR* itself in planta. Thus, although *pehSR* affects *flhDC* expression in the plant, it is not essential for expression.

The global regulator PhcA had little effect on *flhDC* expression in planta or in culture. Even though a *phcA* mutant overexpresses the PehR-regulated polygalacturonases (Brumbley and Denny 1990), and expression of *pehSR* increases 12-fold in a *phcA* mutant background (Allen et al. 1997), *flhDC* was not overexpressed in a *phcA* mutant background in culture. However, the *flhDC* expression peak was shifted from 5×10^6 to 5×10^8 CFU/ml, similar to the altered pattern observed for motility itself in a *phcA* background (Clough et al. 1997). In *E. coli*, FlhDC is tightly controlled at the post-transcriptional level by protein degradation (Claret and Hughes 2000) and by negative feedback inhibition (Furness et al. 1997); a similar mechanism may prevent *flhDC* overexpression in the *phcA* mutant background. In the plant, the *flhD::gus* expression pattern was identical in wild-type and *phcA* mutant backgrounds.

Together, these in planta *flhD::gus* expression results strongly suggest the existence of additional regulatory factors, possibly including plant signals, that control the expression of motility functions during bacterial growth in the host. In the plant, FlhDC cannot be considered the master regulator of motility in *R. solanacearum*. Interestingly, *flhDC* expression is highest and most variable in the plant before disease symptoms occur (Fig. 5). Perhaps during the early stages of infection, bacterial densities are relatively low in most areas of the plant, corresponding to high *flhDC* expression. A few densely colonized vessels and developing biofilms may account for variability. Later in disease, the pathogen may respond to increased bacterial population numbers, plant signals, or disease progress cues by decreasing expression of both the FlhDC and PehSR regulators.

We used an *fliC::gus* reporter construct to study expression of the flagellin gene, which is at the bottom of the motility

regulatory cascade in gram-negative bacteria (Fig. 6). In a wild-type background in minimal medium, *fliC::gus* expression levels responded to cell density and peaked around 1×10^8 CFU/ml. Maximum *fliC* expression occurred at a slightly higher cell density than the peak of motility itself, which is at approximately 5×10^7 CFU/ml in minimal media (Clough et al. 1997; Tans-Kersten et al. 2001). As expected, *fliC::gus* expression was undetectable in the *flhDC* mutant background in culture, indicating that FlhDC is an essential positive regulator of *fliC* expression under these conditions. Because *R. solanacearum* cells rarely are motile in the plant, we expected *fliC* expression to be severely reduced or undetectable in planta (Tans-Kersten et al. 2001). However, to our surprise, we did detect *fliC::gus* expression in the plant, decreasing linearly with increasing cell density. Thus, flagellin gene expression is decoupled from the behavior of motility during bacterial growth in the plant. The *fliC* transcriptional levels measured by our reporter construct may not correspond to actual flagellin synthesis or cell flagellation, because motility genes are subject to post-transcriptional regulation in other systems (Claret and Hughes 2000). Also surprisingly, in the plant, *fliC* still was expressed at wild-type levels in an *flhDC* mutant background. Thus, *fliC* is expressed in planta even in the absence of a major regulator of flagellar biosynthesis, possibly in response to a host signal or to another overriding positive regulator. In enteropathogenic *E. coli*, growth of nonflagellated regulatory mutants in preconditioned tissue culture media restored motility, suggesting that host signals can influence regulation of motility (Giron et al. 2002).

EPS production, a key virulence phenotype that is induced at high cell densities through a distinct regulatory cascade beginning with PhcA, is regulated similarly in culture and in planta (Schell 2000). However, we found that the PhcA-PehR-FlhDC regulatory pathway functioned very differently when *R. solanacearum* grew in a host plant rather than in culture (Table 2). Because most previous regulatory data were obtained from bacteria growing in culture, our regulatory model must be adjusted to incorporate these results. We found that PehR affects *flhDC* expression and motility in both minimal media and in planta, and is a positive regulator of the entire motility cascade. Plant host signals or conditions present in the plant significantly affected expression of two important virulence regulators, PehR and FlhDC. Curiously, although PhcA negatively regulates *pehSR* expression in culture (and PehSR is required for motility in culture and in planta), PhcA did not appear to play any role in regulation of *flhDC* during bacterial growth in the plant. Further, in the plant, the bacterium could bypass FlhDC regulation and express *fliC* when cells were predominantly nonmotile, even in the absence of *flhDC*, which was absolutely required for *fliC* expression in culture. Given that this regulon is largely positively regulated, it seems likely that additional regulators activate transcription of *fliC* in response to the plant environment. Further experiments are needed to identify putative regulators controlling expression of motility and *fliC* in the plant, and to identify signals that activate motility regulatory paths during pathogenesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions.

The bacterial strains and plasmids used in these experiments are listed in Table 1. *Ralstonia solanacearum* strains were grown at 28°C in CPG broth (Hendrick and Sequeira 1984) or on tetrazolium chloride plates containing CPG plus 1.8% (wt/vol) agar and 0.05% (wt/vol) 2,3,5-triphenyltetrazolium chloride (Kelman 1954). Boucher's minimal media (BMM) (Boucher et al. 1985) amended with 0.2% (wt/vol) glucose

was used when minimal medium was required. *E. coli* strains were cultured at 37°C in Luria-Bertani (LB) medium (Miller 1992). Antibiotics were added as required in the following concentrations: ampicillin (Ap) at 50 mg/liter, kanamycin (Km) at 25 mg/liter, tetracycline (Tc) at 15 mg/liter, streptomycin (Sm) at 30 mg/liter, and gentamycin (Gm) at 25 mg/liter. Growth rates of wild-type and mutant strains were compared in BMM amended with 0.2% (wt/vol) glucose and in tobacco leaf tissue (cv. Bottom Special) as previously described (Tans-Kersten et al. 2001).

DNA manipulations.

Isolation of plasmid and chromosomal DNA, restriction site analysis, cloning, and PCR were performed using standard methods (Ausubel et al. 1995). *R. solanacearum* and *E. coli* were transformed by electroporation as previously described (Allen et al. 1991). DNA sequencing and oligonucleotide synthesis were performed at the University of Wisconsin-Madison Biotechnology Center, Madison. DNA sequence was analyzed using the DNASTAR software package (DNASTAR, Inc., Madison, WI, U.S.A.).

Cloning and mutagenesis of *flhDC*.

We designed primers to amplify the *flhDC* operon (5' GCACGGTCTATCCCGTTTCGTT 3' and 5' CCCAGGAAACGGTAGATGTTCA 3') based on the published *R. solanacearum* GMI1000 sequence (Salanoubat et al. 2002). These primers amplified an approximately 1,000-bp product from strain GMI1000 genomic DNA, but not from strain K60 genomic DNA. We used the GMI1000 PCR product to probe a K60 genomic library (Allen et al. 1991) which identified six hybridizing cosmids. A 4.0-kb *EcoRI* fragment from cosmid 15-2 was subcloned into pBluescript SK+ (pBS SK+) to create pflhDC1 (Table 1). Sequencing revealed that this subclone contained the entire *flhD* gene and upstream region, but only part of *flhC*. A *ClaI* recircularization of pflhDC1 deleted a 2.9-kb upstream region, leaving a 1.1-kb insert containing *flhD*; this was named pflhDC2. A 4.7-kb *ClaI* fragment from cosmid 2-89 containing the entire *flhDC* operon was subcloned into pBS SK+ to create pflhDC4.

To create an *flhDC* mutant strain, the 850-bp gentamycin resistance gene cassette (*aacCI*) from pUCGM was cloned into a 96-bp *BalI* deletion within *flhD* in pflhDC2 to create pflhDC3. The *flhD*::Gm mutation was introduced into the K60 chromosome by homologous recombination as previously described (Allen et al. 1991) (Fig. 1). Transformants with gentamycin resistance and ampicillin sensitivity were screened for wild-type growth and loss of motility. Disruption of the *flhD* locus by the gentamycin resistance cassette was confirmed by PCR analysis of genomic DNA. The cassette insertion in *flhD*

caused polar inactivation of *flhC*; the resulting *flhDC* double mutant strain was named K702.

For complementation analysis, we PCR amplified the *flhDC* locus and 372-bp promoter region precisely from cosmid 2-89. The primers were 5' GCTTTA GCGCATGGTCTACC 3' and 5' TTCAGGCGTCGATCGTCTTG3'. The resulting 1,300-bp product was sequenced and A-T cloned into pSTBlue-1 to create pflhDC5. A *BamHI*-*HindIII* fragment was subcloned into pLAFR3 creating pflhDC6.

flhDC and *fliC* reporter gene constructs.

We used sequence information from pflhDC1 to design primers that amplified a 372-bp region of the *flhDC* promoter immediately 5' to the ATG start site (5' GTTGTCCCGTCGTGTGCAG 3' and 5' GCTTTAGCGCATGGTCTACC 3'). This promoter-containing PCR product was A-T cloned into pSTBlue-1 to create pflhDCpro; this construct was confirmed by sequencing. Next, the 1,900-bp *BglII*-*BamHI* fragment from pRTL2-Nia/GUS containing the promoterless *gus* gene was cloned downstream of the *flhDC* promoter to make pflhDCGUS1. The 2,200-bp *BamHI*-*HindIII* fragment from pflhDCGUS1 containing the *flhDC* promoter fused to *gus* was moved into the broad host-range plasmid pLAFR3, creating pflhDCGUS2. Restriction digests confirmed that the promoter-*gus* fusion fragment was in the opposite orientation relative to the pLAFR3 *lac* promoter. This pflhDCGUS2 reporter plasmid was electroporated into *R. solanacearum* strains K60 (wild-type), KB5 (*pehR*::Tn5), and K60-phcA (*phcA*::Sm). *R. solanacearum* strain K60 carrying plasmid pLAFR3GUS, which contains only the 1,900-bp promoterless *gus* gene in the opposite orientation from the vector *lac* promoter, served as a negative control for all GUS activity assays.

To make the *fliC*::*gus* reporter construct, we used *R. solanacearum* strain K60 *fliC* sequence data (GenBank accession number AF283285) (Tans-Kersten et al. 2001) to design primers that amplified a 419-bp region of the *fliC* promoter immediately 5' to the ATG start site (5' GGTTCGACGATGAG GACATTC 3' and 5' TGACGGCTCCTATATTCC 3'). As with the *flhDC* reporter construct (above), the PCR product was cloned into pSTBlue-1 (pflhCpro) and the promoterless *gus* fragment was inserted downstream (pflhCGUS1). The resulting 2,300-bp fragment containing the *fliC* promoter fused to *gus* was moved into pLAFR3 in the opposite orientation relative to the pLAFR3 *lac* promoter to create pflhCGUS2 and electroporated into strain K60 (wild-type) and K702 (*flhDC*).

Two additional *cis*-merodiploid strains expressing *gus* from the *fliC* promoter were created by incorporating the pflhCGUS1 plasmid into the chromosome of strains K60 (wild-type) and K702 (*flhDC*) by single homologous recombination. PflhCGUS1 DNA was electroporated into K60 (wt) and

Table 2. Summary of *flhDC* and *fliC* expression^a

Growth condition	Bacterial cell density ^b			
	In culture		In planta ^c	
	Low	High	Low	High
<i>flhDC</i> expression in wild-type background	Low to moderate	Moderate	Moderate to high	Moderate
<i>flhDC</i> expression in <i>phcA</i> mutant background	Low	Low to moderate	Moderate	Moderate
<i>flhDC</i> expression in <i>pehR</i> mutant background	None	None	Moderate	Low to very low
<i>fliC</i> expression in wild-type background	Low	Low	No data	Low
<i>fliC</i> expression in <i>flhDC</i> mutant background	None	None	Moderate	Low to very low
Bacterial motility ^d	0–30% motile	0–50% motile	0% motile	5% motile

^a Expression levels in pmoles of 4-methylumbelliferone produced per minute per cell. High = 1×10^{-3} to 1×10^{-2} , moderate = 1×10^{-5} to 1×10^{-3} , low = 1×10^{-7} to 1×10^{-5} , very low = 1×10^{-8} to 1×10^{-7} , and none = below background ($<1 \times 10^{-8}$).

^b Low = 1×10^5 to 5×10^7 CFU/ml and high = $>5 \times 10^8$ CFU/ml.

^c Estimated cell density in xylem fluid from infected tomato plants (CFU/ml).

^d Directly observed by light microscopy of cultures or xylem fluid from infected tomato plants (Tans-Kersten et al. 2001).

K702 (*flhDC*) as previously described (Allen et al. 1991) and genotypes of kanamycin-resistant transformants were confirmed by Southern hybridization. Strain K60/*fliC::gus* remained motile on semisolid motility media and K702/*fliC::gus* was nonmotile (data not shown).

Motility assays.

Motility of wild-type and mutant strains was assayed on semisolid motility media containing 1% (wt/vol) tryptone and 0.325% (wt/vol) noble agar (Boles and McCarter 2000). Plates were inoculated with a 2- μ l drop of bacterial culture containing 1×10^6 CFU/ml. Motility was visualized as a white halo of cells moving outward from the original inoculation site after 3 to 5 days of incubation at 28°C. We also observed bacterial motility from culture and xylem samples directly under the light microscope as previously described (Tans-Kersten et al. 2001).

Tomato virulence assays.

Soil-soak and petiole inoculation virulence assays were performed as previously described (Tans-Kersten et al. 2001), using the highly susceptible heirloom tomato cv. Bonny Best for all assays. Soil-soak results are the mean of four assays, each containing 16 plants for each treatment (96 plants total) with inoculum concentrations ranging from 5.38×10^6 to 2.3×10^7 CFU/gram of soil. Petiole assay results are the mean of three experiments containing 56 plants in total, with between 418 and 1,732 cells inoculated per plant. Inoculum concentrations were determined spectrophotometrically (absorbance at 600 nm [OD_{600}]) and confirmed by dilution plating. Plants were coded, inspected daily for wilt symptoms, and scored by a rater blind to treatment identity on a disease index of 0 to 4, where 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.

Arabidopsis virulence assays.

The virulence of *R. solanacearum* strains on *Arabidopsis* Col-0 (ecotype Columbia) plants was measured using a soil-soak inoculation method. *R. solanacearum* strains were grown overnight in CPG broth to mid-log phase. Cells were centrifuged at $6,000 \times g$ for 20 min, resuspended in distilled water, and diluted to an $OD_{600} = 1.0$, corresponding to approximately 1×10^9 CFU/ml. Bacterial suspensions were dilution plated to confirm inoculum cell density. *Arabidopsis* Col-0 plants (3 to 4 weeks old) were grown in Sunshine mix #1 (Sun Gro Horticulture) in 2-square-inch pots at 22°C with 8 h of daylight. Plants were inoculated by wounding the roots with a serrated knife and pouring 10 ml of bacterial suspension on the soil surface. Final inoculum concentrations ranged from 1.8 to 9.6×10^9 CFU/plant. Plants were coded and rated daily on a disease index scale of 0 to 4, where 0 = no symptoms; 1 = one to three inverted leaves (leaves that are turned upside-down), visible anthocyanin production, or both; 2 = three or more inverted leaves and anthocyanin production; 3 = wilting over less than 50% of the leaf surface; and 4 = more than 50% of the leaf surfaces wilted or dead. Soil-soak results are the average of four assays, with a total of 72 to 84 plants.

Statistical analysis.

Disease progress curve results were analyzed using repeated measures analysis of variance over the time course of the experiment (Sokal and Rohlf 1981). Tukey's honestly significant difference multiple comparisons test was used to compare disease index means between strains on specific days during the assay. All analyses were done using Systat (version 5.2; Systat Inc., Evanston, IL, U.S.A.).

GUS activity assays.

GUS activity assays were performed as previously described over a time course of 45 min (Jefferson 1987). For in culture data, bacteria were grown in BMM + 0.2% (wt/vol) glucose at 28°C and harvested by centrifugation at $8,000 \times g$ for 3 to 20 min, depending on culture volume. For in planta data, tomato plants were inoculated by the soil-soak method or, for *phcA* and *pehR* mutant strains, by directly introducing 10 μ l of washed mid-log phase bacterial culture into the lower tomato stem with a needle and syringe. This was necessary because of the reduced ability of these mutants to invade and colonize plants. Plant tissue was harvested two to seven days after inoculation by cutting a 100-mg section of stem immediately above the cotyledons and homogenizing the stem section in 1 ml of sterile distilled water followed by centrifugation at $6,000 \times g$ for 3 min. Cells from either liquid culture or plants were enumerated by dilution plating. Cells were lysed by adding lysozyme at 200 μ g/ml and DNaseI at 1 U/ml and incubating for 30 min at 37°C; lysed cells were used immediately for GUS activity assays. Data were expressed as picomoles of 4-MU produced PMPC and graphed relative to cell density in CFU/ml of either liquid culture medium or tomato xylem fluid.

Estimation of cell density in xylem fluid: comparison of in culture and in planta samples.

R. solanacearum is confined to the host plant vascular system through the early and middle stages of disease; therefore, it is inappropriate to determine cell density in the plant based on CFU/g of plant stem; CFU/ml of xylem fluid is more representative of the cell densities experienced by the bacteria. To estimate the number of bacteria in xylem vessels and directly compare in planta to in culture GUS activity data for gene expression versus cell density, plant tissue cell densities that originally were expressed in CFU/g of tomato stem were converted to CFU/ml of xylem fluid by multiplying by a factor of 13.75. To determine this ratio, we compared bacterial populations in the xylem fluid (CFU/ml) to those in total stem tissue (CFU/g) by inoculating tomato plants by the soil-soak method (Tans-Kersten et al. 2001). Once plants began to show wilt (4 to 5 days post inoculation), plant tissue was harvested by cutting a 100-mg section of stem immediately above the cotyledons and homogenizing the stem in 1 ml of sterile distilled water. Passively exuded xylem fluid (10 μ l) was harvested from the cut stem of the same plant. All samples were dilution plated to enumerate bacteria. Eight plants were harvested, with two at each disease index (1 to 4). Although there was some variability among samples, xylem fluid samples contained on average 13.75 times more bacteria than sectioned stem samples from the same plant. For this analysis, we assumed that most bacteria remain in the plant xylem tissue, even in later stages of disease; the ratio would be slightly lower if this was not the case. Although this multiplier shifts the in planta expression data, the trends remain the same.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Ralstonia solanacearum GMI1000 genome website: <http://histone.toulouse.inra.fr/bioinfo/annotation/iANT/bacteria/ralsto/index.html>