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Isolation of a *Xanthomonas oryzae* pv. *oryzae* Flagellar Operon Region and Molecular Characterization of *flhF*

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An 8.1-kb DNA fragment from *Xanthomonas oryzae* pv. *oryzae* that contains six open reading frames (ORF) was cloned. The ORF encodes proteins similar to flagellar proteins FlhB, FlhA, FlhF, and FliA, plus two proteins of unknown function, ORF234 and ORF319, from *Bacillus subtilis* and other organisms. These ORF have a similar genomic organization to those of their homologs in other bacteria. The *flhF* gene product, FlhF, has a GTP-binding motif conserved in its homologs. Unlike its homologs, however, *X. oryzae* pv. *oryzae* FlhF carries two transmembrane-like domains. Insertional mutations of the *flhF* gene with the omega cassette or the kanamycin resistance gene significantly retard but do not abolish the motility of the bacteria. Complementation of the mutants with the wild-type *flhF* gene restored the motility. The *X. oryzae* pv. *oryzae* FlhF interacts with itself; the disease resistance gene product XA21; and a protein homologous to the PilL protein of *Pseudomonas aeruginosa,* XooPilL, in the yeast two-hybrid system. The biological relevance of these interactions remains to be determined.

Bacterial flagella are important virulence factors for pathogenesis of animals and plants (Finley and Falkow 1997; Moens and Vanderleyden 1996). In some plant–pathogen systems, flagella-driven chemotaxis plays a role in the early interactions with host plants (Vande Broek and Vanderleyden 1995). Motility enables foliar pathogens to reach internal sites in the leaves (Beattie and Lindow 1995). Recently, the bacterial protein flagellin has been found to be a plant elicitor, providing evidence that plants have a sensitive perception system for this protein (Felix et al. 1999).

Xanthomonas spp. bear a single polar flagellum and cause serious diseases to virtually all plant crops. *X. oryzae* pv. *oryzae* is the causal agent of rice bacterial blight, an important disease in the rice-growing areas of the world (Mew 1987). The bacteria infect rice leaves by entering hydathodes, multiplying in the epitheme, and accessing the xylem vessels (Mew et al. 1984). The transmission of the pathogen is favored by intense wind-driven rainfall that facilitates bacterial entry into plant tissues through wounded leaf edges (Ou 1985). Feng and Guo showed that *X. oryzae* pv. *oryzae* chemotaxis occurs toward hydathode exudates of susceptible rice plants but not toward hydathode exudates of resistant plants (Feng and Guo 1975). This result suggests that chemotaxis plays a role in *X. oryzae* pv. *oryzae* pathogenicity before penetration of bacteria into the rice leaf. The flagellation and chemotactic properties of pathovars in the *Xanthomonas* spp., however, have not been extensively studied.

The pathogen–plant relationship in the *X. oryzae* pv. *oryzae*-rice system follows a gene-for-gene pattern (Hopkins et al. 1992). Plants containing resistance genes are resistant to pathogens containing the corresponding avirulence genes. More than 20 resistance genes have been identified by genetic analysis, two of which, *Xa1* and *Xa21*, have been cloned from rice (Song et al. 1995; Yoshimura et al. 1998). Two *X. oryzae* pv. *oryzae* avirulence genes, *avrXa7* and *avrXa10*, and some virulence genes such as *gumG*, *hrpX*, *xpsF*, *xpsD*, *hpa1*, and *hpa2* also have been isolated (Dharmapuri and Sonti 1999; Kamdar et al. 1993; Ray et al. 2000; Tang et al. 1996; Zhu et al. 2000). Although significant progress has been made in the characterization of host and bacterial genes governing the interaction, little is known about the genes governing *X. oryzae* pv. *oryzae* flagellar biosynthesis and movement.

Bacterial flagella are complex structures. Their biosynthesis and locomotion require many genes (Macnab 1992). For example, the entire *Salmonella typhimurium* flagellar system requires at least 40 genes (Jones and Macnab 1990). Except for some of the receptor genes, the flagellar and related genes of *S. typhimurium* and *Escherichia coli* are found at a few chromosomal locations where they form contiguous clusters, namely, flagellar regions I, II, and III. Within each region, there are several operons that contain from one to as many as nine genes. These operons are arranged as a regulon (Macnab 1992; Manson et al. 1998). In contrast, the gram-positive bacterium, *Bacillus subtilis*, has a different organization of flagellar genes, although it is similar to *S. typhimurium* and *E. coli* in gene sequence and flagellar structure (Bischoff and Ordal 1992; Macnab 1992). *B. subtilis* has a large flagellar operon that contains at least 30 genes as well as several other smaller clusters (Bischoff and Ordal 1992). Some of the genes occur in the same order as their enteric counterparts, but in other cases the order is quite different. For instance, in *B. subtilis*, flagellar genes *flhB*, *flhA*, and *flhF* are tandemly linked in the same operon. In *S. typhimurium*, however, following *flhB* and *flhA* is a chemotactic gene, *flhE* (Ditty et al. 1998; Macnab 1992). In contrast with the well-characterized flagella of

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Nucleotide sequence and/or amino acid sequences of the flagellar operon region have been submitted to GenBank, accession no. AF226282. Sequence x*oopilL* also has been deposited in GenBank, accession no. AF226283.

mammalian pathogens, only a few flagellar genes have been cloned and characterized from phytopathogenic bacteria (Chesnokova et al. 1997; Pleier and Schmitt 1989).

In the present study, we isolated a flagellar operon region from *X. oryzae* pv. *oryzae*. The sequenced region contains six genes, among which a gene homologous to *flhF* from *B. subtilis* was further characterized. Insertional mutations of *X. oryzae* pv. *oryzae flhF* retard but do not abolish bacterial motility. The mutants still produce normal flagella on swarm medium. *X. oryzae* pv. *oryzae* FlhF encoded by *flhF* interacts with XA21 and a *X. oryzae* pv. *oryzae* protein similar to PilL, a twitching motility protein from *Pseudomonas aeruginosa*, in the yeast two-hybrid system, which suggests that FlhF plays a role in other cell functions. FlhF also can interact with itself at the C-terminal domain, suggesting that it may function as a dimer.

RESULTS

Identification of *flhF* and isolation of a flagellar gene region in *X. oryzae* pv. *oryzae*.

In a yeast two-hybrid screen of a *X. oryzae* pv. *oryzae* (POX99A) genomic library, we used the XA21 kinase domain as bait in an attempt to isolate gene-encoding protein that interacts with the XA21 kinase domain. A 3.1-kb clone (pCX432) was isolated from five identical clones in two independent screens. The 3.1-kb fragment contains three ORF (Fig. 1). The first ORF, fused in frame to the *gal*4 activation domain of the pPC86 vector, encodes a protein homologous to a flagellar gene, *flhF*, from other organisms such as *P. putida* and *B. subtilis*. The second ORF encodes a protein homologous to a protein of unknown function, and the third has high homology to another flagellar gene, *fliA*, the product of which is a sigma factor controlling the transcription of other flagellar genes (Ohnishi et al. 1990). The above results suggest that the 3.1-kb fragment is a part of the flagellar operon in *X. oryzae* pv. *oryzae*.

Because flagella play important roles in bacterial pathogenicity, we isolated the flanking regions of *flhF* to better characterize the genes controlling flagella in *X. oryzae* pv. *oryzae*. To isolate a larger flagellar operon region, an *X. oryzae* pv. *oryzae* cosmid library was screened with a *Sal*I fragment from *flhF*. Five clones were found to contain the 3.1-kb fragment. One of the clones, pHMX1, was selected for further study. A 5.5-kb *Eco*RI fragment and two *Bam*HI fragments that were 4.4 and 10 kb from pHMX1 were subcloned into pUC18 and pBluescript, respectively. These three plasmids, pXO7, pBSB4.4, and pBSB10, were sequenced, as was 8.09 kb of the flagellar operon region. Sequence analysis showed that the flagellar operon region contains six ORF in the same orientation (Fig. 1). In addition to the three ORF-encoding proteins, FlhF, ORF319, and FliA, there are three ORFs located upstream of *flhF*. The two immediately upstream of *flhF* are homologous to *flhA* and *flhB*, respectively. The ORF preceding *flhB* is ORF234, which encodes a protein homologous to a protein with unknown function from other organisms. These six ORFs are tandemly arranged, with some overlap between ORF234 and *flhB* and ORF319 and *fliA*, and a 588 base gap between *flhA* and *flhF*. The organization of the five ORF downstream of ORF234 is basically the same as that in *P. putida*, *B. subtilis*, and *Borrelia burgdoferi* (Carpenter et al. 1992; Ge and Charon 1997). The identities of the deduced amino acid sequences of the five *X. oryzae* pv. *oryzae* genes range from 27.5 to 57.1% (Table 1). A BLAST search indi-

Table 1. Percent identity of *Xanthomonas oryzae* pv. *oryzae* flagellar proteins to homologs from selected organisms

Organism	FlhB[a]	FlhA	FlhF	ORF 319[b]	FliA
Pseudomonas putida	*[c]	43.7	34.5	57.1	50.8
Escherichia coli	40.9	52.9	*	*	50.5
Salmonella typhimurium	42.7	51.6	*	*	50.0
Yersinia enterocolitica	40.1	50.3	*	*	48.0
Bacillus subtilis	36.3	41.6	32.0	29.8	*
Borrelia burgdorferi	29.4	35.2	27.5	35.9	*

[a] Percents are relative to 100% of *X. oryzae* pv *oryzae*.
[b] ORF = open reading frame.
[c] * = amino acid sequence not available.

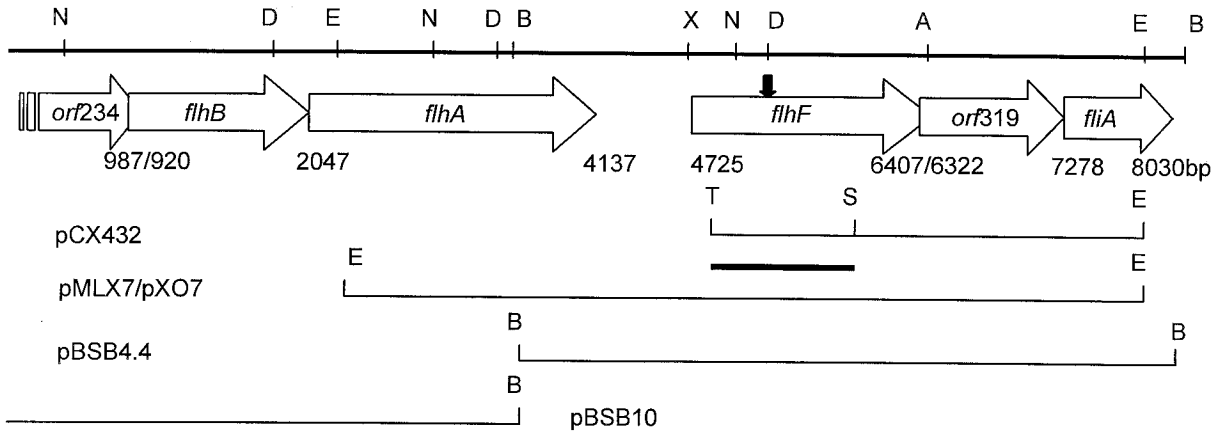

Fig. 1. Flagellar gene region of *Xanthomonas oryzae* pv. *oryzae*. Closed arrow = site of the omega cassette and Kan^R gene insertion for inactivation of the *flhF* gene. Plasmids pXO7, pBSB4.4, and pBSB10 are subclones of the *X. oryzae* pv. *oryzae* PXO99A cosmid clone pHMX1. pMLX7 contains the same 5.5-kb *Eco*RI fragment as in pXO7 and was used to complement *flhF*-deficient mutants. pCX432 was taken from a yeast two-hybrid screen with the XA21 kinase domain as bait. Thick line under pCX432 = 1-kb DNA fragment used as a probe for Southern hybridization or cosmid library screen. N = *Nco*I; D = *Dra*III; E = *Eco*RI; B = *Bam*HI; X = *Xho*I; A = *Apa*I; T = *Tsp*509I.

cated that these flagellar genes are the first cloned from phytopathogenic bacteria.

Analysis of interaction with the XA21 kinase domain and the structure of *X. oryzae* pv. *oryzae* FlhF.

Because FlhF is fused in frame to the GAL4 activation domain, it is the protein that interacts with the XA21 kinase domain in a yeast two-hybrid system. In order to find which part of FlhF is involved in the interaction, fragments of *flhF* with a series of deletions on either side were used to test the interaction of their products with the XA21 kinase domain. It was found that only the full-length FlhF interacts with the XA21 kinase domain, whereas FlhF truncated on either end loses the ability to interact with the kinase domain (Fig. 2).

A putative ribosomal binding site was identified preceding the second ATG start codon, indicating that the FlhF protein translation likely starts from the second methionine. Consequently, the predicted protein is 561 amino acids (aa) in length with a predicted estimated molecular weight of 68 kDa. The deduced amino acid sequence contains a hydrophobic region from aa239 to aa256, which was predicted to be a transmembrane domain by the transmembrane regions and orientation program (TMpred) (Hofmann and Stoffel 1993). The total score of 1,026 is well above the threshold score of 500. There is another putative transmembrane helix from aa192 to aa214 that might be precluded by TMpred, probably as a result of a charged residue (R194). Protein motif analysis revealed motifs that are characteristic of the GTP-binding proteins GPTGAGKT, DTAG, and TKLD. The three consensus sequences of GTP-binding motifs are GXXXXGKS/T, DXXG, and NKXD (Carpenter et al. 1992). The first motif also is generally referred to as a P-loop (Saraste et al. 1990).

The existence of the GTP-binding motifs indicates that FlhF is likely to be a GTP-binding protein (Fig. 3). The deduced amino acid sequence of *X. oryzae* pv. *oryzae flhF* was aligned with the known homologs from other organisms. The alignment revealed extensive identity in the N- and C-terminal regions, particularly the GTP-binding motif in the C terminus. The alignment also displayed dissimilarity between the *X. oryzae* pv. *oryzae* FlhF, and its homologs as the central portion (aa86 to aa341) of the *X. oryzae* pv. *oryzae* FlhF is not conserved. In addition, the predicted transmembrane domains of *X. oryzae* pv. *oryzae* FlhF could not be identified in its counterparts (Fig. 3).

Insertional mutation of *flhF* with antibiotic resistance cassettes.

To create a *flhF*-deficient mutant, an omega cassette (Sp^R–Sm^R) and a kanamycin resistance gene were used to create knockout mutants by inserting them into the unique *Dra*III site of *flhF* (Fig. 1). After electroporation, the revived cells were cultured on Kan or Sp–Sm media to select recombinants resulted from either single- or double-crossover events. Recombination occurred at a high frequency (2.4×10^{-7}). Preliminary experiments showed that *X. oryzae* pv. *oryzae* is sensitive to ampicillin at a concentration of 100 μg of ampicillin per milliliter. As a result, 120 Kan^R or Sp^R–Sm^R colonies were transferred to ampicillin containing polysaccharide (PS) plates. Out of the 120 Kan^R or Sp^R–Sm^R colonies, five and eight, respectively, did not grow on AMP plates, indicating that a double crossover had occurred and the vector DNA was eliminated from the chromosome. Southern analysis confirmed the replacement of wild-type *flhF* with the mutant *flhF*. After insertion of the 1.2-kb Kan^R cassette and the 2-kb omega cassette, a 5.5-kb *Eco*RI fragment band in the wild-type PXO99A cells was shifted to 6.7 and 7.5 kb, respectively (data not shown). One clone for each mutation was selected for further characterization.

Phenotype analysis of *X. oryzae* pv. *oryzae flhF*-deficient mutants.

The *flhF*-deficient *X. oryzae* pv. *oryzae* mutants PXO99DΩ2 and PXO99DK7 were observed for their growth rate in comparison with the wild-type PXO99A cells. There was no difference in the growth rate between the mutants and the wild-type cells in PS liquid medium or on PS solid medium (data not shown). PXO99DΩ2 and PXO99DK7, together with wild-type PXO99A, were used to inoculate 6-week-old rice plants of Xa21 containing IRBB21 and susceptible IR24 with a scissors-dipping method (Kauffman et al. 1973). Twelve days after inoculation, plants were scored by measuring lesion length. The mutants showed no difference in avirulence or virulence on resistant or susceptible rice varieties as compared with the controls (data not shown). Because the scissors-dipping method introduces wounds into the leaves, this procedure may allow *X. oryzae* pv. *oryzae* cells to bypass the hydathode and directly enter the xylem. A spray method was therefore also employed (Srivastava and Rao 1968). No significant difference in lesion length was ob-

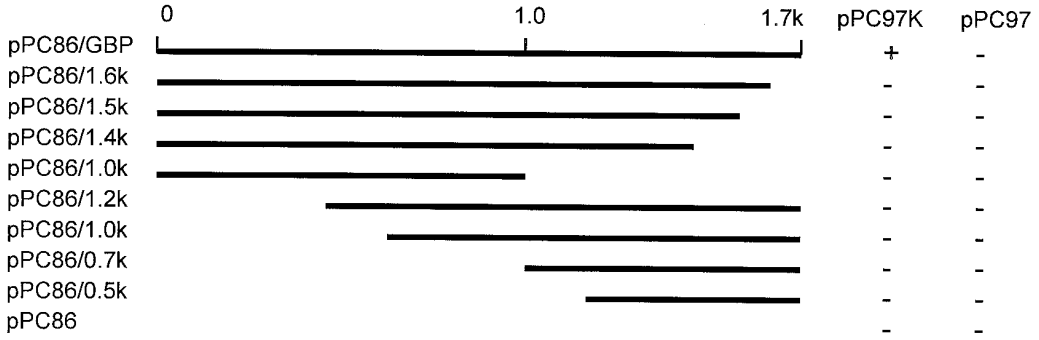

Fig. 2. Interaction between FlhF and the XA21 kinase domain. DNA fragment encoding the XA21 kinase and juxtamembrane domains was in frame ligated to *gal*4 binding domain of bait vector pPC97. Full-length *flhF* (GBP) and *flhF* fragments with different length truncations were in frame and ligated to the *gal4* activation domain of pray vector pPC86. +/– = positive or negative interaction on the basis of the expression of reporter genes LacZ and His3.

served, however, between the wild type and mutant cells (data not shown). These results indicate that *flhF* does not encode a race-specific component, nor does it affect growth in medium or in planta.

Swarm plate analysis showed that cells of the wild-type and mutant *X. oryzae* pv. *oryzae* strains were motile on semisolid swarm medium (Sockett and Armitage 1991). The movement was very slow in the first day of culture, but got faster after 2 days (Fig. 4). This type of movement suggests that motility of *X. oryzae* pv. *oryzae* is inducible, which is seen in other *Xanthomonas* pathovars (Kamoun and Kado 1990). Indeed, electron microscopy (EM) showed that cells of the wild-type and two mutant strains cultured on rich PS agar medium were not flagellated, whereas cells from swarm plates were flagellated (data not shown). Four days after inoculation, the motility zone of the wild-type and mutant cells was measured (Table 2). It was clear that the mutants were still motile, though the motility was severely impaired by the *flhF* mutation. The mutant PXO99DΩ2 carrying two transcription terminators in the Ω cassette swarmed slower than the mutant PXO99DK7.

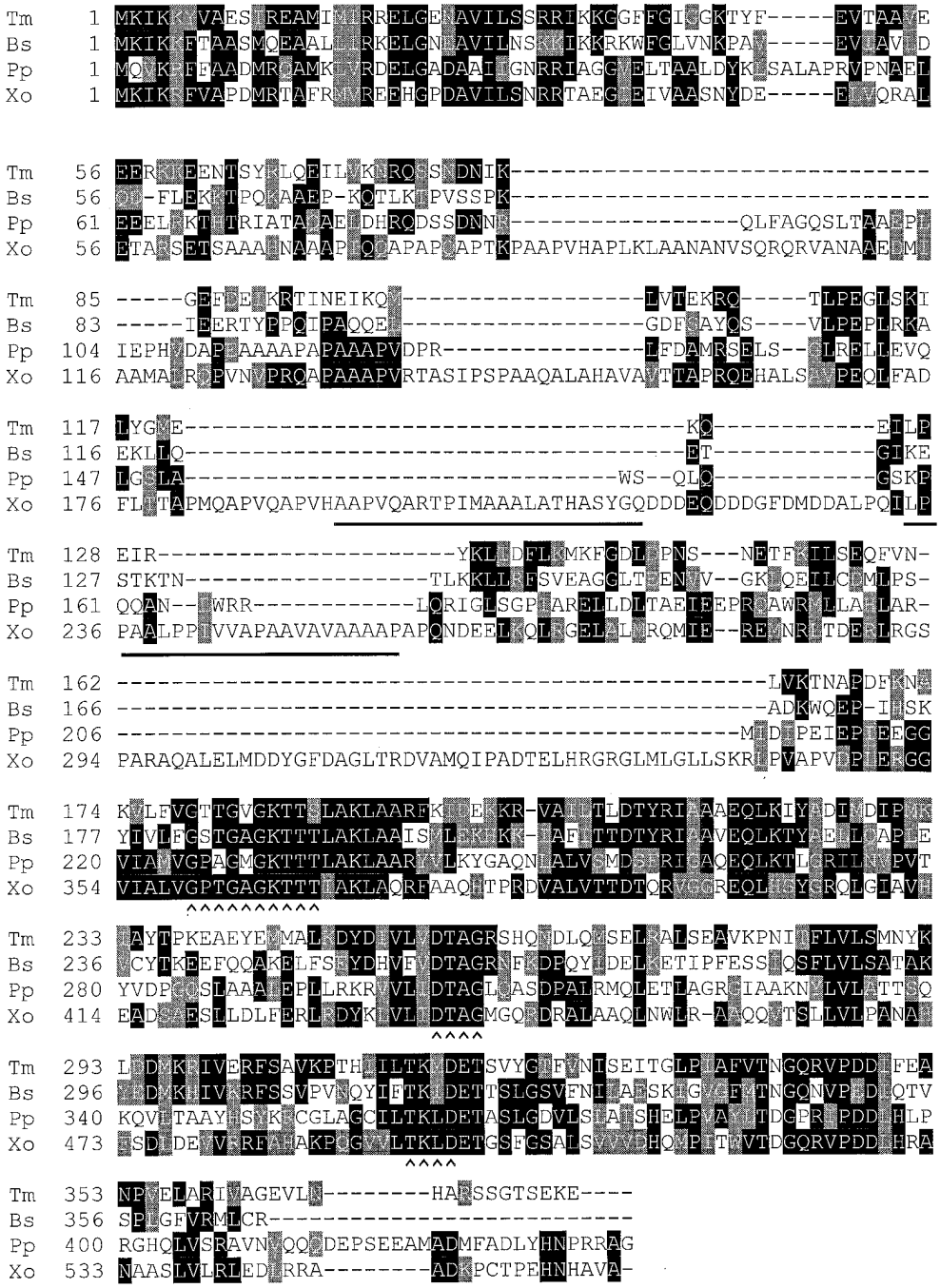

Fig. 3. Multiple alignment of FlhF amino acid sequences. Identical amino acids from all four organisms are shaded with black, whereas similar amino acids are hatched. Predicted transmembrane domains of *Xanthomonas oryzae* pv. *oryzae* FlhF are underlined with a thick line. Conserved GTP-binding motif is indicated with arrows. Tm = *Thermotoga maritima*; Bs = *Bacillus subtilis*; Pp = *Pseudomonas putidas*; Xo = *X. oryzae*.

This result suggests a polar effect on the expression of the downstream genes, although the effect was not severe enough to abolish motility. EM observation of the mutant cells did not show significant difference between the flagellar phenotype and the wild-type cells (data not shown). These results are distinct from the observation on the *flhF*-null mutant of *B. subtilis*, which was nonmotile and nonflagellated (Carpenter et al. 1992).

To complement *flhF*-deficient mutants, a wide-host-range plasmid, pMLX7, was introduced into PXO99DΩ2 and PXO99DK7 by conjugation. The pMLX7 plasmid contains a 5.5-kb *Eco*RI fragment that harbors *flhF*, ORF319, and a part of *flhA* and *fliA*. The complementation restored the mutants to their full motility (Table 2), indicating that *flhF* is a motility-associated gene in *X. oryzae* pv. *oryzae*.

FlhF interactor and FlhF function.

From the above results it is clear that *flhF* plays a role in *X. oryzae* pv. *oryzae* motility. However, virtually nothing is known of its mode of function. To identify FlhF signaling components, *X. oryzae* pv. *oryzae* FlhF was used as bait to screen the *X. oryzae* pv. *oryzae* genomic library with a yeast two-hybrid system. In one screen, more than 20 clones were identified to encode parts of an FlhF-interacting protein. The full-length gene encoding the protein was isolated from a *X. oryzae* pv. *oryzae* cosmid clone. The deduced protein, XooPilL, is composed of 1,030 aa with a predicted molecular weight of 124 kDa (Fig. 5). A BLAST search found that this protein is homologous to PilL encoded by the *pilL* gene from *P. aeruginosa*, with an overall identity of 29.8%. PilL is composed of 836 aa and is thought to play a role in signal transduction of pilus-twitching motility (Mattick et al. 1996). The newly isolated XooPilL homolog has two transmembrane domains (aa209 to aa230 and aa876 to aa896), as predicted by TMpred, with a total score of 644 and 1,244, respectively. Furthermore, it was predicted to be an inner membrane protein by PSORT (a computer program for the prediction of protein localization sites in cells) and has a leucine-zipper motif with four leucine repeats (aa250 to aa271), which may be involved in protein–protein interaction. *P. aerugonisa* PilL, however, does not have a similar transmembrane domain yet does have a leucine-zipper motif with only three leucine repeats.

In the same screen, more than 20 clones contained parts of the *flhF* gene. These clones can be grouped according to the size of the *flhF* gene fragments inserted in the pray plasmid (Fig. 5). There are four groups of clones containing the 3′ parts of *flhF*, which are 1, 567, 1,347, 1,089, and 993 bp in length, respectively. The smallest fragment of 993 bp still includes the GTP-binding motifs. This result suggests that the FlhF may form a dimer and that dimerization occurs in the C-terminal domain.

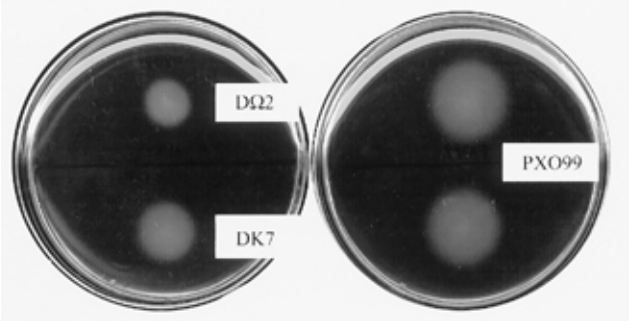

Fig. 4. Swarm plate analysis of PXO99DΩ2 and PXO99DK7 in comparison with wild-type PXO99A. Cells were grown on swarm plates at 28°C for 4 days.

DISCUSSION

In an attempt to isolate XA21 kinase-interacting proteins from *X. oryzae* pv. *oryzae*, we cloned a *X. oryzae* pv. *oryzae* gene homologous to a flagellar gene, *flhF*, from other organisms. An 8.1-kb *X. oryzae* pv. *oryzae* DNA fragment harboring the *flhF* homolog also was cloned and sequenced. Sequence analysis suggested that this region carries a flagellar operon region and encodes six ORF. To our knowledge, this is the first time such a flagellar operon region has been isolated from a phytopathogenic bacterium.

The six ORF in the 8.1-kb *X. oryzae* pv. *oryzae* DNA fragment designated ORF234, *flhB*, *flhA*, *flhF*, ORF319, and *fliA* are arranged in tandem in the same orientation (Fig. 1). All six ORF have homologs from other bacteria, but only the homologs of four ORF (*flhB*, *flhA*, *FlhF*, and *FliA*) have a role in flagellar biosynthesis (Carpenter et al. 1992; Macnab 1992; Ohnishi et al. 1990). The identity of the flagellar protein homologs ranges from 27.5 to 57.1% (Table 1). The four *X. oryzae* pv. *oryzae* ORF were named according to their homologs from other organisms. The function of ORF234 homologues is not yet known. The ORF319 homologue from *B. subtilis* encodes a putative 33-kDa ATP-binding protein and was not required for either motility or chemotaxis (Kirsch et al. 1994). Instead, the ORF319 homolog is thought to be a flagellar biosynthesis switch protein (Kunst et al. 1997). The genomic organization of *X. oryzae* pv. *oryzae* genes is primarily the same as that of their counterparts in *P. putida* and *B. subtilis* (Carpenter et al. 1992; Ditty et al. 1998). There are some discrepancies, however, especially around *fliA*. In *E. coli*, *fliA* is in another flagellar region (Ge and Charon 1997). In *B. subtilis*, *fliA* has not yet been identified, although it is not in the position downstream of the ORF319 homolog ORF298, as it is in the case of *X. oryzae* pv. *oryzae*, because this position is filled by *cheB*, a chemotexis gene. In addition, between *flhB* and the ORF234 homolog ORF219, there are three genes, *fliP*, *fliQ*, *and fliR*. In *P. putida*, *flhA*, *flhF*, *orfC*, and *fliA* are in the same order as their homologs in *X. oryzae* pv. *oryzae*, but genes upstream of *flhA* have not been identified. In *X. oryzae* pv. *oryzae*, there is a 587 base region with no ORF between *flhA* and *flhF*. These results indicate a cer-

Table 2. Swarm plate analysis of *Xanthomonas oryzae* pv. *oryzae* strain PXO99A, its *flhF*-deficient mutants PXO99DΩ2 and PXO99DK7, and mutants complemented with wild-type *flhF*[a]

Strains	Diameter of motility zone (cm)[b]
PXO99A	4.50 ± 0.11
PXO99AC[c]	4.45 ± 0.05
PXO99DΩ2	2.60 ± 0.10
PXO99DΩ2C[c]	3.75 ± 0.25
PXO99DK7	2.85 ± 0.15
PXO99DK7C[c]	3.61 ± 0.30

[a] Cells were incubated at 28°C for 6 days.
[b] Data = mean ± standard error of three replicates.
[c] Strains of PXO99A, PXO99DΩ2, and PXO99DK7 complemented with pMLX7 containing wild-type *flhF* gene.

tain degree of evolutionary differences among these genera in flagellar gene organization.

flhF was further characterized among the six genes cloned from the *X. oryzae* pv. *oryzae* flagellar operon. *flhF* was first isolated from *B. subtilis* and later cloned from other organisms (Carpenter et al. 1992; Ditty et al. 1998; Ge and Charon 1997). It encodes a 41-kDa putative GTP-binding protein (Carpenter et al. 1992), whereas the *flhF* isolated from *X. oryzae* pv. *oryzae* encodes a predicted 67-kDa protein, which is bigger than its homologs from other organisms. Multiple alignment showed that the N- and C-terminal regions of *X. oryzae* pv. *oryzae* FlhF are quite conserved in regard to its homologs, particularly in the GTP-binding motifs, which suggests that it also is a GTP-binding protein. The middle part of the protein, however, is much less conserved. For instance, TMpred predicted a hydrophobic region of 23 aa (aa234 to aa256), a putative transmembrane domain (Fig. 3). There may be another transmembrane helix situated at aa192 to aa214, probably precluded by TMpred as a result of a charged residue, R (198). The region between these two putative transmembrane helices has no positively charged residues, which is in line with the expected situation for a loop located outside the cytoplasm, according to the "positive–inside" rule (Wallin and von Heijne 1998). Both transmembrane helices and the loop are missing in the FlhF homologues. These results suggest that the *X. oryzae* pv. *oryzae* FlhF may be an inner-membrane protein, whereas its homologs might be localized in the cytoplasm.

It has been suggested that FlhF plays a role in the flagellar biosynthesis in *B. subtilis* (Carpenter et al. 1992). A null mutation in *B. subtilis flhF* produced nonmotile cells lacking flagella, as determined by an agglutination test and Western blot analysis. Cells deficient in *flhF* failed to activate transcription of the *hag* and *mot* loci, encoding the flagellar filament structural protein flagellin and the motility protein, respectively (Carpenter et al. 1992). In contrast, although insertional mutations in *X. oryzae* pv. *oryzae flhF* did significantly retard motility of *X. oryzae* pv. *oryzae* and the omega cassette insertion resulted in an even more severe retardation, it did not abolish motility. We noticed that on swarm plates with 0.4% agar the cells of the wild-type and mutant strains move slower than those on swarm plates with 0.3% agar. Moreover, almost no movement was observed for the mutant PXO99DΩ grown on the swarm plates with 0.4% agar, displaying a more significant difference from the wild-type and mutant PXO99DK7 (data not shown). EM, however, showed that cells with insertional mutations in *flhF* still produce normal flagella (data not shown). It is possible that the mutant with the Kan^R insertion in *flhF* had leaky expression, although the mutant with the omega cassette insertion carries a transcription terminator and should disrupt the *flhF* transcription and the downstream genes such as *fliA*, which regulates the transcription of the flagellar capping gene *flhD*. Nonetheless, the normal flagellation of the mutant PXO99DΩ2 indicates that the Ω cassette did not exert a polar effect on *fliA*. The residual motility of the insertion mutants might be a result of a possible gene redundancy.

Because *X. oryzae* pv. *oryzae* FlhF interacts with the XA21 kinase domain in the yeast two-hybrid system, we initially postulated that FlhF might act as an avirulence or a virulence factor to trigger or disturb the XA21-mediated defense pathway, similar to the interaction of *Yersinia* spp. with its host, where Yop proteins can enter animal cells to destroy key functions of immune cells (Galan and Collmer 1999). An emerging theme in host–bacterial interactions is that virulence and accessory proteins that affect host–signal transduction are similar to domains of host proteins. For instance, *Yersinia* spp. YopM carries LRR domains commonly found in eukaryotic host proteins (Leug and Straley 1989). The FlhF protein has a P-loop motif, which is widely conserved in the largest class of plant resistance gene products (Baker et al. 1997). Our results by two methods from rice plant inoculations, with or without the Xa21 resistance gene with the mutants and wild-type PXO99A cells, did not support the above hypothesis. If the FlhF function was instrumental for bacteria virulence to rice plants, the susceptible rice plants, IR24, would show reduced lesion length when inoculated with FlhF mutants. Similarly, if FlhF conferred an avirulence function, increased lesion length would have been observed on Xa21 plants inoculated with the FlhF mutants. Such changes in lesion length were not observed (data not shown). Parallel experiments with *X. oryzae* pv. *oryzae* Korean race 1 (DY87031), which is normally virulent on the Xa21 line (Wang et al. 1998), and two mutants with the same insertional mutations in *flhF* also displayed no change in pathogenicity (data not shown).

In the present study, a protein homologous to *P. aeruginosa* PilL interacted with *X. oryzae* pv. *oryzae* FlhF. The PilL of *P. aeruginosa* is hypothesized to play a role in signal transduction of bacterial-twitching motility (Mattick et al. 1996). XooPilL, like FlhF, contains two predicted transmembrane domains, which suggests it is a membrane protein. Interestingly, analysis of the deduced protein sequence showed that it contains a leucine-zipper motif, which means it may interact with other proteins in a signal-transduction pathway. If XooPilL also plays a similar role in *X. oryzae* pv. *oryzae*, as it does in *P. aeruginosa*, XooPilL may have dual functions. In this scenario, XooPilL would control pili-twitching motility and flagella-driven motility. Such a dual function is reminis-

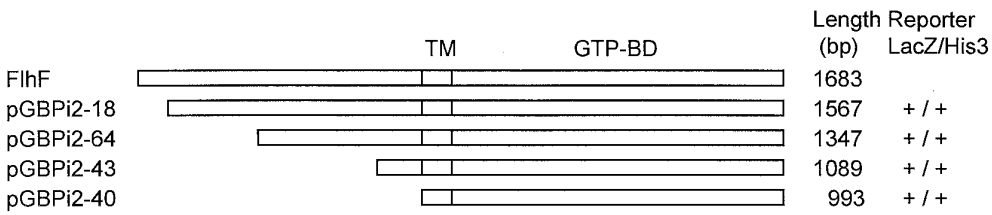

Fig. 5. Self-interaction of FlhF of *Xanthomonas oryzae* pv. *oryzae* in the yeast two-hybrid system. *flhF* is the full-length gene; pGBPi2-18, -64, -43, and -40 are representatives of the *X. oryzae* pv. *oryzae* clone groups that interact with FlhF and contain parts of *flhF*. + = expression of reporter genes LacZ and His3 for protein interaction.

cent of the *E. coli* FlhD, which functions as a positive regulator of the flagellar regulon and acts in other aspects of cell function, including cell division (Manson et al. 1998). Because many type IV pilus proteins have a remarkable similarity to chemotaxis proteins (Darzins and Russel 1997), it is possible that *xoopilL* is a new flagellar gene.

In the search for the *X. oryzae* pv. *oryzae* FlhF interactors, a series of clones that encodes parts of FlhF were isolated. The smallest clone is 993 bp, which encodes 331 aa of the C-terminal portion of the FlhF carrying the GTP-binding domain. This result suggests that the FlhF can interact with itself, forming a dimer. Further work is needed to characterize XooPilL and its relation to FlhF.

In summary, a flagellar operon region containing six genes was isolated from *X. oryzae* pv. *oryzae*. Among the six genes, four have homologs of known functions in flagellar biosynthesis from other organisms, whereas two have homologs of unknown function. *X. oryzae* pv. *oryzae flhF* is significantly different from its counterparts in other organisms in that it has two putative transmembrane domains. Mutations of *flhF* by insertional inactivation do not impair flagellation or affect race specificity, growth in media, or virulence on rice plants. The mutations, however, significantly decrease but do not abolish bacterial motility. The protein encoded by the *flhF* interacts with XooPilL, suggesting a possible role in signal transduction controlling bacterial motility. FlhF also interacted with itself. These results should open new avenues for the study of phytopathogenic bacteria flagella.

MATERIALS AND METHODS

Bacterial strains and culture media.

The bacterial strains and plasmids used in this study are described in Table 3. *X. oryzae* pv. *oryzae* strains were routinely grown in liquid or solid PS media or NB medium (Difco, Detroit, MI, U.S.A) at 28°C. *E. coli* strain DH10B (Gibco BRL, Grand Island, NY, U.S.A.), plasmids pUC19 (New England BioLabs, Beverly, MA, U.S.A.), and pBluescript (Stratagene, La Jolla, CA, U.S.A.) were used for all cloning experiments. *E. coli* cells were cultured in Luria-Bertani broth at 37°C. The antibiotics used for *X. oryzae* pv. *oryzae* and *E. coli* were, per milliliter: 100 µg of ampicillin, 50 µg of gentamycin, 50 µg of kanamycin, 50 µg of spectinomycin, and 50 µg of streptomycin, purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Molecular techniques.

Standard recombinant DNA techniques were used (Sambrook et al. 1989). Restriction enzymes and ligase were obtained from New England BioLabs. DNA phosphatase (from shrimp) was obtained from Amersham Pharmacia Biotech Inc. (Piscataway, NJ, U.S.A.). *X. oryzae* pv. *oryzae* race 6 strain POX99A genomic DNA was prepared according to Wilson (1994). For Southern DNA hybridization, ^{32}P-dCTP (New Life Science Products, Boston, MA, U.S.A.) was used to label DNA probes with a random labeling kit (Amersham Corp., Arlington Heights, IL, U.S.A.). A Midiprep kit (Qiagen, Chatsworth, CA, U.S.A.) was used to prepare large amounts of vector and plasmid DNA for sequencing. DNA sequencing on both strands of DNA fragments cloned in pBluescript or pPC86 was performed by the dideoxy-chain termination method with an automated sequencer (Model 400L; Li-Cor, Lincoln, NE, U.S.A.) (Sanger et al. 1977). M13 reverse and M13-20 primers were used to sequence the inserts in pBluescript. Primers GAL4TA (5′-GGATGTTTAATACCACT-3′) and TADC1 (5′-TTGATTGGAGACTTGACC-3′) were used to sequence the inserts in pPC86. To fill in gaps, a primer walking strategy was used with synthesized primers (Operon, Alameda, CA, U.S.A.). Polymerase chain reaction (PCR) was performed with Vent polymerase (New England Biolabs) and the Programmable Thermal Controller (MJ Research Inc., Watertown, MA, U.S.A.). PCR products were cloned with a TA cloning kit (Invitrogen Corp., Carlsbad, CA, U.S.A.). Bacterial transformation was done with the BRL Electroporator (Life Technologies Inc., Gaithersburg, MD, U.S.A.). DNA and protein sequence analyses and homolog searches were performed with Sequencher (Gene Codes, Ann Arbor, MI, U.S.A.), NCBI BLAST (Altschul et al. 1997), TMpred (Hofmann and Stoffel 1993), and CLUSTAL (Thompson et al. 1994).

Identification of an *X. oryzae* pv. *oryzae* clone containing *flhF*.

The experiment was initially designed to search for interactors with the XA21 kinase domain. An *Xa21* fragment that encodes the cytoplasmic kinase domain, including part of the juxtamembrane and C-terminal domains (Song et al. 1995), was generated with primers Pr822-29 (5′-AGGTCGACCCGGGAATGAAAGGCCACCCATT-3′) and Pr822-20 (5′-GGGGTACCGGATCCTCAAAATTCAAGGCTCCCTTCAC-3′). The PCR product was cloned into the TA cloning vector PCR2.1. After the sequence was confirmed, the insert was released from PCR2.1 vector with *Sal*I and *Bam*HI and inserted into the binding domain vector pPC97 at the *Sal*I–*Bgl*II site. The bait plasmid (pPC97K) was introduced into yeast cells of strain HF7c with the Li–Ac method, described in Clontech's yeast two-hybrid system manual (Clontech, Palo Alto, CA, U.S.A.).

To construct the library for the yeast two-hybrid screen, *X. oryzae* pv. *oryzae* chromosomal DNA from *X. oryzae* pv. *oryzae* race 6, strain PXO99, was partially digested with Tsp509I. Fragments ranging from 0.5 to 5 kb were inserted into the activation domain of pPC86 at the *Eco*RI site. The constructed library contains about 1×10^6 clones, with an average DNA insert size of approximately 2 kb. The HF7c (pPC97K) cells were sequentially transformed with the *X. oryzae* pv. *oryzae* library and were spread on SD-Leu, -Trp, and –His media to select the kinase interactor. Out of 2.4×10^{-7} transformants screened, 156 Leu^+, Trp^+, and His^+ colonies were tested for the expression of another reporter gene, LacZ. The His^+ and $LacZ^+$ colonies were further verified. The prey plasmids were isolated from the His^+ and $LacZ^+$ colonies, amplified in DH10B, and returned to the HF7c (pPC97K) cells to check interaction between the kinase and interactor proteins. The verified positive clones were sequenced. One of the clones, pCX432, encodes a protein homologous to a flagellar protein FlhF from other bacteria (Fig. 1).

Isolation of a flagellar gene region.

To isolate the flagellar gene region, a 1-kb *Sal*I fragment from pCX432 that contains part of *flhF* was used to screen a cosmid library of *X. oryzae* pv. *oryzae* (race 2, strain POX86)

that was constructed with cosmid vector pHM1 (Fig. 1). The positive clones were confirmed by Southern hybridization. One confirmed clone was subcloned into pBluescript or pUC18 with *Bam*HI or *Eco*RI. The subclones containing the flanking regions of pCX432 were sequenced (Fig. 1).

Analysis of the interacting part of FlhF with the XA21 kinase domain and construction of *flhF*-deficient mutants.

To find which part of FlhF is essential for interaction with the XA21 kinase domain, *flhF* fragments with truncations of different lengths were constructed by PCR amplification with pXO7 as template. The fragments were fused in frame with the gal4 activation domain. The constructed plasmids were used for transformation of yeast HF7c cells containing pPC97K or pPC97 (Fig. 2). The interaction test was performed as described above.

A 4.4-kb *Bam*HI–*Eco*RI DNA fragment from pXO7 harboring the *flhF* gene was subcloned into the pUC18 plasmid at the *Bam*HI–*Eco*RI site. The resultant plasmid, PUCX4.4, was linearized in a *Dra*III site internal to *flhF* (Fig. 1). The linearized plasmid DNA was blunt ended by filling it with Klenow enzyme. An omega cassette (Prentki and Krisch 1984) containing a streptomycin–spectomycin resistance gene and a kanamycin resistance gene were inserted into the *flhF* region at the *Dra*III site, described below.

The omega cassette was released from plasmid pHP45Ω (Prentki and Krisch 1984) with *Bam*HI. The protruding 5′ end was filled with T4 DNA polymerase. The omega cassette was then ligated to the linearized pUCX4.4. Because of the presence of a transcription terminator at both ends of the cassette, the insertional mutation with the omega cassette was deemed to cause a polar effect on the transcription of downstream genes in the operon. Therefore, a terminatorless kanamycin resistance gene cassette was used for the insertional inactivation of *flhF*. A 1.28-kb Kan^R fragment was released from pUC4K (Amersham Pharmacia Biotech) with *Bam*HI blunt ended as described above and ligated into pUCX4.4 at the *Dra*III site. The orientation of the *flhF* and Kan^R cassette was determined by restriction digestion with *Xho*I, which cuts at a site upstream of *flhF* and into the Kan^R gene. Only the clones containing Kan^R that are oriented in the same direction as *flhF* were chosen to transform the wild-type PXO99A cells.

The constructed plasmids pUCXDK and pUCXDΩ with insertion of Kan^R and omega cassettes in *flhF* were introduced into PXO99A cells by electroporation. The PXO99A cells were freshly cultured on PS agar plates and single colonies were used to inoculate PS liquid medium. The cells were cultured overnight to an optical density of about 0.6 at 600 nm and washed with cold-wash buffer (10% glycerol in water) three times. Approximately 100 ng of pUCXDK and pUCXDΩ DNA was used for electroporation of 20 µl of the PXO99A competent cells, five electroporations for each plasmid construct. Electroporation was done at 2.0 kv, 4KΩ, 330 µF. After electroporation, the cells were pooled and revived in 5 ml of SOC medium for 6 h. The cells were plated onto PS agar plates containing either kanamycin or spectromycin–streptomycin. After 3 days of culture, a number of the colonies were transferred onto PS agar plates containing Kan–Amp or Spe–Sm–Amp to select recombinants with double-crossover events. To confirm the replacement of the wild-type *flhF* with the insertional mutant gene by allelic exchange, a Southern analysis of the *Eco*RI-digested genomic DNA from the wild-type cell and the mutants was carried out with a 1-kb *Sal*I fragment from pCX432 as probe.

Table 3. Bacterial strains and plasmids used in the study

Strains or plasmids	Description	Source or reference
Escherichia coli		
DH10B		Gibco BRL, Grand Island, NY, U.S.A.
S17-1	294, recA, chromosomally integrated RP4 derivative, Sm^R, Tc^R	Simon et al. 1983
Xanthomonas oryzae pv. *oryzae*		
PXO99A	Philippine race 6 strain; avirulent to rice plants with Xa21	Hopkins et al. 1992
PXO99DΩ2	flhF⁻ Kan^R gene inserted at *Dra*III site of flhF	This study
PXO99DK7	flhF⁻Ω cassette (Sm^R–Sp^R) inserted at *Dra*III site of flhF	This study
PXO99DΩ2c	PXO99DΩ2 complemented with pMLX7	This study
PXO99DK7c	PXO99DK7 complemented with pMLX7	This study
Plasmids or cosmids		
pPC97	GAL4, DNA-BD, Leu2, Amp^R	Chevray and Nathans 1992
pPC86	GAL4, DNA-AD, Trp1, Amp^R	Chevray and Nathans 1992
pBDleu	GAL4, DNA-BD, Leu2, Amp^R	Gibco BRL
pPC97K	pPC97 with XA21 kinase fusion	This study
pCX432	pBC86 with 3.1-kb *X. oryzae* pv. *oryzae* clone	This study
pBDLeu3.1	pBDleu with 3.1-kb *Sma*I–*Spe*I fragment from pCX432	This study
pUC18	OripUC, Amp^R	BioLabs, Beverly, MA, U.S.A.
pML122	OriV, OriT, Mob/rep, Gm^R, Nm^R	Labes et al. 1990
pBluescript SK	OriColE1, Amp^R	Stratagene, La Jolla, CA, U.S.A.
pUC4K	OripBR322, Amp^R, containing Kan^R cassette	Pharmacia, Piscataway, NJ, U.S.A.
pHP45Ω	pUC18 containing Ω cassette Sp^R–Sm^R	Prentki and Krisch 1984
pHMX1	pHM1 *X. oryzae* pv. *oryzae* cosmid clone	This study
pXO7	pUC18 with 5.5-kb *Eco*RI fragment from pHMX1	This study
pMLX7	pML122 with5.5-kb *Eco*RI fragment	This study
pUCX4.4	pUC18 with 4.4-kb *Bam*HI–*Eco*RI fragment from pXO7	This study
pUCXDΩ2	pUCX4.4 with Ω insertion at *Dra*III	This study
pUCXDK7	pUCX4.4 with Kan^R insertion at *Dra*III	This study
pBSB4.4	pBluescript with 4.4-kb *Bam*HI fragment from pHMX1	This study
pBSB10	pBluescript with 10-kb *Bam*HI fragment from pHMX1	This study

Complementation of *flhF*-deficient mutants.

A 5.5-kb *Eco*RI fragment from *X. oryzae* pv. *oryzae* cosmid clone pHMX1 containing *flhF* was subcloned into the wild-host-range vector pML122 in the *Eco*RI site (Fig. 1). The orientation of the insert was checked to ensure that the insert was in the same direction as the *npt* promoter. The resultant plasmid pMLX7 was introduced into *E. coli* strain S-17 by electroporation. The pMLX7 was transferred to the two *flhF*-deficient mutants, PXO99DΩ2 and PXO99DK7, by biparental mating (Hopkins et al. 1992). The transconjugants were then used for phenotype analysis.

Motility analysis.

Fresh colonies from PS agar plates were stabbed into swarm plates composed of 0.03% (wt/vol) Bacto Peptone, 0.03% yeast extract, and 0.3% agar. The inoculated cells were cultured for 4 days or longer at 28°C and examined for bacteria swarming away from the inoculated site (Sockett and Armitage 1991).

Identification of FlhF interactors.

A 3.1-*Sma*I–*Spe*I fragment from pCX432 was ligated in frame into pBDleu (Gibco BRL) at the *Sma*I–*Spe*I site. *flhF* in the 3.1-kb fragment was fused in frame to the *gal*4 binding domain. This bait plasmid was transformed into the yeast strain HF7c. The transformation and *X. oryzae* pv. *oryzae* library screen were done as indicated above.

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