The Flagellar Sigma Factor FliA Is Required for *Dickeya dadantii* Virulence

Courtney E. Jahn,¹ David K. Willis,^{1,2} and Amy O. Charkowski¹

¹Department of Plant Pathology, University of Wisconsin, and ²Vegetable Crops Research Unit, United States Department of Agriculture–Agricultural Research Service, Madison, WI 53706, U.S.A.

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The genome sequence of the *Enterobacteriaceae* phytopathogen *Dickeya dadantii* (formerly *Erwinia chrysanthemi*) revealed homologs of genes required for a complete flagellar secretion system and one flagellin gene. We found that *D. dadantii* was able to swim and swarm but that ability to swarm was dependent upon both growth media and temperature. Mutation of the *D. dadantii* *fliA* gene was pleiotropic, with the alternate sigma factor required for flagella production and development of disease symptoms but not bacterial growth in *Nicotiana benthamiana* leaves. The flagellar sigma factor was also required for multiple bacterial phenotypes, including biofilm formation in culture, bacterial adherence to plant tissue, and full expression of pectate lyase activity (but not cellulase or protease activity). Surprisingly, mutation of *fliA* resulted in the increased expression of *avrL* (a gene of unknown function in *D. dadantii*) and two pectate lyase gene homologs, *pelX* and ABF-0019391. Because FliA is a key contributor to virulence in *D. dadantii*, it is a new target for disease control.

The *Enterobacteriaceae* phytopathogen *Dickeya dadantii* causes wilting and soft rot in a wide range of plants, including ornamental plants and economically important vegetable crops (Ma et al. 2007). *D. dadantii* produces a variety of cell-wall-degrading enzymes, such as pectate lyases (Pels), polygalacturonases, and cellulases. These enzymes cause plant tissue maceration and are the primary pathogenicity factor of this pathogen (Barras et al. 1994). However, many other factors are important for pathogenicity. Iron scavenging, for example, allows for systemic attack, weakening the plant by transferring iron from plant cells to chrysobactin, a *D. dadantii* siderophore (Enard et al. 1988; Persmark et al. 1989, 1992). Other virulence factors include lipo- and exopolysaccharide production (Condemine et al. 1999; Schoonejans et al. 1987), the type III secretion system (T3SS), and proteins involved in environmental sensing and resistance against plant defense (Llama-Palacios et al. 2003; Lopez-Solanilla et al. 1998).

We have observed that *D. dadantii* cells are motile in diseased plant tissue (*unpublished observations*) but whether the production of secondary metabolites, enzymes, and secretion systems that contribute to virulence is coordinated with motility is unknown. As yet, flagellar genes have not been shown to

contribute to virulence for this genus. Many phytopathogenic bacteria are motile by means of flagella and this motility contributes to virulence (Chesnokova et al. 1997; Mulholland et al. 1993; Panopoulos and Schroth 1974; Tans-Kersten et al. 2001). In animal pathogens, such as *Escherichia coli* and *Salmonella* spp., flagella are also an important factor in host–parasite interactions. Approximately 50 genes are known to be involved in constructing and operating the flagellar apparatus, and its transcription is coordinated by a complex regulatory hierarchy that includes both positive and negative regulators (Macnab 1996). Flagella operons are transcribed in three temporal classes: early, middle, and late. The early genes are included in the master flagellar operon, *flhDC*, that directs transcription of the middle and some of the late genes. The middle operons encode structural and assembly proteins required for the biosynthesis of the flagellar basal body and hook. The gene *fliA* encodes an alternative transcription factor, σ^{28} , which is specifically required for class III promoter transcription. Class III promoters control genes that encode for later products in flagella assembly, including the external filament, the flagella motor, and chemotaxis genes (Chilcott and Hughes 2000; Macnab 1996). Mutations in *fliA* lead to aborted flagella biosynthesis that stalls after formation of the flagella basal body and hook (Chilcott and Hughes 2000; Macnab 1996).

In some bacterial species, the same proteins that regulate expression of flagella genes also regulate virulence factors. There are many *Enterobacteriaceae* species that coregulate flagella and the T3SS. For example, in *Yersinia enterocolitica*, the T3SS is downregulated by FliA, probably by downregulation of the T3SS transcriptional activator *virF* (Horne and Pruss 2006). In contrast, the T3SS in *Salmonella typhi* is upregulated by FliA (Eichelberg and Galan 2000). FliA also upregulates the T3SS in *S. typhimurium* but to a lesser extent than is seen in *S. typhi* (Eichelberg and Galan 2000). These differences show that the regulatory effects of FliA on virulence genes cannot yet be predicted solely based upon comparison with related genera; rather, they must be experimentally determined.

Flagella are used for both swimming and swarming motility. Individual swimming cells perceive a chemical signal via methyl-accepting chemotaxis proteins, triggering a chemotactic response to the signal (Yi et al. 2000). Swarming is characterized by movement of bacteria in tight groups of bound cells through bacterial-produced slime on semisolid surfaces (Harshey 2003). Swarming motility requires bacteria to undergo a differentiation process that causes the cells to become filamentous and hyperflagellate (Allison and Hughes 1991; Fraser and Hughes 1999). Repeated events of swarmer differentiation, swarming movement, and reversion to short cells give rise to concentric rings as populations of bacteria colonize a rich solid surface (Shapiro 1998). Some species may also form

Corresponding author: A. O. Charkowski; Telephone: +1.608.262.7911; Fax: +1.608.263.2626; E-mail: amyc@plantpath.wisc.edu

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elaborately shaped colonies with dendritic extensions, but this does not occur with all bacterial species that are capable of swarming.

Because we have observed that *D. dadantii* cells are motile in diseased plant tissue, we hypothesized that *D. dadantii* *flhA* would be required for full virulence and that, as in related pathogens, it will affect expression of genes in addition to flagellar genes. In this work, we found that a *D. dadantii* mutant lacking *flhA* is reduced in virulence, reduced in ability to adhere to plant tissue, reduced in Pel activity, and affected in biofilm development.

RESULTS

The *D. dadantii* flagellar operon structure is encoded in one locus and the flagellin includes the flg22 sequence.

Like *E. coli*, *D. dadantii* produce peritrichous flagella; however, unlike some other *Enterobacteriaceae*, *D. dadantii* encodes only one type of flagella and one type of flagellin. The flagella regulatory proteins found in other *Enterobacteriaceae* are conserved in *D. dadantii*, including the master regulator FlhD/FlhC and the alternate sigma factor, FlhA. The order of the genes within each operon is similar to *E. coli*; however, the arrangement in the chromosome differs, with flagellar operons in *E. coli* and *S. enterica* Typhimurium in three loci and flagellar operons in *D. dadantii*, *Pectobacterium carotovorum*, *P. atrosepticum*, and *Y. enterocolitica* in a single gene cluster (Bell et al. 2004; Horne and Pruss 2006) (Fig. 1A). The *Pectobacterium* spp., unlike *D. dadantii*, encode a second flagellin homolog elsewhere in the chromosome.

A 22-amino-acid portion (flg22) of the flagellin FlhC may be recognized by plants via FLS2 as a pathogen-associated molecular pattern (Morden et al. 1991), resulting in elicitation of

plant defenses (Chinchilla et al. 2006; Felix et al. 1999; Gomez-Gomez and Boller 2002; Hayashi et al. 2001). The flg22 sequences were compared among pathogenic bacteria to determine if the amino acids required for plant perception were present in *D. dadantii*. All five key amino acids previously identified as required for defense elicitation were found in all *Enterobacteriaceae* flagellin proteins examined; thus, plants encoding FLS2 would be predicted to recognize and respond to *Enterobacteriaceae* species. All of the *Enterobacteriaceae* flg22 sequences examined are identical except for that of *D. dadantii* and two of the six *Pectobacterium* flagellin homologs which, based on BLAST search of the National Center for Biotechnology Information protein database, encode flg22 sequences unique among all sequenced flagellins (Fig. 1B).

D. dadantii motility is affected by growth media and temperature.

Bacterial motility can be observed in media with low levels of agar, with 0.2 to 0.4% agar typically used for measuring swimming and 0.45 to 0.8% for swarming (Burkart et al. 1998; Harshey 1994; Niu et al. 2005). In some species, swarming bacteria form elaborate dendritic colonies but *Enterobacteriaceae* species often do not form elaborate swarming patterns (Burkart et al. 1998; Julkowska et al. 2004). We compared four growth media—tryptic soy agar (TSA), Luria-Bertani agar (LBA), salt-optimized broth plus 2% glycerol (SOBG), and swarming media (SM)—at two different agar concentrations (0.3% for swimming and 0.5% for swarming) to examine *D. dadantii* motility. We found that *D. dadantii* 3937 swam in all four media at temperatures ranging from 20 to 36°C (not shown). In contrast, *D. dadantii* swarmed only on TSA and SM but not in LBA or SOBG (Fig. 2). Temperature differentially affected motility on these media, with bacteria swarming

Fig. 1. Organization of flagellar genes and flg22 regions of flagellin from *Enterobacteriaceae* pathogens. Sequences were retrieved from the National Center for Biotechnology Information and ASAP (Glasner et al. 2003, 2006). **A**, Flagella genes are organized into two distinct operon structures. *Dickeya dadantii* and *Escherichia coli* are pictured as representatives. Genes are indicated by blocks and arrowheads. The start of each gene cluster is indicated with a black rectangle and arrowheads indicate direction of gene clusters. Genes are labeled at the end of each operon. Genes shaded gray are hypothetical or unknown function. Group 1 has three separate gene clusters on the chromosome while group 2 genes are in one large cluster. **B**, The flg22 sequences were identified and aligned manually. Stars indicate conserved amino acids. *Enterobacteriaceae* species are separated from non-*Enterobacteriaceae* species by a line. Sun and associates (2006) determined *Xanthomonas campestris* pv. *campestris* polymorphisms between elicitors and nonelicitors of plant defense (shaded) and the arrow indicates the critical amino acid for recognition of flg22 by *Arabidopsis thaliana*.

in TSB at room temperature and 28°C but not at 36°C. In contrast, *D. dadantii* swarmed in SM at temperatures up to 36°C. Surprisingly, although *D. dadantii* 3937 grew in LBA at 39°C it was unable to grow in TSA or SM at 39°C; thus, no swarming was observed.

Flagellar genes are required for pellicle formation.

D. dadantii forms two chemically and genetically distinct types of biofilm in culture: a biofilm ring at the air-liquid-solid surface junction and a pellicle at the air-liquid interface (Yap et al. 2005). The T3SS is required for pellicle formation but the role of motility has not been examined. We used allelic-exchange mutagenesis to delete the flagellar sigma factor *fliA* from *D. dadantii*.

As expected, the *fliA* mutant was not motile (Fig. 3). The biofilm ring that formed in SOBG cultures at the liquid-air-solid surface interface with the *fliA* mutant was only weakly

attached to the glass surface, in contrast to biofilm rings that form with T3SS mutants, which are firmly attached to the surface (Yap et al. 2005). Although small aggregates formed at the air-liquid-surface interface, the *fliA* mutant was unable to grow outward from these aggregates; therefore, no pellicles formed, in contrast to wild-type cultures. *D. dadantii* swims but does not swarm in SOBG, and does form biofilms in SOBG; therefore, swarming motility is not likely to be important for biofilm formation in this medium.

Motility, but not biofilm formation, could be restored by expression of *fliA* from pGEM-T Easy in the *fliA* mutant (not shown). We hypothesized that downregulation of flagella genes is required for biofilm formation. Therefore, *fliA*, under the control of its native promoter, was inserted into the chromosome between *lacY* and *pri* in the *fliA* mutant. With this strategy, we successfully restored biofilm formation and motility to the *fliA* mutant (Fig. 3).

Fig. 2. Effect of temperature and medium on motility. A wild-type *Dickeya dadantii* cell suspension (2 μ l, 1×10^8 CFU/ml) was spotted onto tryptic soy agar (TSA), Luria-Bertani agar (LBA), salt-optimized broth plus 2% glycerol (SOBG), and swarming medium (SM) containing 0.5% agar. The plates were incubated at four different temperatures (room temperature (RT), approximately 25°C, plus 28, 36, and 39°C) and examined the next day to determine motility of *D. dadantii*. Swarming occurred only on some media and only at lower temperatures. Growth at 39°C was observed only on LBA.

D. dadantii *fliA* contributes to bacterial attachment to plant tissue.

Because FliA was required for pellicle formation, we hypothesized that this gene was also important for attachment to plant tissues. In three independent experiments, the *fliA* mutant was significantly reduced in attachment compared with the wild type (Table 1, two-tailed *t* test, $P < 0.001$). In all three experiments, when the *fliA* mutation was complemented by expression of *fliA* from the chromosome, bacterial attachment was restored to the wild-type level (Table 1, two-tailed *t* test, $P > 0.09$).

The *D. dadantii* *fliA* mutant is reduced in symptom development.

When infiltrated into *Nicotiana benthamiana* leaves, *D. dadantii* 3937 wild-type cells first cause a leaf curl and plant

stunting, followed by maceration of the infiltrated region in the inoculated leaf, and sometimes proceeding to maceration of the entire inoculated leaf or the entire plant (Fig. 4). In our assay, over half of the plants inoculated (29 of 40) by infiltrating one leaf with 10^6 CFU/ml of wild-type *D. dadantii* cells developed systemic maceration symptoms (Figs. 4 and 5). We found that the *fliA* mutant typically caused dry necrotic symptoms only in the region where it was inoculated into *N. benthamiana* leaves. Of the 40 plants inoculated with the *fliA* mutant, 3 developed systemic symptoms, with maceration extending down the leaf petiole and, in two cases, into other leaves on the plant. Thus, the *fliA* mutant was capable of causing systemic symptoms but at a much lower incidence than occurs with the wild type. Like wild-type *D. dadantii*, the complemented *fliA* mutant also caused a range of symptoms on *N. benthamiana*

Fig. 3. Motility and biofilm formation of *Dickeya dadantii*. Tryptic soy agar swim and swarm plates were photographed after 24 h of incubation at 28°C. Biofilm formation is demonstrated in column 3; Wt = wild type. The *fliA* mutant was no longer motile and unable to make a pellicle or a complete biofilm ring. The chromosomal complementing clone restored these phenotypes in the *fliA* mutant.

Table 1. Mean log₁₀ and standard error of the mean (SEM) of *Dickeya dadantii* 3937 cells attached to alfalfa sprouts after a 4-h incubation

Strain, experiment ^y	Mean	SEM	Significantly different ^z	<i>P</i> value
3937				
1	1.3	±0.08	...	...
2	2.2	±0.28	...	...
3	2.3	±0.08	...	...
3937 <i>fliA</i> :: <i>Km</i>				
1	0.22	±0.10	Yes	0.0001
2	0.61	±0.24	Yes	0.0007
3	0.72	±0.25	Yes	0.0001
3937 <i>fliA</i> :: <i>Km lacY:fliA</i> ⁺ : <i>Cm:prt</i>				
1	1.4	±0.07	No	0.0983
2	1.8	±0.25	No	0.3179
3	2.4	±0.07	No	0.0983

^y For experiments 1 and 3, *n* = 10; for experiment 2, *n* = 9.

^z Means significantly different from the wild type at $P = 0.05$ as determined by two-tailed *t* test.

plants, with systemic spread into the petiole or other leaves observed in 32 of 40 plants.

The reduced virulence of the *D. dadantii* *fliA* mutant on its host was not explained simply by a reduction in growth. The mutant, the complemented mutant, and the wild-type strains were inoculated at 4.5 log CFU/g of leaf tissue and all grew to an average of 7.5 log CFU/g of leaf tissue in the inoculated leaves over the course of 3 days. No statistically significant difference in bacterial growth could be detected at days 1, 2, or 3 after inoculation as determined by one-way analysis of variance (data not shown). Therefore, disease symptoms and bacterial growth in leaves were not correlated.

FliA regulates Pel production in *D. dadantii*.

The lack of maceration symptoms without a reduction of in planta growth caused by mutation of *fliA* led us to the hypothesis that the decrease in symptom development was due in part to an alteration in expression of plant cell-wall-degrading enzymes. Supernatants from wild-type and *fliA* mutant cultures were assayed for altered levels of Pel, cellulase, and protease activity using plate activity assays. *D. dadantii* flagella mutants were not reduced in cellulase or protease activity but

there was a reduction in Pel production (Fig. 6). Strains with mutations in *pecS* and *pecT*, which are known Pel regulators, were used as controls.

To quantify the effect of FliA on Pel activity, we used a spectrophotometric assay to measure Pel activity in culture supernatants of wild-type, *fliA* mutant, and complemented mutant culture supernatants (Collmer et al. 1988). The *fliA* mutant was decreased by approximately 40% in Pel activity compared with the wild type (Table 2). Expression of *fliA* from the chromosome restored Pel activity to levels slightly above the wild type. A *pecS* mutant was used as a control and mutation of this negative regulator and, as expected, resulted in production of approximately twice as much Pel activity as the wild type (Reverchon et al. 1994).

D. dadantii produces numerous Pels. The reduction in Pel activity could be explained by a large reduction in transcription of one or a few *pel* genes or a minor reduction in transcription of several *pel* genes in the *fliA* mutant. A transcriptional effect of *fliA* on the type II secretion system (T2SS) is unlikely because we saw no effect on cellulase activity and cellulase, like the Pels, is secreted via the T2SS. We used real-time quantitative reverse-transcriptase polymerase chain reac-

Fig. 4. Time course of symptom development with the wild-type (Wt) and *fliA* mutant inoculated into *Nicotiana benthamiana* plants. Plants were infiltrated with *Dickeya dadantii* at 10^6 CFU/ml, placed in plastic bags to maintain high relative humidity, and then photographed daily. Plants inoculated with Wt exhibit typical soft-rot symptoms whereas flagella mutant symptoms are less severe.

tion (qRT-PCR) to determine whether mutation of *fliA* caused a reduction in mRNA accumulation of any of the *pel* genes. Compared with wild-type *D. dadantii*, we saw no reduction in mRNA levels for any of the 11 Pel genes examined (Table 3). These genes included *D. dadantii* homologs of *pelA*, *pelB*, *pelC*, *pelD*, *pelE*, *pelF*, *pelL*, *pelW*, *pelX*, *pelZ*, and ABF-0019391. Rather, we observed a significant increase in mRNA levels of the periplasmic exo-Pel *pelX* (Shevchik et al. 1999) (2.2-fold, $P < 0.0001$), the Pel homolog ABF-0019391 (3.2-fold, $P = 0.0071$), and a gene encoding a T2SS substrate of unknown function, *avrL* (Kazemi-Pour et al. 2004) (1.9-fold, $P = 0.0017$).

Some members of this set of transcripts demonstrated unusually high sample-to-sample variability, making the statistical confirmation of mean differences very difficult (Table 3). For example, the mean relative expression ratio (RER) of *pelA* and *pelW* exceeded twofold but the high standard error of the mean (SEM) prevented establishing this difference as significant by two-tailed *t* test. This is in contrast to transcripts such as *pecT*, *pelZ*, and *pelE* that exhibited very low SEM between the same RNA samples.

DISCUSSION

We found that *D. dadantii* can both swim and swarm in culture media and that swarming is affected by both media and temperature. *D. dadantii* FliA, which encodes the sigma factor that activates class III flagella genes, also contributes to pellicle formation, adherence to plant tissue, symptom development, and Pel activity. Thus, FliA is a positive regulator of multiple traits associated with virulence. Curiously, although disease symptoms and Pel activity were significantly reduced with the *fliA* mutant, both the wild-type and mutant strains grew to equal levels in inoculated leaves. Therefore, leaf symptoms caused by *D. dadantii* are not correlated with bacterial growth in this assay.

In *D. dadantii* and other bacterial species, multiple genes contribute to both biofilm formation and virulence (Koutsoudis et al. 2006; O'Toole and Kolter 1998; Yap et al. 2005). The elimination of pellicle formation in the *fliA* mutant might be

Fig. 6. Phenotypic characterization of an *fliA* mutant using a pectate lyase plate activity assay. Strains were grown overnight in polypectate enrichment medium at 30°C with shaking. Culture supernatants were added to wells in the assay medium, and plates were incubated at 28°C and developed at 24 h. Pectate lyase activity is evident as a white halo or cleared zone surrounding the well. The gene mutated in each strain used is indicated by each well. The assay was repeated three times and representative results are shown.

Fig. 5. Disease rating of *Nicotiana benthamiana* after infiltration with the wild type and the *fliA* mutant. A single leaf was infiltrated with 10^6 CFU/ml, and the plants were placed in plastic bags and evaluated daily for symptom development. Plants were rated using the following scale: 1 = no symptoms, 2 = leaf curl, 3 = maceration at infection site, 4 = maceration into petiole, and 5 = systemic spread. Each bar indicates the mean disease rating for each day post inoculation. Over the course of 5 days, a χ^2 analysis indicated that the *fliA* mutant was significantly different from the wild type ($P < 0.0001$) whereas the complementing clone was not significantly different from the wild type ($P = 0.0635$).

due to the requirement for motility in order to reach the air-liquid interface. However, our data show that the *fliA* mutant formed aggregates on the sides of test tubes at the air-liquid-surface interface. Thus, bacteria were present at the air-liquid interface and outward growth to form a pellicle should be possible if reaching the culture surface was all that was required. The lack of a bacterial aggregation demonstrates that FliA-regulated genes are required for wild-type bacterial aggregation. Flagella can function as adhesins (Arora et al. 1998); however, because FliA has also been shown to regulate pili production in *E. coli* (Claret et al. 2007), the lack of aggregation observed in the *D. dadantii fliA* mutant is not necessarily due to the lack of flagella in the *fliA* mutant.

Several transcriptional regulators have been identified as contributing to both bacterial attachment to plants and biofilm formation in culture (Barak et al. 2005). We used alfalfa sprouts to test bacterial adherence to plants because they can be handled whole, lending themselves well to in situ experiments. Damaged plant tissue is sticky and bacteria that normally do not adhere to intact plant tissue will adhere to wounded tissue; therefore, leaf disks were not used (Wachtel and Charkowski 2002). Also, *Dickeya* spp. cause bacterial sprout rot of alfalfa, making this a relevant model (Moline and Kulik 1997; Pierce and McCain 1985). Mutation of *fliA* reduced, but did not eliminate, the ability of *D. dadantii* to adhere to plant tissue; thus, FliA-regulated genes contribute to adherence of bacterial cells to each other during both biofilm formation and adherence to plant tissue. As with biofilm formation, the contribution of *D. dadantii* FliA-regulated genes to bacterial adhesion to plant tissue is probably due to a bacterial adhesin and not due solely to bacterial motility because the sprouts were agitated in a suspension of bacteria on a rotating incubator during the adhesion assay. It was not surprising that the bacterial adhesion to plant tissue was only partially reduced in the *fliA* mutant. In the related species, *S. enterica*, multiple polymers contribute to bacterial adherence to plant tissue and elimination of any single polymer results in only a partial reduction in bacterial adherence to plant tissue (Barak et al. 2007).

We used the model plant *N. benthamiana* to evaluate the role of FliA in *D. dadantii* pathogenicity. When inoculated into *N. benthamiana* leaves, *D. dadantii* multiplies by up to three log orders in the inoculated leaves, which is comparable to the level of multiplication observed in the plant host from which this strain was initially isolated, *Saintpaulia* (African violet) leaves (Okinaka et al. 2002; Yang et al. 2008). The symptoms caused by *D. dadantii* on *N. benthamiana* progress in a predictable manner. The bacteria first cause the inoculated leaf to curl, then the inoculated leaf decays, and finally the decay spreads down the leaf petiole and to the rest of the plant. Leaf curl and dwarfing symptoms have not been reported previously with *D. dadantii*, perhaps because most virulence assays have been performed with petioles of detached witloof chicory leaves, potato tubers, or fleshy *Saintpaulia* leaves. Although the *fliA* mutant grew as well as the wild type in inoculated leaves, it failed to cause systemic maceration symptoms. Bac-

terial growth without symptom development has been seen with other plant pathogens as well (Willis et al. 1990). However, the *fliA* mutant did cause extensive leaf curling and plant dwarfing. *D. dadantii* produces auxin (Yang et al. 2007) and we have recently found that auxin production is required for the leaf curl and dwarfing symptoms to develop (*unpublished observations*).

The *fliA* mutant grew as well as the wild type in inoculated leaves and caused systemic dwarfing and leaf curl symptoms but did not macerate the inoculated plants; therefore, we hypothesized that mutation of *fliA* affected plant cell-wall-degrading enzyme production. We found that Pel but not cellulase or protease activity was reduced in the *fliA* mutant. The magnitude of the effect of FliA on Pel activity is similar to that of PecS, except in the opposite direction. Mutation of FliA results in slightly less than a onefold decrease whereas mutation of PecS causes a onefold increase in Pel activity compared with wild-type cultures. Because the *fliA* mutant has lower Pel activity, we hypothesized that FliA is likely to regulate one or more of the 11 Pel genes encoded by *D. dadantii* either directly through activation of transcription or indirectly by derepression of *pecT* or another regulator. FliA is not likely to act through the Pel regulators KdgR, CRP, or PecS because, unlike PecT, these regulators also control cellulase activity and cellulase activity was not affected in the *fliA* mutant. FliA is also not likely to act through the acyl-homoserine lactone (AHL) quorum-sensing system because AHL-based quorum sensing plays only a minor role in regulating plant cell-wall-degrading enzymes in *D. dadantii* (Nasser et al. 1998). *E. coli* and *S. enterica* FliA binds to TAAAGTTT-N₁₁-GCCGATAA (Ide et al. 1999) and a similar sequence is found upstream of some *D. dadantii* flagellar operons. No putative FliA binding sites were found in the *pecT* or Pel gene promoters; thus, the *D. dadantii* genome sequence revealed no clues for the mechanism by which FliA affects Pel production.

The reduction in Pel activity observed could be due to a large reduction in expression of 1 or 2 of the 11 Pel genes, but

Table 2. Pectate lyase activity of *Dickeya dadantii* mutant strains compared with the wild type

Strain	Pectate lyase activity (μmol/min) ^z
3937	0.303 ± 0.055 A
WPP 381, 3937 <i>fliA::Km</i>	0.177 ± 0.019 B
WPP382, 3937 <i>fliA::Km lacY::fliA⁺::Cm::prt</i>	0.405 ± 0.036 C
3937 <i>pecS::MudIIIP13</i>	0.615 ± 0.088 D

^z Values followed by different letters are significantly different at *P* = 0.05 according to Tukey's highly significant difference.

Table 3. Quantification of transcripts in the wild type and *fliA* mutants

Characterization, name ^v	Target transcript RER ^w	SEM ^x	<i>P</i> value ^y
Negative regulator			
<i>pecT</i>	0.90	±0.06	0.7542
Major pectate lyases			
<i>pelA</i>	2.51	±1.08	0.2774
<i>pelE</i>	0.92	±0.05	0.4384
<i>pelD</i>	2.07	±0.60	0.1970
<i>pelB</i>	1.12	±0.21	0.6513
<i>pelC</i>	1.18	±0.07	0.6210
Minor pectate lyases			
<i>pelZ</i>	0.81	±0.05	0.1092
<i>pelW</i>	2.78	±0.64	0.0531
<i>pelL</i>	1.39	±0.33	0.4291
<i>pelI</i>	1.73	±0.42	0.1366
<i>pelX</i>	2.24	±0.11	<0.0001 ^z
Predicted pectate lyase			
ABF-0019391	3.22	±0.65	0.0071 ^z
Unknown function			
<i>avrL</i>	1.89	±0.12	0.0017 ^z

^v Gene name or ASAP identification number.

^w RER = relative expression ratio of the target transcript in the *fliA* mutant using the target transcript abundance in wild-type *Dickeya dadantii* as a calibrator.

^x Standard error of the mean.

^y Determine by two-tailed *t* test with a 95% confidence interval using Prism 5.0a software (GraphPad Software, Inc.).

^z A *P* value <0.05 indicates that the mean RER of the target transcript in the *fliA* mutant was significantly different from the target gene mean RER in the wild type.

we found no significant reduction in Pel mRNA accumulation in the *fliA* mutant, disproving this hypothesis. If the reduction in enzyme activity observed is due to a relatively small reduction in mRNA levels of multiple Pel genes, it may not be possible to detect by real-time qRT-PCR. We observed slight (less than onefold) reduction in Pel activity and a concurrent reduction in mRNA levels is a difference of less than one qRT-PCR cycle.

Surprisingly, two Pel homologs, *pelX* and ABF-0019391, and a gene of unknown function, *avrL*, were upregulated in the *fliA* mutant relative to the wild type. PelX differs from the other Pels in that it is a periplasmic exo-Pel that is not solely dependent on calcium ions for activity and is not regulated by PecT (Shevchik et al. 1999). PelX is not secreted and has low enzymatic activity compared with many of the other Pels; therefore, it is unlikely that PelX contributed to the enzyme activity measured. The Pel homolog ABF-0019391 is most similar to PelL and has orthologs in all three sequenced *Pectobacterium* genomes; however, there is no biochemical data available on the enzyme encoded by this gene. AvrL is a T2SS-secreted protein homologous to a *Xanthomonas* protein, AvrXca, a protein that confers an avirulence phenotype when *Xanthomonas campestris* is inoculated onto *Arabidopsis* (Kazemi-Pour et al. 2004; Parker et al. 1993).

Several studies have shown evidence for the disparity between mRNA and protein abundance but few have looked at relative expression levels of mRNA and the corresponding enzyme activities. In cases where both are examined, unpredictable results sometimes have been obtained (Anderson and Seilhamer 1997; Glanemann et al. 2003; Gygi et al. 1999). For example, in *E. coli* LF82, FliA regulates type 1 pili biosynthesis via GGDEF and EAL domain proteins in a complex manner that cannot be simply related to the effect of FliA on transcription of these GGDEF and EAL regulatory proteins (Claret et al. 2007).

The flagellar operons of the soft rot pathogens *Dickeya* and *Pectobacterium* spp. are similar in organization and the soft rot bacterial flagellins are similar in that they all include a flg22 sequence likely to activate defenses in at least some plant species via the resistance protein FLS2 (Chinchilla et al. 2006; Felix et al. 1999; Gomez-Gomez and Boller 2002; Sun et al. 2006). Consistent with this, a FLS2-like response to *Dickeya* and *Pectobacterium* crude extracts was reported in tomato by Felix and associates (1999). Because this motif is conserved in both soft rot genera as well as in closely related human pathogens such as *S. enterica* and *E. coli*, defense responses against

flagellin apparently do not provide enough pressure to select for flagella that lack the eliciting amino acids in the flg22 sequence in the soft rot enterobacteria.

FliA contributes to pellicle formation, aggregation, virulence, and enzyme production and regulates mRNA accumulation of two Pel homologs and *avrL*, suggesting that there is substantial interplay between known *D. dadantii* virulence genes and those required for motility. Our current knowledge only covers small fragments of how these pathways overlap. Unraveling the multiple mechanisms and signals used to coregulate motility and virulence proteins may lead to new control methods for plant-pathogenic bacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Wild-type *D. dadantii* and derivatives were grown in Luria-Bertani (LB) medium or polypectate enrichment medium (PEM) (sodium polypectate at 1.5 g/liter, (NH₄)₂SO₄ at 0.13 g/liter, K₂HPO₄ at 0.17 g/liter, and MgSO₄ · 7H₂O at 0.06 g/liter) (De Boer and Kelman 2001) at 37°C for DNA isolation and mutagenesis. The growth medium used to induce biofilm formation, SOBG, consisted of salt-optimized broth plus 2% glycerol (20 g of tryptone, 5 g of yeast extract, 0.5 g of NaCl, 2.4 g of MgSO₄, 0.186 g of KCl, and 50 ml of 40% glycerol per liter) (Yap et al. 2005). For pellicle assays, bacterial cultures were suspended in SOBG to approximately 10⁶ CFU/ml and incubated at 25°C in stationary 20-mm glass tubes or 100-ml beakers for at least 3 days. For swarm and swim motility assays, 2 µl of an overnight culture was spotted in the middle of a swim plate (0.3% [wt/vol] agar) or a swarm plate (0.5% [wt/vol] agar) and allowed to dry at room temperature. Media used for motility assays included LBA, SOBG, TSA (pancreatic digest of casein at 17 g/liter, enzymatic digest of soybean meal at 3 g/liter, dextrose at 2.5 g/liter, sodium chloride at 5 g/liter, and dipotassium phosphate at 2.5 g/liter), and SM (beef extract at 3 g/liter, peptone at 5 g/liter, and 25 ml of 20% glucose). All plates were incubated at room temperature, 28, 36, and 39°C for 12 to 16 h.

Enzyme plate assays of enzymatic activity were modified from Chatterjee and associates (1995). Briefly, the media compositions were as follows: Pel assay medium = 1% polygalacturonic acid (PGA), 1% yeast extract, 0.38 mM CaCl₂, and 100 mM Tris-HCl, 0.8% agar, pH 8.5; cellulase (Cel) assay medium = 0.1% carboxymethyl cellulose and 25 mM sodium phosphate, 0.8% agar, pH 7.0; and protease (Prt) assay me-

Table 4. Strains and plasmids used in this study

Strains, plasmids	Relevant characteristics ^a	Source
<i>Escherichia coli</i>		
DH5α	<i>supE44 ΔlacU169 (Δ80lacZAM15) hsdR17 recA1EndA1 gyrA96 thi-1 relA1</i>	...
<i>Dickeya dadantii</i>		
3937	Wild-type, <i>Saintpaulia ionantha</i> (African violet) isolate	Lemattre and Narcy 1972
<i>kdgR::miniTn5-Sm</i>	<i>lmrT(con) lacZ2 kdgR::miniTn5-Sm</i>	Nasser et al. 2005
<i>pecS::MudIIPR13</i>	<i>lmrT(con) lacZ2 pecS::MudIIPR13</i>	Reverchon et al. 1994
<i>pecT::uidA Cm</i>	<i>lmrT(con) lacZ2 pecT::uidA Cm</i>	Surgey et al. 1996
Nx3937	3937 Nx ^r derivative	Yap et al. 2008
WPP381	<i>fliA::Km</i>	This work
WPP382	Chromosomal insertion <i>lacY-fliA-Cm-prt</i>	This work
Plasmids		
pGEM-T Easy	Ap ^r , <i>lacZ'</i> , cloning vector	Promega Corp.
pKD4	Km ^r , template plasmid carrying kan cassette	Datsenko and Wanner 2000
pKD3	Cm ^r , template plasmid carrying cat cassette	Datsenko and Wanner 2000
pTCLS _{AD}	Ap ^r , 2.8-kb <i>lacY-prt</i> region in pGEM-T Easy	Yap et al. 2008
pTf <i>fliA::Km</i>	Ap ^r Km ^r , 3.2-kb fragment containing <i>fliA::Km</i> in pGEM-T Easy	This work
pTf <i>fliAc</i>	Ap ^r , 2-kb <i>fliA</i> in pGEM-T Easy	This work
pCLS <i>fliA-Cm</i>	Ap ^r Cm ^r , 4.1-kb <i>lacY-fliA-Cm-prt</i> region in pGEM-T Easy	This work

^a Nx^r, Ap^r, Km^r, and Cm^r = resistant to nalidixic acid, ampicillin, kanamycin, and chloramphenicol, respectively.

dium = 3% gelatin, 0.8% agar, and 0.4% nutrient broth. The best results with the Pel medium were obtained using polygalacturonic acid from ICN Biomedicals Inc. (Aurora, OH, U.S.A.). Each petri plate (100-mm diameter) contained 15 ml of assay medium. Wells were made with a no. 2 cork borer and the bottom of the wells were sealed with molten agarose (0.8% [wt/vol] in water). Overnight culture or culture supernatant (10 µl) was applied to the wells and the assay plates were incubated at 28°C. Cultures were treated with tetracycline to inactivate bacterial cells prior to application to enzyme assay plates. Pel plates were developed with 4 N HCl until zones of clearing formed. Cel plates were developed with 0.1% congo red for 45 min and then washed with 1 M NaCl until zones of clearing appeared. Prt plates developed zones of clearing after incubation of 24 to 36 h without further treatment. Strains used in this study are described in Table 4.

Mutant and plasmid construction.

Primers were obtained from Integrated DNA Technologies, Inc. (Coralville, IA, U.S.A.). To construct plasmids for allelic-exchange mutagenesis and complementation, genes were PCR-amplified with the primer sets listed in Table 5. The products were cloned into pGEM-T Easy (Promega Corp., Madison, WI, U.S.A.). To disrupt *fliA*, a kanamycin resistance cassette was ligated into the unique restriction site *Xma*I in pGEM-T Easy::f*liA*.

These plasmids were electrotransformed into *D. dadantii* strains for allelic-exchange following methods previously described (Ried and Collmer 1987). All mutations were confirmed by PCR and Southern blot analysis. *fliA* was inserted into the chromosome using allelic exchange mutagenesis for complementation of mutations. To construct plasmids to complement the gene mutations, the promoter and coding regions of the genes were PCR amplified. The PCR products were cloned into pGEM-T Easy and then moved into pTCLS_{AD} (Yap et al. 2008). pTCLS_{AD} includes the *lacY-prt* locus plus an engineered multicloning site. This plasmid was designed to insert genes into a neutral chromosomal site. These plasmids were electrotransformed into mutant strains and allelic exchange was used to move the wild-type allele onto the chromosome.

When required, antibiotics were used at the following concentrations: ampicillin (100 µg/ml), chloramphenicol (50 µg/ml), streptomycin (50 µg/ml), and kanamycin (50 µg/ml). Transformation, restriction endonuclease digestion, and other DNA techniques were performed as described (Sambrook and Russell 2001).

Virulence assays.

One-week-old *N. benthamiana* seedlings were transplanted into 4-in. pots. Seedlings were placed in a growth chamber operating at 25°C with 12 h of continuous light per 24-h period. Light was provided by 40-W cool-white fluorescent bulbs at 240 µmol/m/s of photons. Plants were watered once daily and

fertilized once per week with a 50% solution of Miracle Grow (Scotts Co., Marysville, OH, U.S.A.). Plant assays were performed essentially as described (Hirano et al. 1997). Four- to six-week-old *N. benthamiana* leaves were infiltrated with a bacterial suspension of 10⁴ CFU/ml. Infected plants were placed in plastic bags and incubated in a growth chamber at 25°C throughout the course of the assay. At each sampling time, whole leaves were homogenized using a Polytron equipped with a model PTA 20 TS probe (Brinkmann Instruments, Westbury, NY, U.S.A.) in 20 ml of sterile water and the suspension was dilution plated on LBA medium plus appropriate antibiotics.

To evaluate symptom progression, 6-week-old *N. benthamiana* leaves were infiltrated with a bacterial suspension of 10⁶ CFU/ml. One leaf was infiltrated per plant, the potting mix was saturated with water, and the plants were placed in plastic bags and then incubated in a growth chamber at 25°C throughout the course of the assay. Plastic bags were opened daily to evaluate disease progression and to allow for air exchange. Plants were rated using the following scale: 1 = no symptoms, 2 = leaf curl, 3 = maceration at infection site, 4 = maceration into petiole, and 5 = systemic spread. This data was analyzed using a χ^2 test with the general linear model (SAS version 9.1.3; SAS institute, Inc., Cary, NC, U.S.A.).

Alfalfa sprout attachment assays.

Alfalfa sprout assays were performed as described previously (Barak et al. 2005). Alfalfa seed (5 g) was surface sanitized with 50 ml of 3% calcium hypochlorite for 20 min by agitation on a rotary shaker at 250 rpm. Fifty seeds were sprouted in petri plates with 20 ml of sterile water and irrigation water was exchanged daily. Overnight bacterial cultures grown on SOBG were suspended in sterile water at a concentration of approximately 10⁴ CFU/ml for attachment assays. For attachment assays, seed were germinated in sterile water for 3 days with daily water changes. After 3 days, sprouts were transferred petri dishes (11 sprouts per dish) and incubated in bacterial suspensions for 1 h at room temperature with constant shaking at 75 rpm. Sprouts were rinsed three times with sterile water and 10 s of gentle shaking each wash, and then homogenized. The homogenate was plated and the cultures were incubated overnight at 37°C; then, the colonies were enumerated. The experiment was repeated three times with at least nine sprouts analyzed per strain each time. Statistical analysis was performed using a two-tailed *t* test with a 95% confidence interval using Prism version 5.0a (GraphPad Software, Inc., La Jolla, CA, U.S.A.).

Pel activity assay.

Cultures were grown overnight in SOBG; then a 100-µl aliquot of each culture was added to 5 ml of PEM broth prepared with polypectate obtained from Sigma and the cultures were incubated at 30°C for 12 h. Supernatant from the PEM cultures

Table 5. Oligonucleotides used in this study

Primer	Sequence (5'→3')	Restriction site ^z	Amplified region
P0198	CTGCAGGATCCGTGTAGGCTGGAGCTGCTTC	<i>Pst</i> I, <i>Bam</i> HI	...
P0199	CTGCAGGATCCCATATGAATATCCTCCTTA	<i>Pst</i> I, <i>Bam</i>HI	1-kb <i>cat</i> or 1.6-kb <i>kan</i>
P0520	AGAATTCCCGCCAACCTCTATCTTATTG	<i>Eco</i> RI	...
P0519	ATAGCTTGAATTCCTCCCAACTGAACC	<i>Eco</i>RI	3.2-kb <i>fliA</i> spanning region
P0520	ACCCATATTGCGCGGATTGATGC	...	...
P0521	TTCTTCCTGGTTGCTTGAGGCATGAG	...	1.3-kb <i>fliA</i> spanning region
P0522	AATGCGATTGCCTTGTCTAATCTGG	...	...
P0523	AGGAACATCTTCATCTGATTGACG	...	4.0-kb <i>fliA</i> spanning region
P0409	ATGGACATCAGCTCTGCGACC	...	...
P0412	TATCTGATGGGTGCTGGCAT	...	2.8-kb <i>lacY-prt</i>

^z Restriction sites are indicated in the primer sequence by italic or bold text.

was harvested by centrifugation and added to room-temperature Pel activity substrate (0.1% PGA from ICN Biomedical Inc., 100 mM Tris-HCL [pH 8.5], and 0.69 mM CaCl₂). Enzyme activity was measured for 20 min using a Synergy HT Multi-Detection Microplate Reader (BIO-TEK Instruments, Inc., Winooski, VT, U.S.A.). Pel activity was defined as the conversion of 1 µmol of substrate to unsaturated uronide per minute (Nagel and Anderson 1965). Treatments were assessed using the PROC GLM function, and Tukey-Kramer's honestly significant difference (HSD) was used to evaluate multiple comparisons at the treatment level. Statistical analysis was done using Statistical Analysis Software (version 9.1; SAS Inc.). Tukey's HSD was used to assess treatment differences. The experiment was repeated six times.

RNA isolation.

D. dadantii proved to be an extremely difficult organism from which to isolate intact RNA of sufficient quality for real-time qRT-PCR analysis (Jahn et al. in press). Bacteria were cultured for 12 h; then, 1.25 ml of ice-cold ethanol containing 5% water-saturated phenol was added to the culture. Cells were harvested by centrifugation at 6,000 × g at 4°C and then the cell pellets were flash-frozen in liquid N₂. Total RNA was isolated from cell pellets using a modified hot phenol method (Jahn et al. in press). RNA was treated with 2 U of Turbo DNA-free (Ambion, Austin, TX, U.S.A.) for 1 h at 37°C using the rigorous protocol and quantified by UV spectrophotometry (NanoDrop, Inc., Wilmington, DE, U.S.A.). RNA quality was determined with microfluidic capillary electrophoresis using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, U.S.A.). All RNA samples used had an RNA integrity number (RIN) value >7.0 because we found that RNA quality

as determined by an RIN > 7.0 is essential for successful real-time qRT-PCR with *D. dadantii* total RNA (Jahn et al. in press). Residual DNA contamination was quantified using real-time PCR and primers specific for the *Dickeya rplU* gene (Table 6). Those RNA samples showing threshold cycle (C_t) values ≥33 cycles were deemed to be sufficiently free of DNA contamination for further study.

Real-time RT-PCR primer design.

Primers were designed based on *D. dadantii* DNA sequences obtained from ASAP (Glasner et al. 2003, 2006) that were imported into Beacon Designer software (Premier Biosoft International, Palo Alto, CA, U.S.A.); a program designed to generate primer pairs suitable for real-time PCR. The SYBR Green module with program setting "avoid template structure" was chosen to limit primer sequences to regions of little secondary template structure. Primers were obtained from IDT (Integrated DNA Technologies) and their sequences are shown in Table 6. Both reference and target primers exhibited comparable efficiencies as determined using a dilution series of target DNA. Primer efficiencies were determined from dilution curve using the formula $E = 10^{(-1/\text{slope})}$ (Pfaffl et al. 2004), with the slope determined by the MyiQ software (Table 6).

cDNA synthesis and real-time RT-PCR.

First-strand cDNA synthesis was performed using the iScript cDNA synthesis kit according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Briefly, the reaction was performed with 1.0 µg of total RNA in 15 µl of RNase-free water, 4 µl of 5× iScript reaction mix with a blend of oligo dT and random hexamer primers, and 1 µl of iScript reverse transcriptase. The reaction conditions were performed

Table 6. Quantitative reverse-transcriptase polymerase chain reaction primer sequences and efficiencies

Gene name, ASAP ID number	Primer name	Primer sequence (5'→3', forward to reverse)	Efficiency (%) ^z
<i>pecT</i>	P0783	TGGTGGTGCTGGATGAAC	...
	P0784	GCGTAGAAGCGACATAAGAG	100
<i>pelA</i>	P0767	CTGCTGGCTACATTGATC	...
	P0768	AATTCAGTGATATTGGTAACG	99
<i>pelE</i>	P0769	TCAGCATTCCGTCACACAC	...
	P0770	GGCGTTCCAACCATCTCC	90
<i>pelD</i>	P0771	AAAGCAGCACTGAATGGC	...
	P0772	CGATGATGGTGGTGTGG	95
<i>pelB</i>	P0773	CTAAAGATGCGGATATGG	...
	P0774	GATGTAGTTGTAGGATACG	120
<i>pelC</i>	P0775	CAGGATGGCGATATGTTT	...
	P0776	CGTGGATGTTGTTGTAGG	94
<i>pelZ</i>	P0777	TACGACTCCAGCCACTCC	...
	P0778	CAGGTTGCCGATGATTGC	109
<i>pelW</i>	P0785	CGTTATGTGCGGCTTGATG	...
	P0786	ACTTCCGACCATTATTCTGTC	96
<i>pelL</i>	P0763	CGGCAATCATCGCATTACC	...
	P0764	GTTACAGAAGCAGACAGAGAC	104
<i>pell</i>	P0765	TCAGGCTCAGGTCGTATC	...
	P0766	TCATTCCGACAGGAGGTG	109
<i>pelX</i>	P0779	AACAAACGCCGACCTTCC	...
	P0780	TCCTGATGGGTGACTAAATCC	105
ABF-0019391	P0781	AAACACGTCGAATTACAG	...
	P0782	AATTCAGTATCGGAAATCG	86
<i>avrL</i>	P0783	CAGAACACCACCGATCCAATCAG	...
	P0784	CGCCCAATCACCAGAAGAAGTC	101
<i>rplU</i>	P0275	GTTTGACCAAGTTCTGATGGTTGC	...
	P0276	CCAGCCTGCTTACGGTAGTGTTTA	91
<i>gyrA</i>	P0791	CCACCCGTATCCCGAATC	...
	P0792	ACAACCGTCAATCACTTCAG	90
<i>rpoS</i>	P0789	CGCTGTGGATCTGATTG	...
	P0790	ACGATATGGATGGGTAAACG	109
<i>ompA</i>	P0787	CAGACAGCCACGACAATC	...
	P0788	TAGCGTATTCAACACCCACAG	87

^z Primer efficiencies were determined from the dilution curve using the MyiQ Cycler software.

at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min, and the cDNA was stored at 4°C.

Expression of mRNA was analyzed by quantitative real-time PCR using the MyiQ detection system (Bio-Rad Laboratories). Samples were performed in triplicate in 25- μ l reactions; 12.5 μ l of iQ SYBR Green Supermix (Bio-Rad Laboratories), 0.2 μ M forward and reverse primer, and 11.5 μ l of 1:10 diluted cDNA sample. Target gene abundance was initially normalized to combinations of three of four internal reference transcripts (*rplU*, *gyrA*, *rpoS*, or *ompA*) which were shown to be invariant using *BestKeeper* (Pfaffl et al. 2004). For later experiments, only *rpoS* and *gyrA* were used because the four references were behaving similarly and we found that inclusion of a third reference did not affect data interpretation. The RER was calculated as the difference between the C_t values and was determined using the equation $2^{-\Delta\Delta C_t}$ as previously modified (Pfaffl 2001; Rotenberg et al. 2006). PCR conditions were 95°C for 3 min; 40 cycles of 95°C for 10 s and 50°C for 45 s; and 1 cycle of 95°C for 1 min and 55°C for 1 min; followed by a dissociation curve with 80 cycles of 55°C for 10 s with a 0.5°C increase per cycle. The dissociation (Melt) curve performed with each assay certified the absence of primer-dimers and the presence of a single product from each primer reaction. To assure PCR accuracy, PCR reaction products were sequenced directly and compared with the expected target sequence.

For each gene, RNA was isolated from three cultures of the wild type and three cultures of the *fliA* mutant. Two cDNA reactions were prepared per culture for a total of six cDNA reactions. Two RT-PCR reactions were prepared per cDNA for a total of 12 RT-PCR assays per strain. Genes found to be statistically different with these 12 RT-PCR data points were analyzed a second time as described above to confirm the results. Statistical analysis of RER values was performed with GraphPad Prism software using the unpaired two-tailed *t* test function (GraphPad Software, Inc). Data from all cultures tested were combined for these analyses.

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