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Functional Analysis of *pilQ* Gene in *Xanthomanas oryzae* pv. *oryzae*, Bacterial Blight Pathogen of Rice

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Bacterial blight (BB) of rice, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is the most devastating bacterial disease in rice. A virulence-attenuated mutant strain HNU89K9 of *X. oryzae* pv. *oryzae* (KACC10331), with a transposon insertion in the *pilQ* gene was used for this study. The *pilQ* was involved in the gene cluster *pilMNOPQ* of the *Xoo* genome. Growth rate of the *pilQ* mutant was similar to that of wild-type. At level of amino acids, PilQ of *Xoo* showed that a high sequence identities more than 94% and 70% to *Xanthomonas* species and to *Xyllela fastidiosa*, respectively but a low sequence homology less than 30% to other bacterial species. The twitching motility forming a marginal fringe on PSA media was observed on colony of the wild-type strain KACC10331, but not in mutant HNU89K9. Wild-type *Xoo* cells formed a biofilm on the surface of the PVC plastic test tube, while the mutant strain HNU89K9 did not form a biofilm. The results suggest that the *pilQ* gene of *X. oryzae* pv. *oryzae* plays a critical role in pathogenicity, twitching motility, and biofilm formation.

***Keywords*:** *Xanthomonas oryzae* pv. *oryzae*, transposon, *pilQ* mutant, pathogenecity, twitching motility, biofilm

Gram-negative bacterium, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is the causal agent of bacterial blight (BB) on rice (*Oryza sativa* L.). Bacterial blight is a major rice disease in tropical Asian countries where high-yielding rice cultivars are often highly susceptible. BB is a vascular disease resulting in tan-gray to white lesions along the leaf veins (Mew *et al.*, 1993). *Xoo* has been used as a model organism to study plant-pathogen interactions, bacterial race differentiations, and the evolution of plant pathogens. Until now, many virulence genes related to hypersensitive response and pathogenicity (*hrp*), production of extracellular polysaccharides (EPS), extracellular enzymes and lipopolysaccharides (LPS) have been isolated and characterized (Chan and Goodwin, 1999; Shen and Ronald, 2002). However, our understanding of the molecular mechanisms involved in pathogenesis is limited to only a few aspects of pathogenecity. Therefore, it is reasonably able to be assumed that *Xoo* require unique virulence genes for molecular interaction between the pathogen and rice. A mutant library could facilitate the comprehensive identification of genes required for pathogenesis.

Bacterial cells have appendages for movement such as flagellum and pilus that are used for movement in liquid and on solid surfaces, respectively (O'Toole and Kolter, 1998; Mattick, 2002). The movement of pathogenic bacteria on a host is important in disease progression and is associated with the Type IV pili (TFP) system. Type IV pili are flexible, filamentous structures protruding from the cell surface of Gram-negative bacteria (Strom and Lory, 1993; Shi and Sun, 2002). It was revealed that Type IV pili promote the attachment of bacterial pathogens to the specific receptors of host cells during colonization (Bieber *et al.,* 1998). Such attachment is an essential event for the initiation of infection. Limited to colonizing the water-conducting xylem vessels of plants, phytopathogenic bacteria develop biofilms that contribute to the blockage of sap flow resulting in plant stress and disease (Maxwell *et al*, 2003; Meng *et al*., 2005). How bacteria are disseminated in the xylem vessels from the feeding sites has been an important question. Generally, it has been known that Type IV pili play an important role in twitching and social gliding motility for cell movement (Bhaya *et al*., 2000; Mattick, 2002). PilQ is a member of the secretin family of proteins, which functions in Type IV pilus organelle biogenesis. There is also strong evidence for functional role of *pilQ* gene in twitching motility of the phytopathogenic bacterium *Ralstonia solanacearum* based on colony morphologies (Huanli *et al*., 2001). Recently, the twitching motility of *X. fastidiosa* cells has been directly observed *in situ* (Meng *et al*., 2005). Using microfabricated chambers designed to mimic the features of xylem vessels, it was demonstrated that *X. fastidiosa* migrates via Type IV-pilus-mediated twitching motility against a rapidly flowing medium. Furthermore, *pilB* and *pilQ* mutants of *X. fastidiosa* deficient in Type IV pili do not twitch and are thereby inhibited from colonizing upstream vascular regions *in planta*.

A biofilm is a complex aggregation of microorganisms

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marked by the excretion of a protective and adhesive matrix. Biofilms are also often characterized by surface attachment, structural heterogeneity, genetic diversity, complex community interactions, and an extracellular matrix of polymeric substances (Costerton *et al*., 1995). In phytopathogenic bacteria, biofilm formation plays an important role in cell to cell communication between pathogen and host plant and consequently is associated with pathogenecity (Marques *et al*., 2002; Meng *et al*., 2005)

Many bacterial genomes have been completely sequenced and comparative genome analysis using this sequence information provides a powerful tool to provide general insight and specific information regarding the bacterial genome. Whole genome sequences of *Xoo* strains (Lee *et al.,* 2005) and other *Xanthomonas* spp. (Da Silva *et al*., 2002) were determined for gene structure and function of certain genes. A number of the gene orthologs encoding proteins potentially involved in biogenesis and the function of Type IV pili is present in *X. oryzae* pv. *oryzae* genome. Thus, it was reasonably hypothesized that the bacterium actively migrates via twitching motility using the Type IV pili system.

In the present study, a virulence-deficient mutant with transposon insertion in the *pilQ* gene of the *Xoo* strain was isolated from a transposon mutant library and genetic organization of a number of pili genes containing *pilQ* gene was analyzed form whole genome sequence database. Furthermore, *Xoo-pilQ* mutant was assessed for association with twitching motility and biofilm formation.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *X. oryzae* pv. *oryzae* strain KACC10331 and KACC10859 was obtained from the Korean Agricultural Culture Collection (KACC) at the National Institute of Agricultural Biotechnology (NIAB) in Suwon, Korea. *pilQ* mutant HNU89K9 was obtained in different virulence deficient or reduced mutants that were selected from virulence screening of the 24,540 transposon mutant strains of *X. oryzae* pv. *oryzae* KACC10331 (Wang *et al*., 2007). *Xoo* strains were cultured at 28°C on Peptone Sucrose agar (PSA) medium. To culture the mutant, 20 μg/ml Kanamycine was added to the medium. Kanamycin 20 mg/L was supplemented for the selection of transposon mutants. Growth rates of wild-type and mutant *X. oryzae* pv. *oryzae* were determined by transferring 3∼5 day-old cells grown on PSA into liquid PSB medium. The initial optical density for each culture was adjusted to an absorbance of 0.05 at 600 nm. The inoculated cultures were incubated at 28°C on a rotary shaker (200 rpm) and checked every 12 h.

pilQ Mutant and complementation test

pBACF2 Plasmid carrying *pilQ* locus of Xoo was partially digested by *Sau*3A and the DNA fragments ranging from 6 kb from 10 kb were ligated to pML122 vector which was cut by *Bam*HI, and were transformed into *E. coli* DH5α by the MicroPulser^{TM} Electroporation apparatus (Bio-Rad, Micropulser^{TM}, USA). A recombinant clone, pMLPQ containing *pilQ* locus of 6.0 kb was obtained. The pMLPQ was transformed into mutant strain HNU89K9 by the electroporation and plated on PSA medium containing 20 μg/ml gentamicin and 20 μg/ml Kanamycin. After 5 days, the colonies formed on the medium were isolated as complementation clone C89K9.

Southern blot hybridization and PCR amplification

Genomic DNA was extracted from the *pilQ* mutant (HNU 9K9) and wild-type strains KACC10331 and KACC10859 grown in 5 ml PSB for 3 days at 28°C using protocol of Ausubel *et al*. (1987). For Southern blot testing, DNA samples of about 5 μg were digested with the restriction enzyme

Table 1. The bacterial strains and plasmids used in this study

Strains/plasmids	Relevant characteristics	Source
E. coli strains		
DH5α	*f*80d*lac*Z△M15△(*lacAYA-argF)U169 deoR recAl endAl hsdR17(rk- mk-) supE44I- thi-1 gyrA96 reL4*1	Lab. collection
X. oryzae pv. *oryzae* strains		
KACC10331	Wild type strain, Korean race 1	KACC
KACC10859	Wild type strain, Korean race 1	KACC
HNU89K9	KACC10331 *pilQ* :: Tn5 Km^r	This study
C89KB	HNU89K9 harboring pMLPQ, Km^r, Gm^r	This study
Plasmids		
pBACF2	BAC plasmid including *pilQ* gene cluster, chr^r	Lab. collection
pGEM-T- vector	vector, Apm^r, lacZa+	Promega
p89KB	pGEM-T vector harboring 3.2 kb PCR product with transposon and *pilQ* gene from HNU89K9	This study
pML122	Complementation vector, Gen^r, Kan^r	Lab. collection
pMLPQ	pML122 containing *pilQ* gene region of 6.0 kb derived from pBACF2, Gen^r	This study

* Km, Kanamycin; Gen, Gentamycin; Am, Ampicillin; Chr, chroramphenicol; KACC, Korea Agriculture Culture Collection

*Eco*RI, which do not cut in transposon and fractionated on 0.9% agarose gel in TAE buffer (10 mM Tris-acetate, 1 mM EDTA, pH 8.0), transferred to a Hybond^{TM} N^+ nylon membrane (Amersham Bioscience, UK). For Southern hybridization, Kanamycin gene in the transposon was amplified by PCR using primers: KAN-F; 5'-CAATCAGGTGGTGCGAC ATC-3', KAN-R; 5'-TCACCGAGGCAGTTCCAT-3', labeled with AlkPhos Direct Labeling Reagents (GE Healthcare, UK) and used as a probe. Hybridization signals were detected according to the protocol provided by the manufacturer with Detection systems (Amersham Bioscience, UK). All experiments were performed according to the manufacturer's instructions. To confirm transposon insertion in *pilQ* gene, primers: pilQ-F; 5'-CGAGCTGATTGAACTTGTGC-3' and pilQ-R; 5'-GGAGATCGTGAACAGGTTCT-3' were designed from *pilQ* gene of *Xoo* KACC10331 released in the NCBI database. The PCR reaction was performed in a 50 μl mixture containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 μM each dNTP, 100 ng primer and 2.5 units of Taq polymerase (Promega). PCR amplification was carried out in PTC-200TM (MJ Research, Inc.) using the following profile: one cycle of 4 min at 94°C; 30 cycles of 30 sec at 94°C, 30 sec at 60°C, 2 min at 72°C and one cycle of a final extension for 7 min at 72°C. PCR products were separated on a 1.5% agarose gel in TAE buffer and visualized by staining with ethidium bromide (1 mg/L).

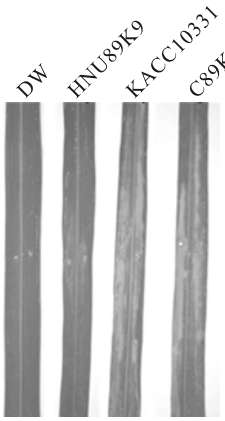


Fig. 1. Pathogenicity of *X. oryzae* pv. *oryzae* strains. The *Xoo* strains were inoculated on leaves of Milyang 23 using the leaf punching method, and pathogenicity was checked after 14 days. DW, distilled water.

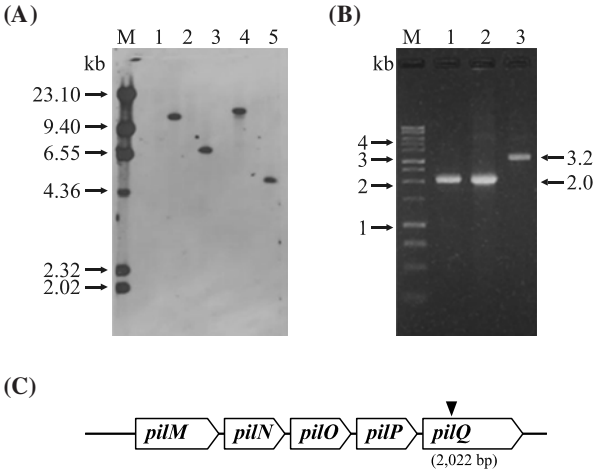


Fig. 2. Transposon insertion in *pilQ* gene of *X. oryzae* pv. *oryzae*. (A) Southern blot hybridization using probe kanamycine gene; M, λ HindIII marker, genomic DNA of wild type strain KACC10331 digested with *Eco*RI (lane 1), Genomic DNA of HNU89K9 digested with restriction enzymes *Eco*RI, *Eco*RV, *Sma*I, and *Sac*I (lanes 2~5). (B) PCR confirmation of wild type strains KACC10331 (lane 1) KACC10859 (lane 2) and mutant strain HNU89K9 (lane 3) using primer set (pilQ-R/pilQ-F). (C) Genetic organization of the *pilQ* gene cluster of *X. oryzae* pv. *oryzae*.

Virulence assay

Virulence assays were executed by punching 60-day-old leaves of the susceptible rice cultivar Milyang 23 with a toothpick soaked in *Xoo* re-suspended cells. Virulence of this mutant strain was investigated 14 days following inoculation

Twitching motility assay

To investigate the twitching motility of *Xoo* cells, the bacterial cells of 1×10^4 of wild-type strain KACC10331 and *pilQ* mutant strain HNU89K9 were spotted in a hole (diameter, 5×5 mm) of 0.5× PS agar plate that were air dried prior to spotting by removing the petri dish lid on a clean bench. Following incubation at 28°C for 3 days, the edge morphology of the colonies was observed under a light microscope (×100). Colonies with a marginal fringe were designated as having a wild-type twitching phenotype, while colonies lacking a marginal fringe were designated as no having a twitching phenotype.

Biofilm formation assay

Each colony from wild-type strain KACC10331 and the *pilQ* mutant strain HNU89K9 was grown in a polyvinylchloride plastic (PVC) tube containing 2 ml PS broth for 3 days at 28°C. The grown cell suspensions were removed from the PVC tube, and the surface of the tube was gently washed with distilled water five times. The tube was stained with 0.1% crystal violet for 5 min and washed with distilled water twice. The biofilm was visualized as blue color bands around the surface of the tube.

Results and Discussion

Characterization of *pilQ* defective mutant

The HNU89K9 showed attenuated pathogenicity on the susceptible rice variety Milyang 23 when compared with the wild-type strain KACC10331. The wild-type strain formed a yellowish lesion on the inoculated leaf and further expanded to the grayish-white blight, while the mutant strain HNU89K9 formed water soaked lesion around the inoculated sites, but the disease symptom of the mutant was not progressed with the pathotype like wild type strain (Fig. 1). On the other hand, the complement clone C89KB recovered pathogenecity comparing to wild type strain KACC10331. The result indicates that the *pilQ* gene is associated with pathogenicity.

It was previously reported that mutations in the *pilQ* gene of *R. solanacearum* (Huanli *et al*., 2001) and *X. fastidiosa* (Meng *et al*., 2005) result in reduction of pathogenicity, showing consistent with our result.

Furthermore, the growth rate of the mutant HNU89K9 was assayed at 600 nm wave-length at different time intervals by comparing it with that of wild-type strain KACC 10331. It was revealed that the growth rate of HNU89K9 is similar to that of the wild-type strain (data not shown), indicating the *pilQ* gene is not caused to reduce the bacterial growth of *Xoo*. To investigate the insertion number of Tn5 in HNU89K9, genomic DNA was extracted from the wild-type strain KACC10331 and the mutant strain HNU89K9 digested with *Eco*RI, *Eco*RV, *Sma*I, and *Sac*I that do not cut the transposon and subjected to southern hybridization analysis using the labeled kanamycine gene as a probe. Each single hybridized band of 11.2 kb, 6.5 kb, 13.2 kb, and 4.5 kb was detected in respective restriction enzymes (*Eco*RI, *Eco*RV, *Sma*I, and *Sac*I)-digested the genomic samples of mutant HNU89K9, but not in *Eco*RI digested the wild-type *Xoo* KACC10331 (Fig. 2A), indicating that the single transposon is inserted in the mutant HNU89K9 genome. The transposon insertion into the *pilQ* gene of the mutant was further confirmed by PCR amplification using a primer set that amplified the *pilQ* gene of *Xoo*. As shown in Fig. 2B, The size of PCR product (3.2 kb) of *pilQ* gene from mutant HNU89K9 was compared to 2.0 kb PCR products of wild type strains, confirming the transposon (1.2 kb) insertion in the *pilQ* gene. For identifying transposon target sequence, the 3.2 kb PCR product was cloned, yielding p89KB and sequenced using a primer (5'-GATTGTCGCACCACCTGATTG-3') designed from end sequences of transposon. The sequencing analysis showed a transposon is inserted in sequence of *pilQ* gene (data not shown).

Genetic organization of *pilQ* region in the *X. oryzae* pv. *oryzae* genome

The whole genome sequence of the *Xoo* KACC10331 strain (Lee *et al.*, 2005) provides the foundation for sequence-based identification of insertion sites in transposon mutants and genetic organization of target genes. The distribution of pili genes in the *Xoo* genome were analyzed according to the *Xoo* sequence information released in NCBI. Interestingly, 26 pili-related genes were present in different regions of the *Xoo* genome including *pilA, pilZ, pilH, pilMNOPQ, pilTU, pilDCE, pilBRS, pilGHIJL, pilF, pilE1Y1*, *pilX*, and *pilV*. The *Xoo* genome analysis suggests that a number of pili gene orthologs may encode proteins involved in the biogenesis and function of Type IV pili. As shown in Fig. 2C, it was revealed the *pilQ* gene is a member of a gene cluster, *pilMNOPQ*, the *pilQ* organization of *Xoo* resembles to that of the *pilMNOPQ* operon in *Myxococcus xanthus* (Sun *et al*., 2000) and *Pseudomonas aeruginosa* (Alm and Mattick, 1997). In *M. xanthus* and *P. aeruginosa, pilQ* is a secretin located in the outer membrane and may form a channel gate through which the pilin subunits travel. Inactivation in the *pilQ* gene prevented the biogenesis of TFP. It was also reported that 14 pili genes (*pilIJKLMNOPQRSTUV*) are involved in Type IV pilus biogenesis of an *E. coli* cell (Sakai and Komano, 2002). Of them, the *pilQ* gene product is a cytoplasmic ATPase and is essential for thin pilus formation (Sakai *et al.,* 2001). The remaining *pil* genes are likely to encode structural proteins that function in the establishment of the pilin transport apparatus and thin-pilus basal body. Most of these pili gene products contain signal sequences or trans-membrane domains, suggesting that they are transported to the peri-plasmic space, inner membrane, and outer membrane.

The amino acid sequences deduced from *pilQ* gene in *Xoo* were compared with other protein sequences from different bacterial species including *X. campestris* pv. *campestris*, *X. campestris* pv. *vasicatoria*, *X. axonopodis* pv. *citri*, *Ralstonia* sp., *Xylella fastidiosa* in GenBank (Table 2). Consequently, *pilQ* of *Xoo* showed high identities more than 90% to amino acids of PilQ from *Xanthomonas* species, showing a closely related genetic relationship among them. Sequence identity to *Xyllela fastidiosa* was 60%, but a low sequence homology of 30% was identified in other bacterial species. The result suggests that *pilQ* is conserved in *Xanthomonas* species, but not in other bacterial species.

Table 2. Amino acid similarity among PilQ proteins of different bacterial species and *X. oryzae* pv. *oryzae* strains

Percent identity

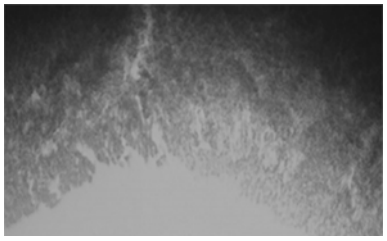
	1	2	3	4	5	6	6	8	9	10	11	12	
1		100.0	69.4	69.2	32.2	35.2	34.8	35.7	92.3	93.5	93.5	94.5	1
2	0.0		68.8	68.7	33.4	36.5	36.1	35.7	92.3	93.5	93.5	94.5	2
3	37.2	36.2			34.7	36.1	36.3	36.9	69.4	69.4	69.4	68.9	3
4	37.5	36.3	1.8		36.3	36.6	37.9	36.8	69.5	69.3	69.3	68.6	4
5	133.8	129.3	127.7	128.2		33.5	38.9	34.1	32.9	33.6	33.6	33.4	5
6	120.1	114.7	117.6	116.0	126.7		44.9	48.0	36.0	35.3	35.3	35.7	6
7	114.4	109.2	113.8	113.8	114.4	94.2		39.9	37.3	35.6	35.6	35.9	7
8	121.8	117.0	124.5	126.0	131.6	78.9	105.0		37.1	36.6	36.6	36.7	8
9	8.2	8.2	36.3	36.4	131.0	115.9	109.4	114.7		90.0	90.0	91.6	9
10	6.6	6.6	36.0	35.5	132.3	113.8	111.1	116.1	10.5		100.0	93.7	10
11	6.6	6.6	36.0	35.5	132.3	113.8	111.1	116.1	10.5	0.0		93.7	11
12	5.6	5.6	36.5	36.5	129.5	115.1	111.1	117.0	8.5	6.5	6.5		12
	1	2	3	4	5	6	6	8	9	10	11	12	

Divergence

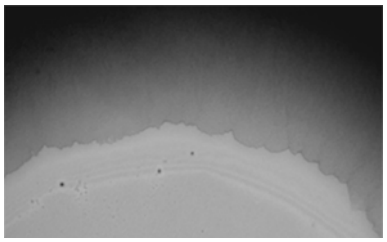
1. *X. oryzae* pv. *oryzae* KACC10331
2. *X. oryzae* pv. *oryzae* MAFF311018
3. *Xylella fastidiosa* 9a5c
4. *Xylella fastidiosa* Temecula
5. *Acinetobacillus* sp.
6. *Methylobacillus flagellatus*
7. *Nitrosococcus oceani*
8. *Ralstonia eutropha*
9. *X. axonopodis* pv. *citri*
10. *X. campestris* pv. *campestris* 8
11. *X. campestris* pv. *campestris* A
12. *X. campestris* pv. *vesicatoria*

pilQ Gene of *X. oryzae* pv. *oryzae* is associated with twitching motility and biofilm formation

It has been reported that Type IV pili is required for pathogenesis and a form of surface-associated movement known as twitching motility. Motility is a consequence of the extension and retraction of Type IV pili, which promotes bacteria across a surface by an undescribed mechanism. In different gram negative bacteria, it has been reported that *pilQ* genes involved in Type IV pili are closely related to twitching motility. Therefore, we assessed the twitching motility phenotype of the mutant HNU89K9 carrying *pilQ*. The bacterial cell suspensions of mutant HNU89K9 and wild-type *Xoo* KACC10331 were spotted in the hole of a PS agar plate and incubated for 2 days. The twitching motility was assessed by light microscopy (×100). Under the microscope, the edge of the colonies of strains that were proficient in twitching motility was highly irregular. This kind of marginal fringe was observed on the wild-type strain, while the smooth-colony edge phenotype was observed on the mutant strain (Fig. 3). This is considered a consequence of the surface movement associated with Type IV pili. Such a marginal fringe can be referred to as twitching motility, because it closely resembles that of TFP-dependent characteristics in well-studied strains of *R. solanacearum* and *X. fastidiosa* (Marques *et al*., 2002; Meng *et al*., 2005). The finding suggests that *Xoo* cells actively migrate to other plant tissue via twitching motility.



KACC10331



HNU89K9

Fig. 3. Twitching motility assay of the wild-type *Xoo* strain KACC 10331 and mutant strain HNU89K9. Bacterial cells were inoculated in PSA for 2 days, and the twitching motility was observed by the formation of marginal fringe on each colony.

KACC10331 HNU89K9

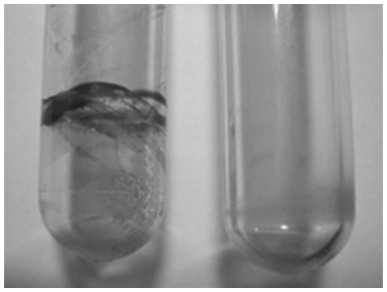


Fig. 4. Biofilm formation assay of wild-type *Xoo* strain KACC 10331 and mutant strain HNU89K9. *Xoo* strains KACC10331 and HNU89K9 cells were grown in PVC tubes containing PS liquid media at 28°C for 7 days. Biofilm formation was observed on the surface of PVC tubes by staining with 0.1% Crystal violet.

Twitching is a form of bacterial movement on surfaces of solid materials and can be used to colonize pathogens on hosts. It is facilitated by Type IV pili that are generally located at one cell pole (Mattick *et al.,* 2002) and is characterized by jerky, intermittent movements that facilitate surface colonization. The cell surface component of the pathogen may play a role in systemic colonization during interactions between host and pathogen, and it has mainly been focused on EPS and LPS biosynthesis related genes as candidates in *Xoo*. Phytopathogenic bacteria may physically be attached on the host plant in virtue of the chemicals. However, movement of the bacterial cell is required for expanding the disease in plant tissue. For example, *R. solanacearum* and *X. fastidiosa,* the causal agent of vascular wilt disease of plants, possess twitching-mediated movement since they were previously shown to exhibit colony morphology characteristic of twitching behavior (Marques *et al*., 2002; Meng *et al*., 2005)

On electron microscopic observation (TEM) of *X. fastidiosa* cells, *pilB*, and *pilQ* mutants result in cells that bore only the short *pili* and did not twitch. Moreover, their ability for the pilus-mediated basipetal movement was relatively reduced when compared to wild type strain, suggesting long Type IV pili play an important role in movement through plant tissue basipetally. We made observations of *Xoo* cells on a transmission electron microscope (TEM). The long and short pili containing single flagella were present in the *Xoo* cells of KACC10331 (data not shown), although we failed to detect the distinct difference of pili between wild-type and mutant HNU89K9 strains on TEM by our technique. A special apparatus is required to capture in detail the twitching motility of each cell. A microfluidic chamber was devised for observing the upstream twitching of *X. fastidiosa* (Meng *et al*., 2005). It was observed that twitching movement propels individual cells in various directions depending on the rate and medium flow. The twitching speed, paths, and cumulative distance were also measured under the chamber.

Since it was reported that Type IV pili have been associated with biofilm formation (O'Toole and Kolter, 1998; Meng *et al*., 2005), a biofilm experiment was performed in mutant HNU89K9 and wild type *Xoo* KACC10331 strains. Both *Xoo* strains were grown in a test tube made of PVC containing PS broth for 7 days to test for its ability to form a biofilm on an abiotic surface. The biofilm was detected by staining with crystal violet (CV), a purple dye that stains bacterial cells. The unattached cells were removed by repeated washing with distilled water. As shown in Fig. 4, the biofilm was observed on a wild-type PVC tube as a ring of CV-stain that forms at the interface between air and medium. In contrast,

mutant HNU89K9 strain did not have such biofilm formation, strongly suggesting the *pilQ* gene is a factor for forming biofilm of *Xoo*. Biofilm formation is critical for bacterial attachment and pathogenicity on hosts (Costerton *et al*., 1995). It was reported that Type IV pili-mediated twitching motility in *Pseudomonas aeruginosa* may be necessary for cells to migrate along the surface to form multi-cell aggregates that lead eventually to the formation of biofilm (O'Toole and Kolter, 1998), showing good agreement with our result. Therefore, it is important to note that the strain defective in *pilQ* genes expresses neither twitching motility nor biofilm formation. It is reasonably assumed that Type IV pili play a direct role in stabilizing interactions with the abiotic surface and forming cell-to-cell interactions required to form biofilm. The *fimA* gene in the *E. coli* code for an essential Type I pilus protein functioning in surface attachments and biofilm formation (Orndorff *et al*., 2004). Mutations in *fimA* gene of *X. fastidiosa* resulted in the cell phenotype with short pili, causing a decrease in biofilm formation (Meng *et al*., 2005). However, in *X. fastidiosa,* long pili absence in *pilQ* mutants without twitching resulted in enhanced biofilm formation and indicated distinct functional roles for two length classes of pili. Long pili prevent biofilm formation, while short pili are important in surface attachment and biofilm development. Most plant-bacterial associations rely upon the physical interaction between bacteria and plant tissue. *Xoo* is a vascular pathogen and is normally restricted to the xylem of leaves of infected rice plants during early stages of the disease. Cells adhere to surfaces and each other through a complex matrix consisting of a variety of exopolysaccharides (Takahashi and Doke, 1984). It was previously shown that the release of bacteria from biofilms can involve enzymatic degradation of the EPS component and promote colonization in the vascular system.

In conclusion, we made the first observation of the twitching motility related to the *pilQ* gene of *Xoo,* depending on colony morphology and biofilm formation on solid media. Thus, it was also reasonably assumed that the *pilQ* gene of *Xoo* functions to form the biofilm on the surface of PVC tubes by using a twitch-mediated mechanism. These findings can provide basic information about how *Xoo* cells migrate to the vascular system from the initial infection site. However, in future studies, additional twitching motility related to TFP genes will be analyzed and characterized for function, and an experiment will be performed to observe the detailed movement of *Xoo* cells.

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