Influence of Flagellin Polymorphisms, Gene Regulation, and Responsive Memory on the Motility of *Xanthomonas* Species That Cause Bacterial Spot Disease of Solanaceous Plants

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Increasingly, new evidence has demonstrated variability in the epitope regions of bacterial flagellin, including in regions harboring the microbe-associated molecular patterns flg22 and flgII-28 that are recognized by the pattern recognition receptors FLS2 and FLS3, respectively. Additionally, because bacterial motility is known to contribute to pathogen virulence and chemotaxis, reductions in or loss of motility can significantly reduce bacterial fitness. In this study, we determined that variations in flg22 and flgII-28 epitopes allow some but not all *Xanthomonas* spp. to evade both FLS2- and FLS3-mediated oxidative burst responses. We observed variation in the motility for many isolates, regardless of their flagellin sequence. Instead, we determined that past growth conditions may have a significant impact on the motility status of isolates, because we could minimize this variability by inducing motility using chemoattractant assays. Additionally, motility could be significantly suppressed under nutrient-limited conditions, and bacteria could “remember” its prior motility status after storage at ultracold temperatures. Finally, we observed larger bacterial populations of strains with flagellin variants predicted not to be recognized by either FLS2 or FLS3, suggesting that these bacteria can evade flagellin recognition in tomato plants. Although some flagellin variants may impart altered motility and differential recognition by the host immune system, external growth parameters and gene expression regulation appear to have more significant impacts on the motility phenotypes for these *Xanthomonas* spp.

Keywords: bacterial pathogenesis, bacterial spot, elicitors, flagellin, MAMPs, motility, tomato, *Xanthomonas*

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Motility is an ancient characteristic of prokaryotes and is closely related to chemotaxis, which is the capacity of the bacteria to orientate along a chemical gradient. These features are essential for bacteria to adapt to changing environmental conditions (Ottemann and Miller 1997). Bacteria move toward favorable environments to acquire nutrients and identify suitable places to colonize, behaviors that are possible due to these motility and chemoattractant capacities. Many bacteria use tail-like structures called flagella to move within their environment. There are additional types of bacterial movements that do not involve flagella such as twitching, actin-based intracellular motility, and gliding motility; however, flagellum-dependent motility that includes swimming and swarming is the best characterized type of motility (Josenhans and Suerbaum 2002).

The flagellum is a rotating organelle that includes a type III secretion system, which includes a molecular motor in the inner bacterial membrane, a hook-basal body complex that acts as a junction element, and a rotating filament that is composed of several thousand flagellin protein subunits (Josenhans and Suerbaum 2002). Flagellated bacteria expend a large amount of energy to produce the flagellum and to power flagellum-dependent movement (Josenhans and Suerbaum 2002; Ottemann and Miller 1997). Thus, expression and synthesis of flagellum components are tightly regulated. Flagellin is encoded by the gene *fliC*, which is usually found as part of the conserved flagellum gene cluster (Ausubel 2005). Flagellin proteins have conserved N-terminal and C-terminal domains that are located within the center of the flagellum structure, whereas regions that are more surface exposed have increased variability between different bacterial species (Fliegmans and Felix 2016).

Plants have evolved multiple cell surface pattern-recognition receptors (PRRs) that can recognize either buried or surface-exposed regions of flagellin. Initially identified in *Arabidopsis thaliana*, FLAGELLIN SENSING 2 (FLS2) is a PRR that is present broadly across the plant kingdom and recognizes the flagellin-derived 22-amino-acid peptide called flg22, considered a microbe-associated molecular pattern (MAMP) (Saijo et al. 2018; Zipfel et al. 2004). In addition to FLS2, other PRRs can recognize regions of flagellin. The solanaceous-specific FLAGELLIN SENSING 3 (FLS3) receptor recognizes the 28-amino-acid peptide called flgII-28, another MAMP (Cai et al. 2011; Hind et al. 2016). Although tomato (*Solanum lycopersicum*) plants have both FLS2 and FLS3 receptors, subtle differences between these PRRs indicate that they may have nonredundant functionality (Roberts et al. 2020). In potato (*S. tuberosum*), flgII-28 treatment induces greater defense responses compared with flg22 (Moroz and Tanaka 2020). Rice (*Oryza sativa*) also

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possess a second flagellin receptor that recognizes a region separate from flg22 (Che et al. 2000; Takai et al. 2008); however, neither the PRR nor the specific MAMP have been identified.

Chemotactic movements are important for plant pathogens, particularly at early stages of infection (Chatterjee et al. 2010; Kamoun and Kado 1990; Moreira et al. 2015; Shen and Ronald 2002; Yaryura et al. 2015). For most phytopathogens, chemotaxis and motility are necessary to find and enter the host plant through natural openings such as stomata, hydathodes, wounds, and roots (Huang 1986), although motility or chemotaxis are not necessary for pathogen virulence if the pathogen is directly inoculated into the host plant (An et al. 2014; Yaryura et al. 2015). Additionally, the flagella can help adhere the pathogenic bacteria to its host (Merritt et al. 2007; Yu et al. 2013). Bacteria are known to switch from motile to nonmotile states after successful host infection, and this phenotypic plasticity occurs, in part, through changes in expression of the flagellum-related genes, including *flhC* (Vinatzer et al. 2014). The foliar pathogen *Pseudomonas syringae* pv. *syringae* decreases expression of flagellar and chemotaxis genes when switching from epiphytic to apoplastic growth (Yu et al. 2013). After the vascular pathogen *Ralstonia solanacearum* enters the xylem, the bacteria reduce their expression of *flhC*, leading to decreased motility (Tans-Kersten et al. 2004). Additionally, *Xanthomonas oryzae* pv. *oryzae* requires *flhC* expression to enter stomata in rice leaves (Kumar Verma et al. 2018). Although many phytopathogenic bacteria with reduced or absent flagella have concomitant reductions in virulence, some nonflagellated or nonmotile bacteria are also virulent on plants (Matilla and Krell 2018).

Bacteria in the genus *Xanthomonas* are Gram negative and rod shaped, and most species have a single polar flagellum. Members of this large group of phytopathogens can infect almost 400 plant species, although individual species or pathogens usually have narrow host ranges (Potnis et al. 2015). There are four species of *Xanthomonas* that cause bacterial spot disease in members of the Solanaceae family, which includes tomato, pepper, and chili pepper plants. These species include *X. euvesicatoria*, *X. vesicatoria*, *X. perforans*, and *X. gardneri*. Bacterial spot symptoms include small necrotic lesions that can occur on all aboveground plant parts. The disease is globally rampant, in part because it is seedborne, but also because these bacteria can infect solanaceous host plants at a wide range of temperatures (Jones et al. 1986).

Better understanding of host-pathogen interactions increases the chances of developing disease-resistant crops. Disease-resistant plants are especially important for crops affected by devastating bacterial diseases such as bacterial spot disease incited by *Xanthomonas* spp., which cause significant annual yield losses. We hypothesized that flagellin polymorphisms may help *Xanthomonas* spp. avoid host recognition but that some of these polymorphisms could reduce bacterial motility. In this study, flagellin proteins were characterized for the four *Xanthomonas* spp. pathogenic on tomato and pepper plants. Six flagellin variants were identified, and a collection of isolates was evaluated for variations in motility as well as for motility shifts under inductive and suppressive conditions. In addition, isogenic strains expressing the flagellin variants were created and evaluated for differences in motility as well as exposed to different environmental conditions in order to evaluate changes in *flhC* expression. Finally, tomato plants were inoculated with the isogenic strains to test for altered virulence caused by differential flagellin recognition.

RESULTS

Xanthomonas flg22 and flgII-28 polymorphisms affect oxidative burst responses in tomato.

Given the importance of bacterial spot disease on worldwide tomato and pepper production, we wanted to know whether

variability in the flgII-28 region of flagellin found in different *Xanthomonas* spp. impacts recognition by host plants. We first sought to test the findings from previous studies that indicated that *Xanthomonas* flagellin was not recognized by tomato plants (Felix et al. 1999). We identified two different flg22 epitopes present in the four *Xanthomonas* spp. that infect tomato and pepper plants. One variant is present in *X. euvesicatoria* and *X. perforans*, while the other variant is found in *X. gardneri*; these peptides differ by four amino acid residues and differ from the canonical flg22 sequence from *P. aeruginosa* by nine and eight residues, respectively (Fig. 1A). Notably, the *Xanthomonas* flg22 epitopes have a valine at position 43 instead of the conserved aspartic acid. This residue is important for full activity of the peptide (Felix et al. 1999) and for binding to the FLS2 receptor (Meindl et al. 2000; Parys et al. 2021; Sun et al. 2013), and the D43V variant leads to loss of flg22 perception in *A. thaliana* (Naito et al. 2008; Sun et al. 2006; Wang et al. 2015), *Nicotiana tabacum* (Naito et al. 2008), and *O. sativa* (Wang et al. 2015). Polymorphisms in the D43 residue can also reduce bacterial motility (Naito et al. 2008; Parys et al. 2021). An additional variant was identified from *X. vesicatoria* that differed by only one residue (position 42) from the *X. euvesicatoria*/*X. perforans* variant; however, because this residue matched the amino acid found in the *X. gardneri* variant (Supplementary Table S1), we decided not to include it in our experiments. Neither flg22 peptide from *Xanthomonas* spp. was recognized by tomato leaves, as measured by oxidative burst assays, whereas the canonical flg22 elicited a strong response (Fig. 1C and D).

Next, we identified several flgII-28 variants from *Xanthomonas* spp., including one that is present in some *X. campestris* pv. *campestris* species, another that is found in strains of many *Xanthomonas* spp., and a third that is uniquely present in *X. euvesicatoria* strains (Fig. 1B). The *X. euvesicatoria* variant differs from the *Xanthomonas* consensus variant by one amino acid residue, and both differ from the *X. campestris* variant by six or five residues, respectively. Compared with the robust activity elicited by the flgII-28 from *P. syringae* pv. *tomato* T1, the oxidative burst response of the *X. campestris* and *Xanthomonas* consensus variants was significantly reduced but still greater than the water control (Fig. 1C and D). However, there was no difference in the response between the water control and *X. euvesicatoria* flgII-28 treatments, indicating that this flgII-28 variant cannot be recognized by tomato FLS3. Together, these results suggest that some but not all *Xanthomonas* spp. can evade both FLS2- and FLS3-mediated oxidative burst responses in tomato.

Flagellin polymorphisms in *Xanthomonas* pathogenic strains that cause bacterial spot disease.

Given the importance of motility for *Xanthomonas* to naturally infect their host plants (An et al. 2014; Yaryura et al. 2015), we wanted to know whether the variations present in flagellin sequences from different *Xanthomonas* spp. affected the motility of the bacteria. First, we performed an *in silico* analysis of flagellin using sequence information deposited in the NCBI. We identified six flagellin protein variants in the four *Xanthomonas* spp. that cause disease in solanaceous species. One variant was found in *X. euvesicatoria* strains (flgXe-1), two variants were identified from *X. perforans* strains (flgXp-1 and flgXp-2), one variant was found in *X. vesicatoria* strains (flgXv-1), and two variants were identified from *X. gardneri* strains (flgXg-1 and flgXg-2) (Supplementary Table S1). The identity between the variants matched the phylogenetic relationships between the species (Jacques et al. 2016), with the greatest similarity between *X. euvesicatoria* and *X. perforans* variants, followed by less similarity between these three variants and the variant found in *X. vesicatoria*, and finally the greatest sequence divergence with the *X. gardneri* strains (Fig. 2).

Fig. 1. Polymorphisms in conserved flagellin regions prevent immune recognition by tomato plants. Comparisons of **A**, flg22 and **B**, flgII-28 sequences. Reference sequences are flg22 from *Pseudomonas aeruginosa* (Felix et al. 1999) and flgII-28 from *P. syringae* pv. *tomato* T1 (Cai et al. 2011). The top line indicates the residue position using the *Xanthomonas* flagellin sequence, the bottom line indicates the residue position within the peptide sequence, and shaded residues indicate polymorphisms relative to the references. **C and D**, Oxidative burst produced by *Solanum lycopersicum* 'Brandywine' tomato plants. Leaf disks were treated with 100 nM peptide or water, and the total amount of reactive oxygen species (ROS) was measured over 45 min and quantified using relative light units (RLUs). Results shown are the means of three independent experiments, with error bars indicating the standard error of the means ($n = 4$ plants per experiment, with each plant consisting of three disks per treatment). Different letters indicate significant differences between treatments ($P < 0.05$, one-way analysis of variance using least squares method).

Next, we tested the motility of a collection of *Xanthomonas* isolates. The collection included 18 *X. euvesicatoria* isolates, 8 *X. perforans* isolates, 4 *X. vesicatoria* isolates, and 5 *X. gardneri* isolates (Supplementary Table S2). The *flhC* gene was sequenced for all strains and the results confirmed that we had isolates with all of the six possible flagellin variants. We performed motility assays for each of the strains and, although we observed that motility differed greatly between the strains, there was no clear pattern of differences based on the flagellin variant that each strain possessed (Fig. 3).

It has been shown previously that *X. oryzae* pv. *oryzae* bacteria grown on solid (>1%) agar media are not flagellated (Shen et al. 2001). However, *Xanthomonas* spp. can switch from non-motile to motile states, with the increased motility related to chemotactic behavior (Kamoun and Kado 1990; Shen et al. 2001). We hypothesized that growth on solid media may have unequally altered the motility for the strains in our collection and, therefore, we decided to test whether bacterial motility could be induced using chemotactic attraction assays. Because methionine has been shown to induce motility for *Xanthomonas* spp. (Kumar Verma et al. 2018), we initially evaluated the chemoattraction potential for all isolates using methionine and observed that 17 of the 35 isolates showed increased motility after treatment (Supplementary Tables S2 and S3). To select additional compounds to test for chemoattraction, we used KBase (Arkin et al. 2018) to predict potential auxotrophies using a reference genome for each *Xanthomonas* sp. We performed chemoattraction assays using nine additional compounds, with varying success (Supplementary Table S3). Some of the isolates that did not respond to any of the treatments were already fairly motile (e.g., X0008 and X0010); thus, it was likely that they could not further increase their motility. Other isolates, including the reference *X. euvesicatoria* 85-10 strain (X0001), did increase their motility in response to treatment with other compounds (Supplementary Tables S2 and S3; Supplementary Fig. S3C and D), although there was not a pattern in this response. Interestingly, the *X. gardneri* isolates did not increase their motility in response to any of

the chemoattractant compounds tested (Supplementary Table S3), with all isolates showing less motility when compared with isolates of the other three species (Figs. 3 and 4).

Once we were able to induce bacterial motility, we wanted to test whether we could shift the isolates from motile to non-motile states using growth on minimal media (MM) (Kamoun and Kado 1990). We observed reduced motility of all *Xanthomonas* isolates except one (X0034) after 4 days of growth on MM (Fig. 3; Supplementary Table S2). When the motilities of isolates with identical flagellin variants were grouped together, each of the four groups showed significantly reduced motility after growth on MM (Fig. 4), indicating that motility phenotypes can be altered depending on the growth media. Because we had only one isolate each for flagellin variants flgXp-2 and flgXg-2, we were unable to determine the average motility for these variants. When we compared the motility of individual strains under inductive versus suppressive conditions, we saw more variability for the strains with the flgXe-1 and flgXp-1 variants as compared with those with the flgXv-1 and flgXg-1 variants (Supplementary Fig. S4). This is likely due to an overall lower number of strains with the flgXv-1 and flgXg-1 variants as well as the lower induced motility of these strains.

To test whether prior motility induction conditions affect the motility phenotype of the isolates, we assessed motility of the isolates before and after storage at ultracold temperatures. We hypothesized that, because these isolates came from different sources, they could have undergone different handling over time and thus have “remembered” these different conditions, despite our uniform handling and preservation methods. The ability of bacteria to “remember” past events to influence current behavior is a phenomenon termed “response memory” and has been documented in other bacteria, including altered virulence phenotypes in *P. aeruginosa* (Kordes et al. 2019), changes in stimuli-induced chemotactic behaviors in *Escherichia coli* (Gosztolai and Barahona 2020), and persistence of gene expression after removal of the carbon inducer in *E. coli* (Lambert and Kussell 2014). In order to test this, motility was induced and isolates

Fig. 2. Phylogenetic relationship and sequence similarity of flagellin variants from four *Xanthomonas* spp. **A**, Phylogenetic analysis of six flagellin protein variants from *Xanthomonas* spp. that are pathogenic for tomato and pepper. Variants include flagellin sequences from *Xanthomonas euvesicatoria* (flgXe-1), *X. perforans* (flgXp-1 and flgXp-2), *X. vesicatoria* (flgXv-1), and *X. gardneri* (flgXg-1 and flgXg-2). Branch lengths are measured in the number of substitutions per site from a total of 399 positions, and the branches are annotated with a number representing the percentage of trees in which the associated taxa clustered together. **B**, Identity matrix showing the percent similarity between the amino acid sequences of the flagellin variants. Green shading indicates high similarity, yellow shading indicates moderately high similarity, and orange shading indicates moderate similarity.

Fig. 3. Comparison of motility of *Xanthomonas* isolates under inductive and suppressive conditions. For each isolate, motility was assessed prior to any intervention (original), after growth on nutrient media containing chemoattractant chemicals (induced), or after growth on minimal nutrient media (suppressed). Motility was quantified at 24 h (for suppressed) or 48 h (for original and induced) after inoculation by measuring the diameter of the circular halo formed in the media by the swimming bacteria. Data shown are means of three independent experiments, with error bars indicating the standard error of the means ($n = 4$ replicates per experiment). Motility of **A**, 18 *Xanthomonas euvesicatoria* isolates with the flgXe-1 variant; **B**, 7 *X. perforans* isolates with the flgXp-1 variant; **C**, 1 *X. perforans* isolate with the flgXp-2 variant; **D**, 4 *X. vesicatoria* isolates with the flgXv-1 variant; **E**, 4 *X. gardneri* isolates with the flgXg-1 variant; and **F**, 1 *X. gardneri* isolate with the flgXg-2 variant. Specific chemoattractants used for each isolate can be found in Supplementary Table S2.

were either immediately evaluated using motility assays, or suspended in 20% glycerol and stored at -80°C . Isolates stored at ultracold conditions were streaked onto solid agar media (peptone sucrose media [PSA] with 1.2% agar); then, motility was evaluated. No differences were observed between the motility of the strains before and after ultracold storage (Fig. 5), suggesting that growth conditions prior to storage influenced the motility phenotypes of bacteria. We attempted similar experiments for motility suppression using growth on MM; however, we observed that poststorage growth of isolates on solid agar nutrient media was sufficient to induce some motility of the strains (data not shown). Together, these results suggest that it is possible to switch the motility phenotypes of *Xanthomonas* isolates using artificial bacteriological media to induce motility, and that the variability in motility that we originally observed was likely due to past growth conditions.

Motility and *fliC* expression of *X. euvesicatoria* isogenic strains.

In order to directly compare the motility potential of the different flagellin variants, we constructed isogenic strains that varied only in their *fliC* genes. Using biparental mating and double recombination of the pK18*mobsacB* suicide plasmid, we generated a *fliC* deletion mutant in the reference strain *X. euvesicatoria* 85-10 (85-10 Δ *fliC*) by biparental mating. The

fliC gene was replaced in-frame with the tetracycline resistance gene, and replacement was confirmed using PCR (Supplementary Fig. S2B). As expected, the 85-10 Δ *fliC* strain was nonmotile (Supplementary Fig. S2). Next, the tetracycline resistance gene was replaced by one of six alleles (*fliC*_{Xe-1}, *fliC*_{Xp-1}, *fliC*_{Xp-2}, *fliC*_{Xv-1}, *fliC*_{Xg-1}, or *fliC*_{Xg-2}), and gene replacement for all strains was confirmed using PCR (Supplementary Fig. S2D). All isogenic strains had similar induced motility after treatment with the chemoattractant thymidine (Fig. 6A), which was used because *X. euvesicatoria* 85-10 (X0001) was not responsive to treatment with methionine (Supplementary Table S3). Motility was significantly suppressed in the isogenic strains after growth on MM, although the level of suppression was more variable compared with motility induction (Fig. 6A).

Significant differences in motility induction between isogenic strains were present after 48 h of growth in soft agar plates (Fig. 6B). Surprisingly, unlike the results observed for the isolate collection, the strains with the *X. gardneri* flagellin variants were more motile than some of the other alleles, with the strain expressing the flgXg-2 variant having motility comparable with that observed for both *X. perforans* variants (flgXp-1 and flgXp-2) (Fig. 6B). These results could be explained by differences in the intrinsic motility activity of each variant or by differences in expression of the different alleles.

We expected that expression of *fliC* should have been similar in all isogenic strains, given that the promoter and terminator sequences were identical in each strain. Surprisingly, we observed significant differences in expression of the different alleles, with *fliC* alleles from *X. perforans* having greater expression and those from *X. vesicatoria* have less expression when compared with the *fliC* allele from *X. euvesicatoria* (Fig. 7A; Supplementary Fig. S5A). These expression results correspond to the greater and lesser motility of the corresponding strains (Fig. 6B). However, although the *fliC* alleles from *X. gardneri* were significantly less expressed compared with the strain with the *fliC* allele from *X. euvesicatoria* (Fig. 7A), this difference did not correlate with the observed motility of the strains (Fig. 6B). This discrepancy suggests that factors other than flagellar expression may affect motility for strains containing

Fig. 4. Comparison of motility for each *Xanthomonas* sp. under inductive and suppressive conditions. For each species, the average motility for each isolate was assessed prior to any intervention (original), after growth on nutrient media containing chemoattractant chemicals (induced), or after growth on minimal nutrient media (suppressed). Motility was quantified at **A**, 24 h and **B**, 48 h after inoculation by measuring the diameter of the circular halo formed in the media by the swimming bacteria. Data shown are the mean motility of isolates for each species, with error bars indicating the standard deviation ($n = 18$ isolates for flgXe-1, 7 isolates for flgXp-1, 4 isolates for flgXv-1, and 4 isolates for flgXg-1). Asterisks indicate significant differences between inductive and suppressive treatments for each species ($P < 0.05$, one-way analysis of variance using Kruskal-Wallis test followed by Dunn's posthoc test). Specific chemoattractants used for each isolate can be found in Supplementary Table S2.

Fig. 5. Evaluation of motility changes after storage at ultralow-temperature conditions. For each species, the average motility for each isolate was assessed after growth on nutrient media containing chemoattractant chemicals. Motility was quantified either before (prestorage) or after (poststorage) bacteria were stored at ultralow-temperature conditions (-80°C). Data shown are the mean motility of isolates for each species, with error bars indicating the standard deviation ($n = 18$ isolates for flgXe-1, 7 isolates for flgXp-1, 4 isolates for flgXv-1, and 4 isolates for flgXg-1). No significant differences were observed between the treatments for each species ($P < 0.05$, one-way analysis of variance using Kruskal-Wallis test followed by Dunn's posthoc test). Specific chemoattractants used for each isolate can be found in Supplementary Table S2.

the *X. gardneri* variants. As anticipated, the expression of *fliC* was significantly less in all strains after growth on MM (Fig. 7B; Supplementary Fig. S5B), supporting the corresponding motility changes observed for the strains (Fig. 6A).

Altered virulence phenotypes of *X. euvesicatoria* isogenic strains.

Finally, in order to assess the effects of the flagellin polymorphisms on plant immunity, we performed in planta bacterial virulence assays using vacuum infiltration. Plants of tomato cultivar 'Brandywine' were inoculated with suspensions containing either one of the six isogenic strains, the reference strain *X. euvesicatoria* 85-10, or the *X. euvesicatoria* $\Delta fliC$ mutant strain, and bacterial populations were determined at 0, 6, and 9 days postinoculation

(dpi). We observed larger bacterial populations of the reference, mutant, and flgXe-1 isogenic strains as compared with the other five isogenic strains at both 6 and 9 dpi (Fig. 8). Together with the reactive oxygen species (ROS) results from Figure 1, these findings indicate that the single amino acid polymorphism in the flgII-28 sequence from *X. euvesicatoria* 85-10 allows for evasion of flagellin recognition by the FLS3 receptor in tomato plants.

DISCUSSION

There have been significant efforts to characterize the variability of flg22 recognition by FLS2, including several studies involving *Xanthomonas* spp. (Bhattarai et al. 2016; Felix et al.

Fig. 6. Comparison of motility for *Xanthomonas euvesicatoria* isogenic strains expressing different flagellin variants. For each strain, the average motility was assessed prior to any intervention (original), after growth on nutrient media containing the chemoattractant thymidine (induced), or after growth on minimal nutrient media (suppressed). Motility was quantified at **A**, 24 h and **B**, 48 h after inoculation by measuring the diameter of the circular halo formed in the media by the swimming bacteria. Data shown are the means of three independent experiments with error bars indicating the standard error of the means ($n = 4$ replicates per experiment). Asterisks indicate significant differences between original and inductive treatments or inductive and suppressive treatments for each isogenic strain ($P < 0.05$, pairwise comparison using Student's *t* test), while different letters indicate significant differences between the isogenic strains after inductive treatment ($P < 0.05$, one-way analysis of variance using least squares method).

1999; Shi et al. 2016; Sun et al. 2006; Trdá et al. 2014; Wang et al. 2015); however, there have not yet been similar efforts for flgII-28 recognition by FLS3. We found that the oxidative burst response of tomato plants to treatment with *Xanthomonas* flgII-28 peptides is significantly less than that observed after treatment with a peptide from *P. syringae*, and the flgII-28 variant from *X. euvesicatoria* was completely inactive. In addition, there are many examples of altered flg22 sequences that permit phyto bacteria such as *R. solanacearum*, *P. cannabina* pv. *alisalensis* (syn: *P. syringae* pv. *maculicola*), and *Agrobacterium tumefaciens* to avoid host recognition by FLS2 (Clarke et al. 2013; Felix et al. 1999; Pfund et al. 2004). Our results indicate that some *Xanthomonas* spp. may evade both FLS2- and FLS3-mediated immunity, which would support previous work where tomato suspension-cultured cells did not respond to treatment with flagellin derived from *X. euvesicatoria* (syn. *X. campestris* pv. *vesicatoria*) (Felix et al. 1999). However, our results indicate that tomato plants can still recognize flgII-28 from other *Xanthomonas* spp., including *X. perforans*, *X. vesicatoria*, and *X. gardneri*, and that this recognition can reduce the virulence of bacterial strains with these flagellin variants. Given the known differential robustness of ROS responses to flgII-28 treatment (Bhattarai et al. 2016; Cai et al. 2011; Veluchamy et al. 2014), it is possible that

some tomato varieties or other solanaceous species, including potato and pepper, have FLS3 receptor variants that are able to perceive and respond to flgII-28 from *X. euvesicatoria*. Future work could include screening additional germplasm for natural variations in FLS3 activity; if FLS3 variants are discovered, then they could be used to help identify potential ligand binding sites.

Initially, the motility of the *Xanthomonas* isolates could not be correlated with their flagellin variant; however, less variation was observed after bacterial motility was induced using chemoattractants, with *X. euvesicatoria* and *X. perforans* isolates showing greater motility overall when compared with *X. vesicatoria* and *X. gardneri* isolates. It is known that bacterial motility in soft media (0.2 to 0.4% agar) is related to chemotaxis (Adler 1966), and a previous study observed that *X. oryzae* pv. *oryzae* isolates were not flagellated when grown on solid agar media but they had flagella visible via electron microscopy when grown on swarming media (0.3% agar) (Shen et al. 2001). Our chemoattractant assays mirrored these observations, because we also found that bacteria isolated from the ring boundaries of soft agar (0.2%) media were more motile compared with when the bacteria were previously grown on solid media. The *Xanthomonas* isolates in our study did not respond equally to the different potential chemoattractants, although most did respond to treatment with methionine. Surprisingly, the *X. gardneri* isolates did not show chemoattractant behaviors to any of the 10 treatments tested, even though KBase predicted that the *X. gardneri* reference strains JS749-3 (NCBI RefSeq NZ_CP018728) and ICMP 7383 (NCBI RefSeq NZ_CP018731) are likely auxotrophic for 4 to 6 of these compounds (i.e., leucine, isoleucine, cysteine, methionine, and potentially arginine and proline). Therefore, we cannot confirm whether the *X. gardneri* isolates exhibited their maximum potential motility, although the greater motility of the isogenic strains expressing the flagellin variants flgXg-1 and flgXg-2 indicates that the reduced motility of *X. gardneri* isolates is not due to reduced activity of flagellin itself.

Bacterial growth under nutrient starvation conditions can result in the loss of motility due to downregulation of flagellum-related genes (Santander et al. 2014). Similar findings were observed for the wheat pathogen *Azospirillum brasilense*, where bacterial motility was greatly reduced upon exposure to metabolic stress conditions and nutrient starvation (Sadasivan and Neyra 1985). Previous work found that *Xanthomonas* swarming motility could not be suppressed using minimal nutrient bacteriological media but, instead, only could revert motile bacteria back to nonmotile after infection and reisolation from a host plant (Kamoun and Kado 1990). In contrast, using the same media recipe described by Kamoun and Kado (1990), we observed that isolates of the four *Xanthomonas* spp. tested in our study had significantly reduced motility. Although we cannot definitively elucidate the reasons underlying this discrepancy, it is possible that the differences could be due to suppression of swimming (0.2% agar) instead of swarming (0.3% agar) motility. Regardless, this additional in vitro method provided an efficient way to evaluate motility suppression phenotypes for a large collection of isolates and isogenic strains.

There are several examples where swarming behaviors have been lost in common laboratory strains due, in part, to the conditions the bacteria experience when grown on standard nutrient agar media with >1% agar (Kearns 2010). We have observed swarming behavior on 0.3 to 0.7% agar media for some of our *Xanthomonas* isolates that had only limited swimming motility were grown on soft agar (0.2%) media. However, this motility was not observed when the same strains were grown on 1.2% agar media, suggesting that *Xanthomonas* swarming motility is suppressed by the agar concentrations normally present in growth media.

Fig. 7. Comparison of *fliC* gene expression for *Xanthomonas euvesicatoria* isogenic strains expressing different flagellin variants. For each strain, bacterial samples were collected prior to any interventions (original) or 24 h after growth on minimal nutrient media (suppressed). Transcript abundance of *fliC* was determined by reverse-transcription quantitative PCR analysis using *gyrB* as the reference gene for quantification using the $2^{-\Delta\Delta CT}$ method. **A**, Relative ratio of *fliC* expression of isogenic strains compared with the *X. euvesicatoria* flgXe-1 strain. **B**, Relative ratio of *fliC* expression in strains after suppressive treatments compared with growth under original conditions. Data shown are the means ($n = 2$ biological replicates with 4 technical replicates per biological replicate), with error bars indicating the standard deviation. Different letters indicate significant differences between isogenic strains ($P < 0.05$, one-way analysis of variance using least squares method) while asterisks indicate significant differences between isogenic and suppression treatments for each variant ($P < 0.05$, pairwise comparison using Student's *t* test).

We believe that some of the motility differences observed between the isogenic strains can be explained by differences in the expression of *fliC* but we cannot explain why the *fliC* alleles would be differentially expressed when each gene was integrated into the genome under the control of the native promoter and terminator sequences. Previously, increased *fliC* expression and greater motility was observed for *E. coli* present in the outer ring boundary for bacteria grown in soft agar media (Koster et al. 2012); however, each isogenic strain was handled similarly, and the motility and *fliC* expression were comparable after independent chemoattractant assays, suggesting that experimental variability or error alone cannot account for these differences. We attempted to evaluate the abundance of flagellin proteins in each isolate; however, there are only a few commercial anti-flagellin antibodies available, none of which were generated using flagellin that was similar to *Xanthomonas*. We tested two of the available antibodies, both of which are known to recognize flagellin from human pathogens, including *Salmonella enterica*, *E. coli*, and *Lactobacteria acidophilus* (BioLegend number 629701, discontinued; Abcam ab93713); however, neither antibody appeared to recognize flagellin proteins from *Xanthomonas* spp. (data not shown). Another explanation is that other factors differentially influenced the abundance of *fliC* transcripts in the isogenic strains, including altered transcription due to RNA secondary structure (Chelkowska-Pauszek et al. 2021), differential posttranscriptional regulation by small RNAs or RNA-binding proteins (Christopoulou and Granneman in press; Djapagne and Oglesby 2021), or altered RNA processing or degradation of the transcripts (Trinquier et al. 2020). Future work could include additional experiments to test for the presence of other factors differentially influencing the abundance of the different *fliC* transcripts.

It has been proposed that the history-dependent behaviors of bacterial cells such as “remembered” responses to prior environmental stress could be described as a form of memory (Wolf et al. 2008). The term “response memory” was used to explain the behavior of cells that continued to respond after removal of the stimulus; more specifically, the continued expression of *lac* protein in *E. coli* after the removal of lactose from the environment (Lambert and Kussell 2014). Indeed, memory may function as

one component of a broader framework of the adaptive behavior observed in how bacteria interact with their ever-changing environment (Pessione 2021). In our study, the similar motility of isolates prior to and after ultracold storage suggests that *Xanthomonas* spp. may “remember” earlier environmental conditions and have adapted their phenotypes accordingly. These response memories may have differed between isolates obtained from different sources or maintained under different conditions. For example, some isolates may have been preserved soon after collection from plants, while others may have been grown for longer periods of time on standard laboratory (>1% agar) media, both of which could have resulted in altered motility. Our results indicate that future experiments regarding bacterial motility should take prior growth conditions into account.

Conclusions.

We provide evidence that some *Xanthomonas* spp. can evade both FLS2- and FLS3-mediated immunity, and that variability in the flagellin sequence may impact bacterial motility. In addition, variable motility between bacterial isolates may be due to external growth parameters, and motility can be induced by chemoattracts while it can be suppressed by nutrient-limiting media. We provide evidence that bacteria can “remember” responses to prior environmental conditions, and that these response memories influence the motility phenotypes of *Xanthomonas* bacteria.

MATERIALS AND METHODS

If not specifically noted, experiments were repeated three times with similar results.

Bacterial strains, plasmids, and growth conditions.

Xanthomonas and *E. coli* strains and plasmids used in this study are listed in Table 1 and Supplementary Table S2. *E. coli* strains were cultivated in Luria-Bertani medium at 37°C. *Xanthomonas* strains and isolates were grown in PSA medium (2% [wt/vol] sucrose, 0.5% [wt/vol] peptone, 0.05% [wt/vol] K₂HPO₄, 0.05% [wt/vol] MgSO₄·7H₂O, and 0.2% or 1.2% [wt/vol] agar, depending on the assay), MM (0.7% [wt/vol] NaCl, 0.115% [wt/vol] K₂HPO₄, 0.02% [wt/vol] KH₂PO₄,

Fig. 8. In planta virulence of *Xanthomonas euvesicatoria* isogenic strains. Six-week-old ‘Brandywine’ tomato plants were inoculated by vacuum infiltration with suspensions of each isolate of 5×10^4 CFU/ml. Bacterial populations were measured at 0, 6, and 9 days after inoculation. Data shown are the means of three independent experiments, with error bars indicating the standard error of the means ($n = 3$ plants per experiment, with each plant consisting of the average of three replicate samples). Different letters indicate significant differences between populations ($P < 0.05$, one-way analysis of variance using least squares method).

0.02% [wt/vol] KCl, 0.01% [wt/vol] yeast extract, and 1.2% [wt/vol] agar, and nutrient agar (NA) (0.5% [wt/vol] peptone, 0.3% [wt/vol] beef extract, 0.5% [wt/vol] NaCl, and 1.5% [wt/vol] agar). The antibiotics kanamycin (Km, 50 µg/ml), rifampicin (Rif, 25 to 50 µg/ml), and tetracycline (15 µg/ml) were added as required.

Materials.

Peptides were synthesized by Genscript (Piscataway, NJ, U.S.A.) at >90% purity. Primers were synthesized by Integrated DNA Technologies (Coralville, IA, U.S.A.) and are listed in Supplementary Table S4. All peptides were resuspended in water. Seeds of tomato (*Solanum lycopersicum* 'Brandywine') were obtained from Peaceful Valley Farm & Garden Supply (Grass Valley, CA, U.S.A.). Seedlings were grown in Berger BM6 soilless mix supplemented with vermiculite and osmocote 15-9-12 and kept in a greenhouse (25 and 21°C day and night temperatures, respectively) without supplemental lighting.

Sequence comparisons.

A phylogenetic analysis was performed using six flagellin protein sequences obtained in October 2017 from the NCBI: flgXe-1 (WP_007962409.1), flgXp-1 (WP_008575952.1), flgXp-2 (WP_046935349.1), flgXv-1 (WP_011037106.1), flgXg-1 (WP_046932599.1), and flgXg-2 (WP_006452268.1). Protein alignments were made using ClustalW and a maximum-likelihood tree was generated using MEGA X v10.0.2 (Kumar et al. 2018), with pairwise distances estimated using a JTT model and with 1,000 bootstrap replicates. An estimation of the pairwise percent similarity between six flagellin protein sequences was performed and an identity matrix was generated using MEGA X.

Oxidative burst assays.

Quantitation of ROS production for tomato plants was performed as previously described (Hind et al. 2016). Leaf disks of 6-week-old tomato plants were punched with a number 2 cork borer (4 mm in diameter) and floated adaxial side up overnight in 200 µl of distilled water in individual wells of a 96-well microassay plate. Four plants were used for each treatment, with three replicate disks sampled per plant. The water was replaced 16 h later with a solution containing 100 nM peptide (or water for the controls), luminol at 34 µg/ml (Millipore Sigma-Aldrich, St. Louis, MO, U.S.A.), and horseradish peroxidase at 20 µg/ml (type VI; Millipore Sigma-Aldrich). The luminescence was measured using a FilterMax F5 plate reader (Molecular Devices, San Jose, CA, U.S.A.) for 45 min at an interval of 3 min per read, for a total of 16 reads. Each read had an integration time of 1,250 ms. For each treatment, the total ROS production was calculated by summing the relative light units from the 16 reads. The peptides used for the treatments include three variants of flg22: the standard variant from *P. aeruginosa* (Felix et al. 1999); a consensus variant from *X. euvesicatoria*, *X. perforans*, and *X. vesicatoria*; and the variant from *X. gardneri*; and four variants of flgII-28: the standard variant from *P. syringae* pv. *tomato* T1 (Clarke et al. 2013), one variant from *X. campestris* pv. *campestris*, the consensus variant from multiple *Xanthomonas* spp., and one variant from *X. euvesicatoria*.

Bacterial assays.

Motility assays (outlined in Supplementary Fig. S1) were performed using PSA, and plates were inoculated from individual colonies of bacteria grown for 3 days on PSA 1.2% (wt/vol) agar plates at 28°C. Four individual spots per plate were penetrated using sterile toothpicks inoculated with bacteria. Plates were incubated at 28°C for 48 h and motility was assessed quantitatively by measuring the diameters of the circular halos formed within the media by the swimming bacteria. Each

experiment was repeated three times, for a total of 12 colonies assessed per treatment per isolate. Box plots were used to confirm the lack of outliers, and the Shapiro-Wilk normality test was used to confirm that all data were normally distributed.

Attraction assays were performed using the following chemoattractants: methionine, glutamic acid, thiamine, thymidine, arginine, alanine, leucine, isoleucine, cysteine, or proline. To prepare attraction plates, 5 µl of each 0.1 M chemoattractant solution was added to the center of the PSA 0.2% (wt/vol) agar plates before agar had completely solidified. Three equidistant positions were marked 2 cm away from the chemoattractant spot and the media were inoculated with bacteria at these locations. Bacteria were grown for 72 h; then, cells at the leading edge of the swimming halo were used to streak single colonies on PSA 1.2% (wt/vol) agar plates. Swimming assays were then performed using the resulting single colonies in order to measure postinduction motility.

Suppression assays were performed using MM (Kamoun and Kado 1990) with 1.2% (wt/vol) agar. Single colonies from PSA agar plates were used to inoculate the MM plates and then incubated at 28°C for 96 h. The resulting single colonies were used for motility assays in order to measure the effects of postattraction suppression of motility.

Construction of gene deletion mutant and complementation strains of *Xanthomonas*.

To create the *fliC* knockout mutant ($\Delta fliC$), a marker exchange strategy was applied using the suicide vector pK18*mobsacB* via allelic homologous recombination (Schäfer et al. 1994). The upstream (712 bp) and downstream (516 bp) regions adjacent to the *fliC* coding sequence were amplified from *X. euvesicatoria* 85-10 genomic DNA using the two primer pairs (P0071/P0074 and P0072/P0073) (Supplementary Table S4). The tetracycline resistance gene was used for allelic exchange and amplified from plasmid pRK415 using the primer pair P0075/P0076. The DNA was amplified using Platinum SuperFi DNA Polymerase (Invitrogen, Carlsbad, CA, U.S.A.), the PCR products were purified (E.Z.N.A. Microelute Cycle-Pure Kit, Omega Bio-Tek, Norcross, GA, U.S.A.), and the purified DNA was digested with the corresponding restriction enzymes for cloning into pK18*mobsacB*, resulting in the plasmid pK18 $\Delta fliC$ (Table 1). After sequence confirmation, the plasmid was conjugated from *E. coli* S17-1 λ pir into *X. euvesicatoria* 85-10 by biparental mating. NA plates containing Rif and Km were used to select bacteria that had undergone the first crossover event. Colonies were then plated on PSA containing 10% (wt/vol) sucrose to select for those colonies having undergone the second crossover event. The putative *X. euvesicatoria* 85-10 $\Delta fliC$ candidates were confirmed by colony PCR (Supplementary Fig. S2B), Sanger sequencing of the modified genomic region, and motility assays (Supplementary Fig. S3A and B).

For complementation, the upstream (712 bp) and downstream (516 bp) regions adjacent to the *fliC* coding sequence were amplified from *X. euvesicatoria* 85-10 genomic DNA using the two primer pairs P0071/P0074 and P0072/P0073 (Supplementary Table S4). Each of the *fliC* alleles were amplified using the following primer pairs for the corresponding strains: P0065/P0066 for *fliC_{Xe-1}* from *X. euvesicatoria* 85-10, for *fliC_{Xp-1}* from *X. perforans* 91-118, and for *fliC_{Xp-2}* from *X. perforans* 17-12; P0070/P0066 for *fliC_{Xv-1}* from *X. vesicatoria* ATCC 35937; and P0067/P0068 for *fliC_{Xg-1}* from *X. gardneri* SM177-10 and for *fliC_{Xg-2}* from *X. gardneri* ATCC 19865. The DNA was cloned into pK18*mobsacB* and the plasmids were conjugated into the *X. euvesicatoria* 85-10 $\Delta fliC$ strain, as described above. After selection for the second recombination event, candidates for the isogenic *fliC* complemented strains were confirmed by colony PCR (Supplementary Fig. S2D) and Sanger sequencing of the modified genomic region.

RNA extraction and reverse transcription of RNA templates.

Bacteria were treated as described above and as outlined in Supplementary Fig. S1. Bacteria were harvested and stored at -80°C until RNA was extracted using TRIzol Reagent (Invitrogen), and potential DNA contamination was removed using Turbo DNA-free Kit (Invitrogen). Total RNA (1 μg) was used to prepare cDNA using SuperScript III First-Strand Synthesis System for reverse-transcription PCR (RT-PCR) (Invitrogen).

Quantitative RT-PCR.

Primers used to amplify the target *fliC* gene were designed as follows: the alleles that represent the six variants were aligned, and primers were designed to target consensus sequences while avoiding any polymorphisms. Primers used to amplify the endogenous housekeeping genes *tmRNA* and *gyrB* were described previously (Yan et al. 2019). Primer sets P0236/P0237, P0247/P0248, and P0275/P0276 (Supplementary Table S4) were used to amplify *fliC*, *gyrB*, and *tmRNA*, respectively. Each 10- μl quantitative PCR

mixture contained 5 μl of PowerUp SYBR Green 2 $\times$ Master Mix (Applied Biosystems), 1 μl each of the primers (final concentration of 800 nM), 2 μl of nuclease-free water, and 1 μl of 1:10 diluted cDNA template. Quantitative RT-PCR was performed on a QuantStudio 3 Real-Time PCR System (Applied Biosystems) using fast cycling conditions. Data were normalized to *tmRNA* or *gyrB*, and analyzed using the $2^{-\Delta\Delta\text{CT}}$ method (Schmittgen and Livak 2008). Relative ratios of *fliC* expression were calculated by comparing the motility under different treatments or by comparing the motility of the different isogenic strains to the control strain.

Bacterial virulence assays.

Bacterial isolates were grown on PSA medium for 2 days prior to inoculation, and bacterial suspensions containing bacteria at 5×10^4 CFU/ml were prepared in a solution containing 10 mM MgCl_2 and 0.02% (vol/vol) Silwet L-77 (bioWORLD, Dublin, OH, U.S.A.). Six-week-old tomato plants were immersed in the bacterial suspension and vacuum infiltrated twice for 5 min

Table 1. Bacterial strains and plasmids used in this study, with their relevant characteristics and the reference or source

Strains and plasmids	Relevant characteristics ^a	Reference, source
Strains		
<i>Escherichia coli</i>		
DH5 α	λ -f80d <i>lac</i> ZDM15 D (<i>lac</i> ZYA- <i>arg</i> F) <i>U169</i> <i>recA1</i> <i>endA</i> <i>hdsR17</i> (r_K^- m_K^-) <i>supE44</i> <i>thi</i> <i>l</i> <i>gyrA</i> <i>relA</i>	Laboratory collection
S17-1 λ pir	RP4-2 Tc::Mu-Km::Tn7 <i>pro</i> <i>hdsR</i> <i>recA</i> Tra+ used as mobilizing strain	Simon et al. 1983
<i>Xanthomonas euvesicatoria</i>		
85-10	Wild type; <i>fliC</i> ₈₅₋₁₀ , Rif ^r	Laboratory collection
85-10 Δ <i>fliC</i>	85-10 Δ <i>fliC</i> :: Ω Tet, Rif ^r	This study
85-10 <i>fliC</i> _{Xc-1}	85-10 Δ <i>fliC</i> :: Ω <i>fliC</i> ₈₅₋₁₀ , Rif ^r	This study
85-10 <i>fliC</i> _{XP-1}	85-10 Δ <i>fliC</i> :: Ω <i>fliC</i> ₉₁₋₁₁₈ , Rif ^r	This study
85-10 <i>fliC</i> _{XP-2}	85-10 Δ <i>fliC</i> :: Ω <i>fliC</i> _{XP17-12} , Rif ^r	This study
85-10 <i>fliC</i> _{Xv-1}	85-10 Δ <i>fliC</i> :: Ω <i>fliC</i> ₁₁₁₁ (ATCC 35937), Rif ^r	This study
85-10 <i>fliC</i> _{Xg-1}	85-10 Δ <i>fliC</i> :: Ω <i>fliC</i> _{SM177-10} , Rif ^r	This study
85-10 <i>fliC</i> _{Xg-2}	85-10 Δ <i>fliC</i> :: Ω <i>fliC</i> ₁₀₁ (ATCC 19865), Rif ^r	This study
<i>X. vesicatoria</i>		
1111 (ATCC 35937)	<i>fliC</i> ₁₁₁₁	Potnis et al. 2011
<i>X. perforans</i>		
91-118	<i>fliC</i> ₉₁₋₁₁₈	Potnis et al. 2011
XP17-12	<i>fliC</i> _{XP 17-12}	Schwartz et al. 2015
<i>X. gardneri</i>		
SM177-10	<i>fliC</i> _{SM177-10}	Schwartz et al. 2015
101 (ATCC 19865)	<i>fliC</i> ₁₀₁ (ATCC 19865)	Potnis et al. 2011
Plasmids		
pK18 <i>mobsacB</i>	Allelic exchange suicide vector, Km ^r , <i>sacB</i> , Tra ⁻ , Mob ⁺	Schäfer et al. 1994
pK18 Δ <i>fliC</i>	Km ^r , Tc ^r , pK18 <i>mobsacB</i> , Δ <i>fliC</i> (in frame) replaced with Tc	This study
pK18 <i>fliC</i> ₈₅₋₁₀	pK18 <i>mobsacB</i> containing <i>fliC</i> ₈₅₋₁₀ , including 712 bp upstream and 516 bp downstream of the <i>fliC</i> ₈₅₋₁₀ coding region	This study
pK18 <i>fliC</i> ₉₁₋₁₁₈	pK18 <i>mobsacB</i> containing <i>fliC</i> ₉₁₋₁₁₈ , including 712 bp upstream and 516 bp downstream of the <i>fliC</i> ₈₅₋₁₀ coding region	This study
pK18 <i>fliC</i> _{XP17-12}	pK18 <i>mobsacB</i> containing <i>fliC</i> _{XP17-12} , including 712 bp upstream and 516 bp downstream of the <i>fliC</i> ₈₅₋₁₀ coding region	This study
pK18 <i>fliC</i> ₁₁₁₁ (ATCC 35937)	pK18 <i>mobsacB</i> containing <i>fliC</i> ₁₁₁₁ (ATCC 35937), including 712 bp upstream and 516 bp downstream of the <i>fliC</i> ₈₅₋₁₀ coding region	This study
pK18 <i>fliC</i> _{SM177-10}	pK18 <i>mobsacB</i> containing <i>fliC</i> _{SM177-10} , including 712 bp upstream and 516 bp downstream of the <i>fliC</i> ₈₅₋₁₀ coding region	This study
pK18 <i>fliC</i> ₁₀₁ (ATCC 19865)	pK18 <i>mobsacB</i> containing <i>fliC</i> ₁₀₁ (ATCC 19865), including 712 bp upstream and 516 bp downstream of the <i>fliC</i> ₈₅₋₁₀ coding region	This study
pRK415	Broad-host-range vector, RK2 <i>ori</i> , Tc ^r	Keen et al. 1988

^a Rif^r, Km^r, and Tc^r indicate resistant to rifampicin, kanamycin, and tetracycline, respectively.

each. Inoculated plants were kept in a greenhouse (12-h day length, with day and night temperatures of 27 and 21°C, respectively) until sampled to determine bacterial populations. Leaf disks were punched with a number 3 cork borer (9 mm in diameter) from fully expanded leaflets; leaf 2 was used for 0-dpi samples, leaf 3 for 6-dpi samples, and leaf 4 for 9-dpi samples. Two sets of leaf disks were sampled from each plant for replication at each time point, and three plants were inoculated for each treatment. Leaf disks were macerated in 10 mM MgCl₂, serially diluted, and plated on PSA medium supplemented with Rif at 25 to 50 µg/ml. Bacterial colonies were counted 2 days after plating.

Data availability.

Datasets supporting the conclusions of this article are included within the article and its supplementary files. Bacterial isolates will be made available upon request.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

NCBI: <https://www.ncbi.nlm.nih.gov/>

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