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Proteomic and Functional Analyses of a Novel Porin-like Protein in *Xanthomonas oryzae* pv. *oryzae*[§]

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Proteomic analysis is a useful technique for postulating and elucidating protein functions. In the present work, a shotgun proteomic analysis was used to identify functions of the *PXO_03968* gene (previously known as the *ax21*) from *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), a causal agent for bacterial blight disease in rice. Structural prediction performed on the protein sequence encoded by *PXO_03968* reveals that it encodes a putative porin-like protein, possessing a *β*-barrel domain with 10 *β*-strands and a signal peptide at the N-terminus. We renamed the gene as an *omp1X* (outer membrane protein 1 in *Xoo*), generated its knock out mutant (*XooΔomp1X*), and compared the protein expression level in the mutant to that in the wild type. A total of 106 proteins displayed more than 1.5-fold difference in expression between the mutant and the wild type strains. COG analysis revealed that these proteins are involved in cell motility as well as signal transduction. In addition, phenotypic analysis demonstrated that motility and biofilm formation in *XooΔomp1X* are lower than the wild type. These results provide new insights into the functions of outer membrane proteins in Gram-negative bacteria.

Keywords: outer membrane protein, proteomics, motility, biofilm formation

Introduction

The outer membrane in Gram-negative bacteria protects the cell against harsh environmental conditions. About half of the mass in the outer membrane mainly consists of two different types of proteins; integral membrane proteins and lipoproteins (Koebnik *et al.*, 2000). These outer-membrane proteins (OMPs) are not only indispensable for the structural stability of the outer membrane, but also responsible for diverse functions including transporters, membrane pores, recognition receptors, and membrane-bound enzymes (Wang *et al.*, 2002; Kostakioti *et al.*, 2005; Bishop, 2008; Lee *et al.*, 2013; Smiley *et al.*, 2013). For example, an OMP in *Xanthomonas campestris* pv. *campestris*, XcMopB, is known to be involved in pleiotropic effects, including production of extracellular polysaccharides and pathogenicity (Chen *et al.*, 2010).

In bacteria, some OMPs, including outer membrane porins, possess a *β*-barrel, a secondary structure consisting of an even number (14, 16, or 18) of *β*-strands (Nikaido, 2003) in this case. One of the most extensively studied OMPs is a porin-family protein abundant in Gram-negative bacteria (Zeth and Thein, 2010). Porins are large enough in size to allow passive diffusion of water, ions, and small molecules. Some porins, however, are substrate-specific. For example, LamB, PhoE, and Tsx porins form pores for the transport of maltopentaoses, phosphates, and nucleosides, respectively (Korteland *et al.*, 1982; Maier *et al.*, 1988; Denker *et al.*, 2005).

In addition to porins, many other *β*-barrel-containing membrane proteins are required for diverse functions such as signaling, iron uptake, motility, biofilm formation, and virulence in Gram-negative bacteria (Koebnik *et al.*, 2000; Hahn *et al.*, 2013; Noinaj *et al.*, 2013). The TolC protein, one of the outer membrane efflux proteins containing the *β*-barrel structure, contributes to the secretion of proteins, biosynthesis of polysaccharides, and antimicrobial resistance (Cosme *et al.*, 2008). Moreover, the TolC-HlyD-HlyB complex in *Escherichia coli* has been well-characterized for decades (Wandersman and Delepelaire, 1990). In this complex, TolC is thought to be an integral protein on the outer membrane and an element of the type I secretion system for the secretion of *α*-hemolysin. Another notable example is the EstE protein from *Xanthomonas vesicatoria* (Talker-Huiber *et al.*, 2003). EstE also contains a smaller number of *β*-strands (eleven) at the C-terminal and functions as an esterase.

Proteomics-based approaches have been widely used in recent years to predict functions of proteins/genes of interest, particularly for organisms whose genome information is readily available. Although genetic approaches working with RNA have been developed, the investigation in expression of RNA is not always correlated with this of proteins. In many cases, additional processes prior to and/or post transcription are required for protein expression (Gry *et al.*, 2009). Therefore, approaches that use a combination of proteomic and genomice information have an advantage in being able to directly and efficiently characterize genes and their related functions at the protein level (Peng and Gygi, 2001).

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The present report details efforts to characterize a porin-like protein encoded by the *PXO_03968* gene from *X. oryzae* pv. *oryzae* (*Xoo*), a yellow-pigmented Gram-negative bacterium. *Xoo* is known to cause bacterial leaf blight disease in rice, one of the most destructive diseases in lowland rice-growing areas worldwide (Mew, 1987). The encoded protein had been known as activator of XA21-mediated immunity (Ax21); however, a recent study showed that Ax21 did not possess the activity triggering immune response in rice carrying XA21 pattern recognition receptor (Bahar *et al.*, 2014). Therefore, we re-examined the function of the protein and renamed the protein as Omp1X (outer membrane protein 1 in *Xoo*, NCBI Accession No. KJ818481) in this study. Structural analyses predicted that the protein possesses a β-barrel domain. Shotgun proteomics and a subsequent COG analyses were used to determine the function of Omp1X. Results from these analyses indicate that Omp1X plays a role in cell motility. Phenotypic assays confirmed that the protein is indeed required for motility and biofilm formation in *Xoo*. These results allow us to elucidate protein functions through a combination of proteomic and phenotypic analyses in Gram-negative bacteria.

Materials and Methods

Bacterial strains and growth conditions

Xanthomonas oryzae pv. *oryzae* (*Xoo*) PXO99Az was used as the wild type (Hopkins *et al.*, 1992). *Xoo* were cultured at 28°C in peptone sucrose (PS) medium (peptone 10 g/L, sucrose 10 g/L, L-glutamic acids 1 g/L) or tryptic soy (TS) medium (Tryptic Soy Broth Soybean-Casein Digested 30 g/L). *Escherichia coli* DH5α was used as a host for all vectors required. DH5α was grown in Luria-Bertani (LB) medium at 37°C. Antibiotics were used when required for selection: cephalexin (20 μg/ml), kanamycin (50 μg/ml), gentamicin (10 μg/ml), and ampicillin (100 μg/ml). To check the effect of *omp1X* on bacterial growth, *Xoo* (approximately 10^5 cells/ml) strains were incubated in PS media for three days. Incubated cells were collected at 12 h interval, serially diluted, and quantified by a colony-dotting method on PS media containing 1.5% agar and proper antibiotics.

In silico modeling

The 198-amino acid sequence of Omp1X was submitted to the I-TASSER server (Roy *et al.*, 2010) for structural prediction. The putative 3D structure was visualized using PyMOL (Bramucci *et al.*, 2012) with a Protein Data Bank file from the I-TASSER server.

Construction of *omp1X* gene knockout and complemented strains

Standard protocols described by Sambrook *et al.* (1989), were used for all genetic manipulations. *omp1X* gene knockout mutant (*XooΔomp1X*) was generated by marker-exchange mutagenesis using a kanamycin-resistance cassette and the suicide vector pGEM-T easy (Promega, USA). A DNA fragment *omp1X* was amplified using gene-specific primers, 5′-gagagagtcgccttgcagtt-3′ and 5′-agcgctagagcgtcacattt-3′. The plasmid was digested with *Spo*I, and the kanamycin resistance cassette was inserted. The construct carrying was introduced into wild type cells by electroporation using Bio-Rad Micropulser™ (2.5 kV). After electroporation, the cells were incubated for 3 h at 28°C, and spread on PS plates containing kanamycin. Colonies that grew on the kanamycin plates, but not on the kanamycin/ampicillin, were collected and confirmed by PCR with the same primers used for cloning. To construct the plasmid for complementation of *XooΔomp1X*, a putative promoter region and an open reading frame of *omp1X* gene were amplified using two sets of primers (5′-ctgcagccatggtcgacgatgcagctccatccgtgtg-3′ and 5′-ctcgaggtttcagcagcggaggccgcaacggca-3′ for a promoter, and 5′-gtcgacttaccagctgaagcgcgggc-3′ and 5′-ctcgagcatcatcatcatcat catgctgaaaacctctcctacaac-3′, carrying a region for 6× His-tag). Two fragments were sequentially inserted into the pML122 (Labes *et al.*, 1990) for expression of Omp1X and introduced into *XooΔomp1X* by electroporation. After incubation in PS broth, the cells were spread onto PS plates containing gentamycin and kanamycin [*XooΔomp1X*(Omp1X)]. Expression of the recombinant Omp1X was verified by western blot analysis using the Omp1X antibody generated by Pacific Immunology (Supplementary data Fig. S1).

Protein extraction and peptide preparation

All reagents were purchased from Sigma-Aldrich unless otherwise specified. The wild type and *XooΔomp1X* were grown to 0.6 at OD_{600}, and harvested. After washing twice, the pellets were dissolved in 1 ml lysis buffer (6 M guanidine-HCl, 10 mM DTT, and 50 mM Tris-HCl; pH 7.8). Resuspended cells were disturbed by sonication using an Ultrasonic Processor (Cole Parmer, USA). After collecting supernatants, protein concentration was measured by BCA assay kit (ThermoFisher, USA). The extracted proteins (1,000 μg) were incubated at 60°C, alkylated with 100 mM iodoacetamide, and incubated again with 20 mM DTT. To precipitate the proteins, 0.3 volumes of ice-cold trichloroacetic acid were added, and incubated at 4°C for 12 h. After centrifugation, pellets were dissolved in 1 ml of acetone. After centrifugation, the pellets were dried and then dissolved in 50 mM ammonium bicarbonate.

For tryptic digestion, 5 μg of trypsin (Promega) were added to 200 μg of extracted proteins and the mixture was incubated for 24 h at 37°C. The trypsin-treated proteins were acidified with 0.4% trifluoroacetic acid (TFA, Sigma, USA), and loaded into the Sep-Pak tC18 1 cc Vac Cartridge (Waters, USA) to remove unnecessary molecules. After washing the cartridges with 0.1% TFA, the samples were eluted with 1 ml of elution buffer (0.1% TFA, 50% acetonitrile). The eluted samples were then dried by Speed Vac concentrator (Vision, Korea). The dried samples were resuspended in 30 μl of 0.4% acetic acid, and quantified by BCA assay kit (ThermoFisher).

Mass spectrometry analysis

A split-free nano liquid chromatograph (EASY-nLC II, ThermoFisher) connected to a LTQ Velos Pro dual-pressure linear ion trap mass spectrometer (ThermoFisher) running in nanospray ionization mode was used to analyze the digested proteins (2 μg). The samples were separated by a

column with 7.5 cm of MAGIG C18AQ 200A (5 μm) material (Michrom, USA). The peptides were eluted over a 420-min gradient (300 nl/min) by a water/acetonitrile gradient (Solvent A, water with 0.1% formic acid; Solvent B, 100% acetonitrile with 0.1% formic acid) consisting of 5-min gradient to 7% B, 380-min gradient to 35% B, and 10-min gradient to 80% B, with a final hold at 7% B for 25 min. Full MS spectra were obtained in 6 data-dependent MS/MS scans over m/z 300–2000 mass range. Dynamic exclusion was enabled with 1 repeat count, 0.5-min repeat duration, and 3.0-min exclusion duration, with charge state selection allowed for the selection of 2^+ and 3^+ ions. Peptides were transferred to the ionizer with a 1.8 kV spray voltage and a desolvation capillary temperature of 200°C. In each full MS scan, up to 6 most intense ions were consecutively fetched for fragmentation and analyzed in a centroid mode within the linear ion-trap part of the instrument. Three biological replicates were carried out for all samples.

Protein/Peptide identification and quantification

For interpretation of MS/MS spectra, Thermo Proteome Discoverer 1.3 (ver. 1.3.0.399) with SEQUEST search algorithm was used. The spectra were investigated against the *Xoo* strain PXO99Az database. Trypsin was set as an enzyme, and up to two missed cleavages were acceptable. All peptides possessed 0.01 of a false discovery rate with reversed database searches, and 100 ppm of precursor mass accuracies were accepted. Probability scores of all peptides were over 20. The oxidation of methionine was considered as a possible modification. Proteins that matched to at least two unique peptides were considered to be present in the sample. For comparison of protein expression in the wild type and *XooΔomp1X*, the peptide spectra count (PSM) method was employed. PSMs from each protein were normalized against the total number of PSMs from all proteins in each sample. Proteins detected in all biological replicates were selected and used for comparison. The average of normalized PSMs in three biological replicates was calculated per protein and used as a comparison value for the wild type and *XooΔomp1X* to verify differently expressed proteins.

Motility assay

To test Omp1X function on bacterial motility, the semi-solid plate was used (Lu *et al.*, 2009). *Xoo* strains were grown in TS plates containing 1.5% agar, harvested, and resuspended to 5×10^8 CFU/ml, and 3 μl of cultures were dropped onto the center of the assay plate (TS plates containing 0.3% agar), and incubated at 28°C for 7 d. The diameters were measured, and the mean values were obtained from four biological replicates. This experiment was repeated at least four times. Twitching motility function was observed under a light microscope as described previously (Lim *et al.*, 2008). This experiment was repeated at three times with eight biological replicates. All replicates showed similar patterns.

Biofilm assay

To measure biofilm formation, 96-well polyvinyl chloride (PVC) microplate assay was used (Zimaro *et al.*, 2013). The wild type, *XooΔomp1X*, and *XooΔomp1X* (Omp1X) were grown on PS agar. Bacterial cells were harvested, and diluted to a concentration of 10^5 CFU/ml in minimal media (Schulte and Bonas, 1992). After incubation for 5 d in 96-well PVC (COSTAR, USA), the planktonic cells were removed. The remaining cells were stained by 0.1% crystal violet solution for 30 min, and the excess dye was removed. The dye that stained the attached cells was dissolved in 95% EtOH, the optical density was examined at 590 nm, and average values from 16 biological replicates were calculated. This experiment was repeated at least three times.

Results and Discussion

We generated a structural prediction of Omp1X using the I-TASSER server and PyMOL molecular graphics system (Roy *et al.*, 2010; Bramucci *et al.*, 2012) (Fig. 1). The predicted structure has significant structural homology with an N-terminal transmembrane domain of KpOmpA from *Klebsiella pneumoniae* whose structure has been solved by X-ray crystallography (Renault *et al.*, 2009). The template modeling (TM)-score is an efficient way to measure the similarity between the structure of KpOmpA solved by X-ray crystallography and the predicted structure of Omp1X. A TM-score of more than 0.5 indicates that the structures share the same SCOP/CATH fold (Zhang and Skolnick, 2005; Csaba *et al.*, 2009); the TM-score between Omp1X and KpOmpA was 0.68822, indicating that the predicted model contained the correct topology because over 0.5 TM-scores reveal the structures share the same SCOP/CATH fold (Zhang and Skolnick, 2005; Csaba *et al.*, 2009). The predicted structure of Omp1X has a structure of porins in Gram-negative bacteria: a *β*-barrel domain with 10 *β*-strands, without any domains/secondary structures near the C-terminus (Fig. 1). The *β*-barrel structure is unique to the proteins of the porin family (Zeth and Thein, 2010); hence, the fact that Omp1X contains this structure implies that it belongs to the porin

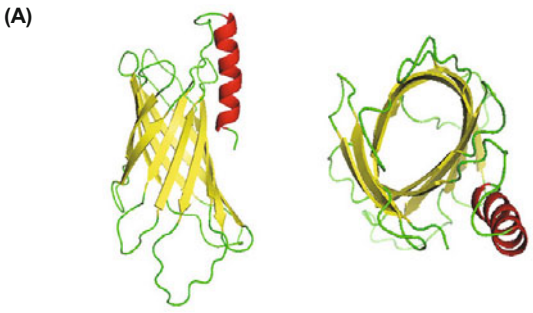

Fig. 1. Predicted structure of Omp1X. (A) 3D structures were generated using the PyMOL molecular graphics system. (B) Amino acid sequences of Omp1X. α-Helices and β-strands are indicated in red and yellow, respectively.

Table 1. Detected numbers of proteins and peptide spectral matches (PSM) in three biological replicates from Liquid chromatography-mass spectrometry experiments

Strains	1st		2nd		3rd		Shared proteins in 3 biological replicates
	Protein	PSM	Protein	PSM	Protein	PSM	
Xoo	708	20,204	601	13,889	656	17,375	540
XooΔomp1X	656	18,085	655	16,422	662	18,858	554

family. The structure prediction provided by I-TASSER suggests that Omp1X may be a novel type or an atypical porin because it possesses a smaller number of β-strands than typical porins. Typical porins are β-barrels consisting of 14, 16, or 18 β-strands (Nikaido, 2003). In addition to a β-barrel domain, the predicted model of Omp1X contains a signal peptide on the N-terminus in a form of α-helix structure (Fig. 1). A prediction made using the SignalP 4.1 server (Petersen *et al.*, 2011) showed that the Omp1X sequence contains a putative signal peptide for a sec-dependent secretion pathway from the N-terminus. These data suggest that the N-terminal sequence of the Omp1X is processed and the mature protein is localized at the outer membrane in *Xoo*. A recent study has shown that Omp1X is indeed localized at the outer membrane in *Xoo* (Bahar *et al.*, 2014), thus supporting our prediction.

Next, a proteomic analysis was carried out to investigate functions of the protein and to identify the proteins whose expression is affected by Omp1X. To create a comparative analysis at the protein level, the *omp1X* gene knockout mutant (*XooΔomp1X*) in the *Xoo* strain PXO99Az was generated using a marker change mutagenesis. An open reading frame of the gene was interrupted by a kanamycin cassette, creating the *XooΔomp1X* strain. The wild type and *XooΔomp1X* strains showed similar growth patterns in PS media (Supplementary data Fig. S1). A shotgun proteomic approach was used to analyze expression patterns of proteins in the wild type and *XooΔomp1X* strains. In total, 708, 601, and 656 proteins were detected from 20204, 13889, and 17375 peptide spectral matches (PSMs), respectively, in three biological replicates of the wild type strain and 656, 655, and 662 proteins from 18085, 16422, and 18858 PSMs were observed from the *XooΔomp1X* strain (Table 1). Among these proteins, 540 and 554 proteins found in all three biological replicates of the wild type and the *XooΔomp1X* strains, respectively, were used for comparison of protein expression patterns. The expression patterns of 106 proteins were associated with the presence of Omp1X (Fig. 2A). Among these changed proteins, 48 and 58 proteins were only found or highly expressed (over 1.5 fold) in the wild type and *XooΔomp1X* strains, respectively, suggesting that about 20% of the detected proteins were specifically affected by Omp1X in *Xoo*.

We used COG analysis to categorize 106 Omp1X-associated proteins (Tatusov *et al.*, 2000). Interestingly, the preponderance of highly expressed proteins in the wild type strain displayed COG results opposite to that of the same proteins in the *XooΔomp1X* strain (Figs. 2B and 2C). Among the highly expressed proteins in the wild type strain, the proteins involved in cell motility were abundant (Fig. 2B). This group contained 17 proteins including flagellin, pilus biogenesis/ assembly proteins, and chemotaxis-related proteins (Supplementary data Table S1). In addition, this group also contained proteins involved in signal transduction mechanisms (Fig. 2B). In contrast to the wild type, only 2 proteins related to cell motility or signal transduction were detected in *XooΔomp1X* (Fig. 2C, Supplementary data Table S2). Instead, *XooΔomp1X* expressed an abundance of proteins involved in replication, recombination, and repair (Fig. 2C). These data suggested that Omp1X is mainly involved

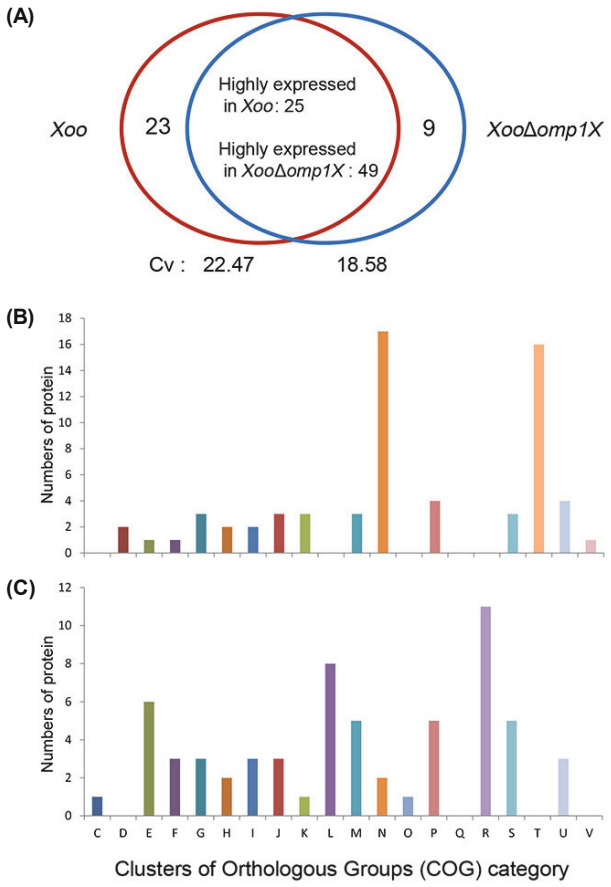

Fig. 2. Comparison and COG analysis of proteins regulated by Omp1X. (A) Venn diagrams illustrate numbers of differently regulated proteins from wild type (red circle) and *XooΔomp1X* (blue circle) in shared proteins from three biological replicates. There are 23 proteins detected only in the wild type; 25, highly expressed in the wild type; 9, only detected in *XooΔomp1X*; and 49, highly expressed in *XooΔomp1X*. Cv, Coefficient of Variation. COG analysis of proteins that were highly expressed or only detected in the wild type (B), and proteins that were highly expressed or only detected in *XooΔomp1X* (C). Abbreviations: C, Energy production and conversion; D, Cell cycle control and mitosis; E, Amino acid metabolism and transport; F, Nucleotide metabolism and transport; G, Carbohydrate metabolism and transport; H, Coenzyme metabolism; I, Lipid metabolism; J, Translation; K, Transcription; L, Replication and repair; M, Cell wall/membrane/envelop biogenesis; N, Cell motility; O, Post-translational modification, protein turnover, chaperone functions; P, Inorganic ion transport and metabolism; Q, Secondary structure; R, General functional prediction only; S, Function unknown; T, Signal transduction; U, Intracellular trafficking and secretion; V, Defense mechanisms.

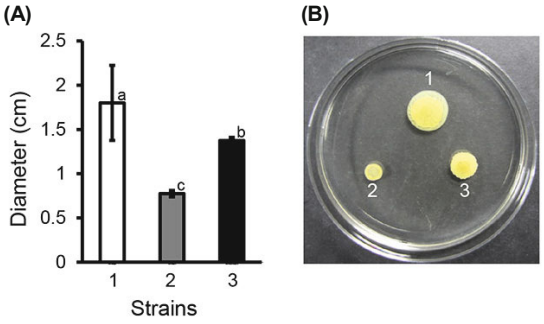

Fig. 3. Effect of Omp1X on motility. (A) Motility of wild type (1), *XooΔomp1X* (2), and *XooΔomp1X*(Omp1X) (3) strains were quantified by measuring the diameter of colony expansion in motility assays. Bars represent the mean of at least four biological replicates with standard deviations. Different letters represent significant differences ($P < 0.01$). (B) A photograph was taken 6 d after incubation. Statistical analysis was carried out using one-way anova with SPSS statistics 21 (IBM).

in bacterial movement and its regulation mechanism. In addition to bacterial motility, these results also suggest that Omp1X is partly associated with other cellular processes such as replication and amino acid metabolism.

Proteomic analysis implies that Omp1X seems to positively affect bacterial motility. To test the degree to which Omp1X affects *Xoo* motility, we carried out a motility assay using semi-solid media. *XooΔomp1X*(Omp1X), a complemented strain was generated, expressing the full length, recombinant Omp1X protein (Supplementary data Fig. S2) controlled by a native promoter on a plasmid in *XooΔomp1X* strain. The motility of the *XooΔomp1X* strain was significantly reduced (2.3-fold) compared with that of wild type strain (Fig. 3). Since a host vector with a broad range was used to carry the *omp1X* gene for the complemented strain, the modification may not be expected to fully restore the motility function. In addition, a twitching motility in terms of a pilus-dependent surface motility was also examined on solid-media containing 1.5% agar under a light microscope (Supplementary data Fig. S3). The marginal regions of colonies of the wild type strain were significantly irregular, while the mutant showed very smooth style at the marginal site.

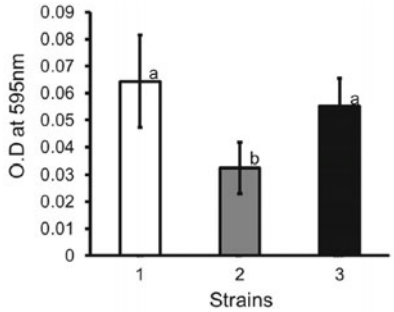

Fig. 4. Involvement of Omp1X in biofilm formation of *Xoo*. Biofilm formation of wild type (1), *XooΔomp1X* (2), and *XooΔomp1X*(Omp1X) (3) strains were measured according to absorbance at 595 nm (OD_{595}) using the polyvinyl chloride plate assay. Bars represent the mean of 16 biological replicates with standard deviations. Statistical analysis was carried out using one-way anova with SPSS statistics 21 (IBM). Different letters represent significant differences ($P < 0.01$).

In the support of our observation, a similar phenotype was also shown in the *piliQ*, which is required for Type IV pili synthesis, knockout mutant of *Xoo* (Lim *et al.*, 2008).

Bacterial motility is closely related with biofilm formation, a self-protection mechanism that helps bacterial cells survive in harsh environments as well as propagate in new niches (O'Toole and Kolter, 1998). To assess if Omp1X is also involved in biofilm formation, a plate adherence assay using a 96-well polyvinyl chloride plate was carried out. As expected, biofilm formation of *XooΔomp1X* was significantly impaired (2-fold) compared with those of the wild type and the complemented strain (Fig. 4). These results indicate that Omp1X is indeed involved in *Xoo* biofilm formation in a positive manner. This is in agreement with a previous work demonstrating that an ortholog of Omp1X is required for motility and biofilm formation in the human pathogen *Stenotrophomonas maltophilia* (McCarthy *et al.*, 2011). In addition, another study reported that an ortholog of Omp1X in *X. oryzae* pv. *oryzicola* whichs is a causal agent of bacterial streak disease on rice was also indispensible for biofilm formation (Qian *et al.*, 2013).

In conclusion, this study elucidates the function of an OMP using *in silico* structural prediction, a shotgun proteomic analysis, and phenotypic observation. Our findings suggest that proteomic data has high correlations with phenotypic changes and the functions of proteins, suggesting that a proteomic approach is a very useful for predicting the role of proteins. Although Omp1X was previously thought to be a rice immunity-triggering effector secreted via type I secretion system, results of our report echo those of a recent work indicating that Omp1X functions as an OMP in *Xoo* (Bahar *et al.*, 2014). In addition, the present study finds that Omp1X, a unique type of porin-like protein, possesses diverse roles in bacterial biology, including motility and biofilm formation. Although motility and biofilm formation of *XooΔomp1X* were significantly impaired and expression of proteins related with both functions were highly changed in the mutant, it is unclear that how expression of those proteins and the phenotypic changes of the mutant are affected by Omp1X. One possible explanation is the phenotypic alteration of the mutant may be affected by metabolic changes related with Omp1X function as an OMP. Therefore, further analyses will be carried out to test whether or not Omp1X functions as a channel, like a porin, and to examine that other phenotypes in *Xoo* are affected by the protein.

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