Novel Insights into Tat Pathway in *Xanthomonas oryzae* pv. *oryzae* Stress Adaption and Virulence: Identification and Characterization of Tat-Dependent Translocation Proteins

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ABSTRACT

Xanthomonas oryzae pv. *oryzae*, an economically important bacterium, causes a serious disease in rice production worldwide called bacterial leaf blight. How *X. oryzae* pv. *oryzae* infects rice and causes symptoms remains incompletely understood. Our earlier works demonstrated that the twin-arginine translocation (Tat) pathway plays a vital role in *X. oryzae* pv. *oryzae* fitness and virulence but the underlying mechanism is unknown. In this study, we used strain PXO99^A as a working model, and identified 15 potential Tat-dependent translocation proteins (TDTP) by using comparative proteomics and bioinformatics analyses. Combining systematic mutagenesis, phenotypic characterization, and gene expression, we found that multiple TDTP play key

roles in *X. oryzae* pv. *oryzae* adaption or virulence. In particular, four TDTP (PXO_02203, PXO_03477, PXO_02523, and PXO_02951) were involved in virulence, three TDTP (PXO_02203, PXO_03477, and PXO_02523) contributed to colonization in planta, one TDTP (PXO_02671) had a key role in attachment to leaf surface, four TDTP (PXO_02523, PXO_02951, PXO_03132, and PXO_03841) were involved in tolerance to multiple stresses, and two TDTP (PXO_02523 and PXO_02671) were required for full swarming motility. These findings suggest that multiple TDTP may have differential contributions to involvement of the Tat pathway in *X. oryzae* pv. *oryzae* adaption, physiology, and pathogenicity.

Xanthomonas is an important genus of gram-negative bacteria that infects hundreds of economically important crops worldwide (Rodríguez-R et al. 2012; Royer et al. 2013). *X. oryzae* pv. *oryzae* causes one of the most serious diseases in rice, known as bacterial leaf blight, in many rice-growing areas (Chatterjee and Sonti 2002; Lee et al. 2005). A variety of virulence determinants, including adhesins, extracellular polysaccharides (EPS), cell motility, and stress tolerance, have been shown to play critical roles in promoting *X. oryzae* pv. *oryzae* infection (Büttner and Bonas 2010; Niño-Liu et al. 2006; Salzberg et al. 2008). Expression of these virulence determinants is differentially controlled by multiple regulatory pathways, including two-component signal transduction systems (Büttner and Bonas 2010), the cyclic di-GMP signaling system (Ham 2013), the quorum sensing system (Ham 2013), the interkingdom signaling system (González et al. 2013), and transcriptional regulators such as GamR (Rashid et al. 2016), Clp (Jeong et al. 2008), and KdgR (Lu et al. 2011).

Protein secretion systems have also been shown to promote bacterial infection during the interactions between *X. oryzae* pv. *oryzae* and rice (Büttner and Bonas 2010). In particular, the *X. oryzae* pv. *oryzae* type II, type III, and type V secretion systems are most extensively studied. Cell-wall-degrading enzymes such as lipase/esterase (LipA), xylanase, and cellulase are secreted through the type II secretion system (T2SS) and have been demonstrated to contribute to the promotion of *X. oryzae* pv. *oryzae* infection and virulence (Aparna et al. 2009; Büttner and Bonas 2010; Jha et al. 2007). The type III secretion system of *X. oryzae* pv. *oryzae* primarily

serves to deliver effector proteins such as AvrXa27, PthXo6, and PthXo7 that are required for bacterial full virulence and modification of host immune defenses (Block et al. 2008). The *X. oryzae* pv. *oryzae* adhesins such as XadA are type V secretion system (T5SS)-secreted proteins that function in bacterial attachment to the leaf surface and colonization in xylem vessels (Das et al. 2009). In addition, our earlier works show that the twin-arginine translocation (Tat) pathway, which is involved in the translocation of secretory proteins across the cytoplasmic membrane, is required for *X. oryzae* pv. *oryzae* virulence (Chen et al. 2009); however, the underlying mechanism (i.e., what are the Tat-dependent translocation proteins [TDTP] and their functions) remain to be elucidated in *X. oryzae* pv. *oryzae*.

In most gram-negative bacteria, the Tat pathway consists of a key component protein with six transmembrane helices, called TatC, and two structurally related TatA family proteins with a single transmembrane helix, called TatA and TatB (Patel et al. 2014). Substrate proteins that are translocated by the Tat pathway usually possess highly conserved twin-arginine (S/T-R-R-x-F-L-K) motifs in their N-terminal signal peptides (Kudva et al. 2013; Patel et al. 2014). Proteins that are translocated by the main secretion (Sec) pathway are usually in an unfolded state (Kudva et al. 2013). However, before Tat substrates are translocated into the periplasm, they are completely folded in the cytoplasm and most of them bind to function-related cofactors such as molybdopterin, FeS, and NiFe centers (Ochsner et al. 2002). The indispensable roles of the Tat pathway in pleiotropic phenotypes and the virulence of various pathogens have been investigated in several studies (Bronstein et al. 2005; González et al. 2007; Pradel et al. 2003; Rodríguez-Sanz et al. 2010). In many cases, it is considered challenging to link the pleiotropic phenotypes of the Tat pathway to particular Tat substrate proteins (De Buck et al. 2008). Nevertheless, with the development of proteomic studies (Hitchcock et al. 2010; Joshi et al. 2010) and bioinformatics prediction programs (Bagos et al. 2010; Bendtsen et al. 2005; Rose et al. 2002), an increasing number of previously

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uncharacterized proteins are currently identified as Tat substrates or TDTP. Most of these proteins play crucial roles in various physiological and biological processes, including iron acquisition and detoxification, electron transport and oxidation-reduction, and the biogenesis of the cell envelope and pathogenicity, in several human- and plant-pathogenic bacteria (Ball et al. 2016; González et al. 2007; Hitchcock et al. 2010;). For example, the phospholipases PlcH and PlcN secreted by the Tat pathway degrade host cellular lipids and contribute to the virulence in *Pseudomonas aeruginosa* (Ochsner et al. 2002). The β -lactamase BRO-2 has been demonstrated to be Tat dependent and to play a key role in virulence by protecting the peptidoglycan cell wall of *Moraxella catarrhalis* from damage caused by β -lactam antibiotics (Balder et al. 2013). In *Escherichia coli*, defects in the cell envelope can be observed in Tat-disrupted mutant strains because the incorrect export of the Tat substrates AmiA and AmiC affects the integrity of the bacterial outer membrane (Ize et al. 2003).

To the best of our knowledge, the TDTP in *X. oryzae* pv. *oryzae* and their functions are still unknown. Investigating this point will facilitate understanding whether the Tat pathway controls *X. oryzae* pv. *oryzae* stress adaption and virulence via its dependent translocation proteins. In the present work, we applied both comparative proteomics and bioinformatics analyses to discover the potential TDTP in *X. oryzae* pv. *oryzae*. This led to identification of 15 candidate TDTP. Combining multiple genetic and phenotypic approaches, we dissected the role of each tested TDTP in *X. oryzae* pv. *oryzae* virulence, growth, colonization, and adaption to multiple stress. This not only identifies new *X. oryzae* pv. *oryzae* virulence determinants but also establishes a previously uncharacterized connection between regulatory effect of the Tat pathway and its dependent translocation proteins in *X. oryzae* pv. *oryzae*. The latter point also helps to strengthen our understanding of how the Tat pathway controls *X. oryzae* pv. *oryzae* physiology, stress adaption, and virulence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The details regarding the bacterial strains and plasmids used in this work are provided in Supplementary Table S1. The wild-type (WT) strain PXO99^A of *X. oryzae* pv. *oryzae* and its derivatives were cultured at 28°C in liquid nutrient broth (NB) (beef extract, 3 g/liter; yeast extract, 1 g/liter; poly-peptone, 5 g/liter; and sucrose, 10 g/liter) or NB agar (NA) plates. *E. coli* DH5 α used for plasmid constructions was grown at 37°C in Luria-Bertani (LB) broth or LB agar plates. When required, the media were supplemented with antibiotics at the following concentrations for *E. coli* and *X. oryzae* pv. *oryzae*: ampicillin at 100 μ g/ml, kanamycin (Km) at 50 μ g/ml, and gentamicin at 5 μ g/ml.

Bioinformatics analyses. The sequence information was obtained from the PXO99^A genome database (GenBank accession NC_010717.2) (Salzberg et al. 2008). Predictions of protein subcellular localization were performed with PSORTb v3.0 (Yu et al. 2010) and CELLO v2.5 (Yu et al. 2006). To predict potential Tat substrates, 4,375 protein sequences in strain PXO99^A were analyzed using the following three major Tat prediction programs: PRED-TAT (Bagos et al. 2010), TatP v1.0 (Bendtsen et al. 2005), and TATFIND v1.4 (Rose et al. 2002). The putative signal peptide cleavage sites, which are normally located downstream from the Tat motif, for the predicted Tat substrates were identified using SignalP v4.0 (Petersen et al. 2011). In the present study, if a protein contains a putative signal peptide cleavage site, which is predicted to be located upstream from the Tat motif, then this protein is defined as a controversial Tat substrate for easy understanding. Cluster of orthologous groups analyses were performed using eggNOG v4.5 (Huerta-Cepas et al. 2016) and Protein Basic Local Alignment Search Tool (BLAST) (Mount 2007). Computation of the theoretical protein isoelectric point and molecular weight for

selected proteins was performed using the ExPASy tool (Artimo et al. 2012).

Preparation of *X. oryzae* pv. *oryzae* extracellular proteins. Extracellular samples for comparative proteome analysis were prepared as described previously, with minor modifications (Qian et al. 2013b). Briefly, the WT PXO99^A and the *tatC* in-frame deletion mutant (Δ *tatC*) were cultured in 250 ml of NB medium at 28°C to the midexponential phase (optical density at 600 nm [OD₆₀₀] = 3.0) (Supplementary Fig. S1). Then, 0.25 ml of bacterial protease inhibitor cocktail purchased from Sigma-Aldrich was added into each bacterial culture. Bacterial cells were removed via centrifugation (6,000 $\times$ g at 4°C for 30 min), and the supernatant was filtered through 0.22- μ m filter membranes. The cell-free supernatant was frozen at -80°C and lyophilized to complete dryness. This dryness was dissolved in double-diluted H₂O and purified using a protein Clean-up Kit (GE Healthcare Life Sciences). Isoelectric focusing buffer containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 40 mM dithiothreitol, and 2% (vol/vol) immobilized pH gradient buffer (pH 4 to 7) was used to dissolve the pellet. The concentration of each protein was determined using the QuickStart Bradford Protein Assay Kit (Bio-Rad Laboratories) according to the manufacturer's instructions. The resulting protein was then stored at -80°C for later use in two-dimensional gel electrophoresis (2-DE). Three biological experiments were conducted for the comparative proteomics in this study.

2-DE and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis. The methods used for 2-DE and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis were applied according to our earlier works (Qian et al. 2013b; Zhao et al. 2011). The protein samples were adjusted to the appropriate loading amount (200 μ g) and separated via 2-DE. Silver staining was performed according to a published procedure (Zhao et al. 2011). The comparative analysis of the resulting images was performed using the PDQuest v7.2 software (Bio-Rad Laboratories). The spots of differentially secreted proteins were excised manually from the 2-D gels. Destaining of silver-stained gels and in-gel trypsin digestion were performed as previously described (Calhoun et al. 2010). The masses of tryptic-digested peptide were determined using a MALDI-TOF-TOF 4700 mass spectrometer (Applied Biosystems). The resulting data were analyzed using the Data Explorer software package (Applied Biosystems), and the identification was performed with the MASCOT program (Matrix Science) using the probability-based Mowse score and a threshold of $P < 0.05$. Similarity searches were performed using the BLAST and the genome database of strain PXO99^A (Salzberg et al. 2008).

Genetic methods. To generate in-frame deletion mutants of a target gene, WT PXO99^A was used as a parental strain and allelic homologous recombination was applied using the suicide vector pK18mobsacB, as described previously (Qian et al. 2013a). Briefly, two flanking regions of the target gene were amplified with specific primer sets (Supplementary Table S2). The two polymerase chain reaction (PCR) fragments were digested with the corresponding restriction enzymes and ligated into pK18mobsacB. The resulting recombinant vectors for each target gene were validated by sequencing and introduced into WT PXO99^A via electroporation. Transformants were selected on NA plates without sucrose containing Km at 50 μ g/ml for the first crossover event. Positive colonies were then plated on NA plates containing 10% (wt/vol) sucrose to screen for a second crossover event. After two rounds of recombination, the resulting in-frame deletion mutants were confirmed by PCR analysis (Supplementary Table S3). For complementation, each target gene with its predicted promoter region was amplified by PCR using the designated primers and cloned into the broad-host-range vector pUFR034 (DeFeyer et al. 1990) or pUFR047 (Andrade et al. 2014). The resulting plasmid was then transferred into the corresponding mutant via electroporation to generate the complemented strains.

Pathogenicity assay. Pathogenicity assays were performed on susceptible 'IR24' rice using the standard leaf-clipping method, as previously described (Yang and Bogdanove 2013). All rice plants were maintained in a greenhouse at 28°C with approximately 70% relative humidity. The *X. oryzae* pv. *oryzae* strains were grown in NB medium at 28°C. All tested strains were adjusted to a concentration of OD₆₀₀ = 0.5 using a BioPhotometer (Eppendorf). Inoculations were performed by immersing scissors in freshly prepared bacterial suspensions and clipping approximately 2 cm from the tips of fully expanded leaves of 5- to 6-week-old rice plants. The lesion lengths (length of grayish symptoms from the inoculated tip to the leading edge) were measured 8 and 16 days postinoculation (dpi), and representative images of IR24 leaves were photographed at the indicated times. At least 10 leaves were inoculated with each tested strain in each replicate. The biological experiments were performed three times.

Growth assay. The growth dynamics of *X. oryzae* pv. *oryzae* strains in susceptible IR24 rice were determined using previously described methods (Yang and Bogdanove 2013), with minor modifications. Leaves of 5- to 6-week-old rice plants in the greenhouse were inoculated with the tested strains at a concentration of OD₆₀₀ = 0.5 using the leaf-clipping method described above. Samples of inoculated leaves were collected every 8 days (0, 8, and 16 dpi), followed by surface sterilization in 70% ethanol for 1 min and 0.5% (vol/vol) sodium hypochlorite solution for 1 min. The leaf samples were washed twice with sterile water for 5 s. The leaf samples were then sliced into small pieces and homogenized in sterile water using sterilized mortars and pestles. The resulting homogenates were diluted serially and plated in triplicate on NA plates supplemented with the appropriate antibiotics. After incubation at 28°C for 3 to 4 days, the CFU on each plate were estimated for each tested strain and converted into the 1 g of CFU per leaf for further data analysis.

To determine bacterial growth in nutrient medium, *X. oryzae* pv. *oryzae* strains were grown in NB medium at 28°C, pelleted by centrifugation at 6,000 × g for 5 min, and resuspended in sterilized distilled water to a concentration of OD₆₀₀ = 1.0. Then, 0.5 ml of each bacterial suspension was inoculated into 50 ml of NB medium to an initial concentration (OD₆₀₀ = 0.01). Growth was monitored by measuring the OD₆₀₀ against the NB medium blank every 4 h after inoculation, and all inoculated samples were grown at 28°C until the stationary phase was achieved. The biological experiments were performed three times and each involved three replicates.

EPS production and swarming motility assays. Assays for testing swarming motility and EPS production were performed according to previous studies (Guo et al. 2012; Qian et al. 2013b; Zheng et al. 2016), with minor modifications. Briefly, to measure EPS production, fresh cultures of *X. oryzae* pv. *oryzae* strains were inoculated into 50 ml of NB medium at a starting OD₆₀₀ of 0.01. After shaking at 220 rpm at 28°C for approximately 5 days, the supernatants were collected via centrifugation at 12,000 × g for 10 min, and EPS was precipitated from the supernatant by the addition of two volumes of ethanol. The EPS pellets were then dried at 60°C to a constant weight, which was recorded as an estimate of the EPS. All values were expressed relative to the volume. The biological experiments were performed three times and each involved three replicates.

For the swarming motility assays, *X. oryzae* pv. *oryzae* strains were cultured in NB medium at 28°C, and the concentration of each culture was adjusted to OD₆₀₀ = 1.0. Next, 3 µl of the bacterial culture were spotted onto the semisolid NA plates with 0.6% (wt/vol) agar. The plates were incubated at 28°C for approximately 5 days, and the diameter of the motility halo was recorded as an estimate of cell motility. The biological experiments were performed three times and each involved three replicates.

Stress tolerance assays. Agar plate-based assays for determining the stress tolerance were performed as described previously, with minor modifications (Li and Wang 2012; Qian et al. 2008). Briefly, *X. oryzae* pv. *oryzae* strains were cultured in NB medium at

28°C to the exponential phase; then, bacterial suspensions were adjusted to a concentration of OD₆₀₀ = 1.0, which was defined as the initial cultures (1×, OD₆₀₀ = 1.0). After diluting the initial cultures 5-fold (5×) and 25-fold (25×), 3 µl of the initial and diluted cultures for each strain were spotted onto NA plates or NA plates containing H₂O₂ at concentrations of 0.1 and 0.15 mM, sodium dodecyl sulfate (SDS) at concentrations of 0.01 and 0.0125% (wt/vol), CuSO₄ at concentrations of 0.25 and 0.5 mM, and NaCl at concentrations of 0.2 and 0.3 M, respectively. The growth phenotype of the WT PXO99^A on each plate were used as a control. All plates were incubated at 28°C for 3 to 5 days until the colony formed by strain PXO99^A at the lowest dilution (25×) was visible on each plate, followed by photography. The experiments were performed three times and each involved three replicates.

Statistical analysis. All data were analyzed using SPSS v.19.0 (SPSS Inc.). Significant differences in virulence, bacterial growth, EPS production, swarming motility, and gene expression among different strains were determined via the hypothesis test of percentages (*t* test).

RESULTS

Identification of 15 potential TDTP in *X. oryzae* pv. *oryzae*. To identify potential TDTP in *X. oryzae* pv. *oryzae*, the extracellular proteins from the WT and the *tatC* in-frame deletion mutant (*ΔtatC*) were prepared for comparative proteomics, as described in detail in Materials and Methods. Strain *ΔtatC* was generated in this study and its virulence deficiency was validated, in agreement with our previous finding derived from the *tatC*-disrupted mutant (Chen et al. 2009). The results of 2-DE showed that, in total, 48 secreted protein spots that displayed higher levels (>twofold) in WT compared with *ΔtatC* were identified (Supplementary Fig. S3). Among them, 19 protein spots with upregulated levels above the threshold ratio of 5.0 were further analyzed via MALDI-TOF-MS. This led to identification of 10 proteins according to the genome of strain PXO99^A (Table 1). Two programs, PSORTb v3.0 and CELLO v2.5, were used to predict the cellular localization of the identified 10 proteins, and the results demonstrated that all of them were located in the outer membrane or extracellular space, thus verifying the reliability of our results. Further bioinformatics analyses revealed that functions of the 10 proteins were classified into six categories (Table 1), including intracellular trafficking, secretion, and vesicular transport; extracellular structures (1 protein); inorganic ion transport and metabolism (2 proteins); cell motility (1 protein); lipid catabolic process (1 protein); cell wall, membrane, and envelope biogenesis (1 protein); and function unknown (4 proteins). Surprisingly, 8 of the 10 proteins were predicted to be T2SS-related proteins with Sec signal peptides at their N-terminal regions, whereas PXO_02047, a type VI secretion system (T6SS)-associated Hcp family protein, was predicted to lack the N-terminal signal peptide using the PRED-TAT and SignalP v4.0 bioinformatics servers. It should be noted that only PXO_02671 (XadA), a T5SS-associated adhesin protein, possessed a predicted twin-arginine motif sequence (RRHRLA) in its N-terminal region when analyzed using the PRED-TAT server (Table 1; Supplementary Table S4). It is worth mentioning that the transcriptional levels of the 10 TDTP-encoding genes were not affected in the *tatC* mutant compared with the WT as determined by quantitative reverse-transcription PCR (Supplementary Fig. S4), suggesting their changes in extracellular protein levels are not due to their alternation in transcription under the tested conditions.

In addition to the comparative proteomics described above, we also employed detailed bioinformatics analyses to further predict TDTP in *X. oryzae* pv. *oryzae*. For this purpose, the genome sequence of strain PXO99^A was analyzed to discover potential TDTP via three major programs, including PRED-TAT, TATFIND v1.4, and TatP v1.0. In all, 25, 52, and 30 proteins with Tat motifs

were predicted as TDTP using TATFIND v1.4, PRED-TAT, and TatP v1.0, respectively (Fig. 1). However, due to the limitation and possibility of each program in generating a degree of false-positive or false-negative predictions (Bagos et al. 2010; Bendtsen et al. 2005; Joshi et al. 2010), the distributions of predicted TDTP from the three programs exhibited large variation and only partial overlap (Fig. 1). Five proteins (PXO_02203, PXO_03132, PXO_01547, PXO_04621, and PXO_01470) were all predicted by use of each program and recognized as reliable or confident TDTP candidates (Fig. 1; Table 2). Such a strategy has been previously adopted and experimentally validated in other bacterial systems for predicting TDTP (McDonough et al. 2008; Widdick et al. 2006). Among them, PXO_03132 is a copper resistance protein A (CopA) with a homolog in *P. syringae* pv. *tomato* (Bronstein et al. 2005) that is important for copper homeostasis. PXO_01547, a nonhemolytic phospholipase C, is predicted to be a secreted protein in PXO99^A, and its homolog (PlcN) has been demonstrated to function in *P. aeruginosa* pathogenesis (Ochsner et al. 2002). PXO_04621 is homologous to glucose-fructose oxidoreductase, which has been demonstrated to have a physiological role in the production of sorbitol for osmoprotection in *Zymomonas mobilis* (Nurizzo et al. 2001). More importantly, the homologs of PXO_01547, PXO_03132, and PXO_04621 in the abovementioned bacteria have also been experimentally confirmed to be Tat dependent (Bronstein et al. 2005; Nurizzo et al. 2001; Ochsner et al. 2002), supporting the reliability and high performance of our prediction approaches.

To conclude, in this study, 10 and 5 proteins were identified as *X. oryzae* pv. *oryzae* TDTP candidates by using comparative proteomics and bioinformatics analyses, respectively. These 15 proteins were selected for further study (see below).

Fig. 1. Venn diagram indicating the distribution of potential twin-arginine translocation (Tat) substrates predicted using three major bioinformatics programs (TATFIND v1.4, TatP v1.0, and PRED-TAT) in *Xanthomonas oryzae* pv. *oryzae* PXO99^A. In total, 77 predicted Tat substrates were successfully annotated. Only five proteins (indicated with arrow) were simultaneously predicted by all three bioinformatics programs.

TABLE 1. Identification of 10 extracellular proteins with a greater than fivefold difference in *Xanthomonas oryzae* pv. *oryzae* compared with Δ tatC

Cat ^a	Spot	Score ^b	Cov (%) ^c	Fold change (WT/ Δ T) ^d	Function, similarity ^e	Functional categorization ^e	Accession number	Protein code	Signal peptide ^f	Pred ^g	pI (cal) ^h	<i>M_r</i> (cal) KD ^h
1	B01	212	17	27.6335	<i>Xanthomonas</i> adhesin-like protein A (XadA)	Intracellular trafficking, secretion, and vesicular transport; extracellular structures	gi 188523050	PXO_02671	Tat	OM, Ex	5.01	119.7
	B02	207	18	1000000	...	...	...	...	Tat	...	...	...
	B03	218	16	1000000	...	...	...	...	Tat	...	...	...
	B04	199	9	1000000	...	...	...	...	Tat	...	...	...
	B05	335	20	1000000	...	...	...	...	Tat	...	...	...
	B06	378	22	20.7356	...	...	...	...	Tat	...	...	...
2	B07	404	35	5.30965	TonB-dependent outer membrane receptor	Inorganic ion transport and metabolism	gi 188520202	PXO_04865	Sec	OM	5.14	87.7
3	B08	371	30	6.20421	TonB-dependent receptor	...	gi 188522758	PXO_02415	Sec	...	5.02	87.4
4	B19	258	38	5.62549	Flagellin (FliC)	Cell motility	gi 188521216	PXO_01001	Sec	Ex, OM	5.08	39.4
	B24	124	31	5.04918	...	...	...	...	Sec	...	...	...
5	B20	277	47	11.452	Secretory lipase/esterase (LipA)	Lipid catabolic process	gi 188523317	PXO_02951	Sec	Peri, Ex	6.07	45.4
	B22	143	35	5.15227	...	...	...	...	Sec	...	...	...
6	B26	165	12	6.85856	Tetratricopeptide repeat domain protein	Unknown function	gi 188518729	PXO_03477	Sec	...	8.73	43.2
7	B28	388	40	5.62907	Hypothetical protein	Unknown function	gi 188519088	PXO_03841	Sec	...	5.82	31.1
8	B39	283	41	9.7861	Predicted methyltransferase	Unknown function	gi 188523596	PXO_03273	Sec	...	9.13	30.8
9	B44	269	81	6.88195	Hcp family protein	Unknown function	gi 188522385	PXO_02047	None	Ex, Peri	5.76	18.1
	B45	293	80	20.9071	...	...	...	...	None	...	...	...
	B46	360	76	16.325	...	...	...	...	None	...	...	...
10	B30	287	31	6.0626	OmpA family outer membrane protein	Cell wall, membrane, envelope biogenesis	gi 188522869	PXO_02523	Sec	OM	4.88	39.5

^a Catalog number.

^b Scores are based on the Mowse search. Protein scores greater than 82 are significant ($P < 0.05$).

^c Sequence coverage.

^d WT = wild-type PXO99^A and Δ T = *tatC* mutant.

^e The functional catalog was performed using protein BLAST and eggNOG v4.5. Names and codes of identified proteins are according to genomic annotation of *X. oryzae* pv. *oryzae* PXO99^A.

^f Twin-arginine (Tat) and secretory (Sec) signal peptides was predicted by PRED-TAT, TatP v1.0, and SignalP v4.0.

^g Bacterial protein subcellular localization prediction was performed using PSORTb v3.0 and CELLO v2.5. OM = outer membrane, Ex = extracellular, and Peri = periplasmic.

^h Computation of the theoretical protein isoelectric point (pI) and molecular weight (*M_r*) for selected proteins was performed using the ExPASy tool.

Four TDTP are involved in *X. oryzae* pv. *oryzae* virulence.

To dissect the roles of 15 proteins described above in *X. oryzae* pv. *oryzae* virulence, each of their encoding genes was in-frame deleted, resulting in generating a library comprising 15 mutants. When observed 16 dpi on susceptible IR24 rice, the pathogenicity test showed that the average lesion lengths after infection with ΔPXO_02523 , ΔPXO_02203 , ΔPXO_03477 , and ΔPXO_02951 were 5.6 ± 0.82 , 11.1 ± 1.33 , 12.3 ± 2.0 and 15.0 ± 1.22 cm, respectively, which were significantly shorter than those of the WT (17.7 ± 0.97 cm, $P < 0.01$) (Fig. 2). Plasmid-based in trans complementation fully or partially rescued the deficiency of each mutant described above in virulence to WT levels. However, under similar test conditions, the lesion lengths caused by the remaining 11 mutants were not significantly different compared with the WT (Fig. 2). In addition, BLASTP searches reveal that *PXO_02951* encodes a secreted lipase, and its homologs in both *X. oryzae* pv. *oryzae* BXO43 (Aparna et al. 2009) and *X. campestris* pv. *vesicatoria* 97-2 (Tamir-Ariel et al. 2012) are also reported to be required for virulence.

Next, to explore whether the growth capacity of ΔPXO_02203 , ΔPXO_02523 , ΔPXO_03477 , and ΔPXO_02951 contributed to their impaired virulence in rice leaves, in planta colonization assays were conducted after 0, 8, and 16 dpi. At the beginning (0 dpi), there were no remarkable differences in colonization ability among all of the tested strains (Fig. 3). However, at 8 and 16 dpi, the colonization abilities of ΔPXO_02203 , ΔPXO_02523 , and ΔPXO_03477 were significantly decreased compared with those of the WT. In trans complementation fully or partially rescued each mutant in this function to WT levels. Under similar test conditions, there were no significant differences in colonization ability among the WT, ΔPXO_02951 , and its complemented strain at all tested time points. All tested mutants except ΔPXO_02523 displayed a WT growth pattern in NB broth, whereas ΔPXO_02523 showed a decreased growth ability compared with that of the WT (Supplementary Fig. S5). These results suggested that mutation in genes *PXO_02203*, *PXO_03477*, and *PXO_02523* impaired their respective in planta colonization abilities, thus representing at least one of the mechanisms underlying their involvement in virulence.

Fifteen TDTP contribute differentially to multiple stress tolerance in relation to *X. oryzae* pv. *oryzae* infection. To successfully infect the host, the phytopathogenic bacteria (i.e., *X. oryzae* pv. *oryzae*) must overcome the challenge of various

stresses during different stages of infection (Li and Wang 2012). To investigate whether the Tat pathway and 15 TDTP mentioned above are involved in stress tolerance of *X. oryzae* pv. *oryzae*, four mimicking stresses, including H_2O_2 , SDS, copper, and sodium, were selected according to previous reports (Bronstein et al. 2005; Li and Wang 2012; Qian et al. 2008). For the H_2O_2 tolerance assay, only the mutant ΔPXO_02523 exhibited impaired tolerance against H_2O_2 compared with the WT on NA plates containing 0.1 mM H_2O_2 . When the concentration of H_2O_2 increased to 0.15 mM, three mutants ($\Delta tatC$, ΔPXO_02951 , and ΔPXO_02523) exhibited impaired tolerance against H_2O_2 compared with the WT (Fig. 4B), whereas in trans complementation could fully or partially rescue their corresponding deficiency in this ability. Other remaining mutants were not affected in this assay. For the SDS tolerance assay, the $\Delta tatC$ strain exhibited visually impaired tolerance against SDS compared with the WT on NA plates containing 0.0125% SDS (wt/vol). The ΔPXO_02523 strain was almost deprived of tolerance against SDS when grown on NA plates containing 0.01 or 0.0125% SDS (wt/vol) (Fig. 4C). The deficiency of both mutants in this ability could be fully or partially rescued by in trans complementation. Other remaining mutants were not affected in this function. For the copper tolerance assay, when compared with the WT, the tolerance of $\Delta tatC$ and ΔPXO_03132 against $CuSO_4$ was completely abolished on NA plates containing 0.25 and 0.5 mM $CuSO_4$ (Fig. 4D), and their deficiency could be fully or partially rescued by in trans complementation. Other remaining mutants had no visible changes in this ability compared with that of the WT. In the sodium tolerance assay, compared with the WT, the tolerance of ΔPXO_02523 against NaCl was completely abolished on NA plates containing 0.2 and 0.3 M NaCl, whereas the tolerance of ΔPXO_03841 was nearly abolished only on NA plates containing 0.3 M NaCl (Fig. 4E). Their deficiency of both mutants could be partially rescued by in trans complementation. Similarly, mutations in other remaining TDTP-encoding genes did not cause visible changes in this assay. As a control, all tested mutants exhibited similar growth phenotype compared with WT on NA plates without any mimicking stress (Fig. 4A). Collectively, our results demonstrate that 15 TDTP contribute differentially to multiple stress adaptation associated with *X. oryzae* pv. *oryzae* infection.

Fifteen TDTP play differential roles in swarming motility and EPS production in *X. oryzae* pv. *oryzae*. EPS and swarming motility are known to play a key role in bacterial epiphytic survival

TABLE 2. Potential twin-arginine translocation (Tat) substrates of *Xanthomonas oryzae* pv. *oryzae* PXO99^A identified simultaneously using three major bioinformatics programs

Functional categorization ^a	Protein code ^a	Length (aa) ^b	Identified by programs ^c	Annotation/similarity ^a	Predicted localization ^d	Tat motif identified in the N-terminal signal sequence ^e
General function prediction only	PXO_02203	334	TF; TP; PT	Putative hydrolase	Periplasmic, cytoplasmic	MTAFQPSRSRRLLRTALTASALTALGQLGA
Secondary metabolites biosynthesis, transport, and catabolism	PXO_03132	602	TF; TP; PT	Copper resistance protein A (CopA)	Periplasmic	APSSSTRHAPAKPDPAPA $\dagger$ AS MSFDSPCSDASSNGLSRRRFVQGLALGGIA VAGGLWRRDARA $\dagger$ AT
Cell wall, membrane, envelope biogenesis	PXO_01547	220	TF; TP; PT	Nonhemolytic phospholipase C	Periplasmic, extracellular	MIDLPRRRLLQAGLTSGAAALLPSIARA $\dagger$ AA
Carbohydrate transport and metabolism	PXO_04621	369	TF; TP; PT	Glucose-fructose oxidoreductase (GFOR)	Periplasmic	MSVESITRRRLLAAFGGAGILLAAPTWA $\dagger$ VP
	PXO_01470	783	TF; TP; PT	Hypothetical protein	Periplasmic, cytoplasmic,	MTCNHTPSLSRRRFLAWSSLAVAAGFLRFP QASA $\dagger$ AQ

^a Functional categorization was performed using protein blast and eggNOG v4.5. Annotations and codes of identified proteins are according to the genomic annotation of *X. oryzae* pv. *oryzae* PXO99^A.

^b Length in amino acids (aa).

^c Tat substrates were predicted using several bioinformatics tools: TF = TATFIND v1.4, PT = PRED-TAT, and TP = TatP v1.0. Twin-arginine motifs are indicated with bold underline.

^d Bacterial protein subcellular localization prediction was performed using PSORTb v3.0 and CELLO v2.5.

^e Putative TAT motifs (in bold and underlined) were identified by checking the first 70 residues of all predicted Tat substrates. Cleavage sites of the N-terminal signal sequence were identified using the SignalP v4.0 and are indicated by arrows and dotted underline.

and disease symptom development in *Xanthomonas* spp. (Büttner and Bonas 2010; Zheng et al. 2016). Moreover, our previous findings also documented that the disruption of *tatC* significantly reduced EPS production and cell motility in strain PXO99^A (Chen et al. 2009). These phenomena prompted us to investigate whether 15 TDTP are also involved in EPS production and swarming ability. Under test conditions, only Δ PXO_02671 exhibited significantly increased EPS production compared with that of the WT, whereas this change could be rescued to WT levels by in trans complementation (Fig. 5A). Compared with the WT, the remaining 14 mutants had small changes in this function. As a control, the *tatC* deletion mutant (Δ *tatC*) exhibited a remarkable decrease in EPS production, in agreement with our earlier findings (Chen et al. 2009). Collectively, these results suggested that PXO_02671 was involved in regulating EPS production in strain PXO99^A.

The results of the swarming motility assay showed that the halo diameters of motility zones from Δ PXO_02523 and Δ PXO_02671 were significantly less than that of the WT. This deficiency from both mutants could be fully or partially rescued to WT levels by in trans complementation (Fig. 5B). These results indicated that PXO_02523 and PXO_02671 are involved in modulating the swarming motility in strain PXO99^A. Furthermore, we also showed that TatC plays a regulatory role in *X. oryzae* pv. *oryzae* swarming motility. The remaining mutants showed only slight changes in swarming motility compared with that of the WT under the test conditions.

Fig. 3. Bacterial populations of wild-type PXO99^A, four virulence-deficient mutants, and their complemented strains in susceptible rice leaves. *Xanthomonas oryzae* pv. *oryzae* strains were inoculated onto 5- to 6-week-old IR24 leaves at a concentration of optical density at 600 nm = 0.5 using the leaf-clipping method. Inoculated leaves for extracting bacteria were harvested every 8 days (0, 8, and 16 days postinoculation), homogenized in sterile water, and serially diluted to count the CFU on nutrient broth agar plates. Bacterial populations were presented as 1 g of CFU per leaf. Each value represents the average bacterial population $\pm$ standard deviation from three independent experiments. Asterisks indicate significant differences compared with the wild type (*t* test, ** indicates *P* < 0.01).

Fig. 2. Virulence tests of wild-type PXO99^A and 15 twin-arginine translocation-dependent translocation protein-coding gene mutants on susceptible rice IR24. **A**, Lesion lengths caused by *Xanthomonas oryzae* pv. *oryzae* strains on IR24 rice leaves. Leaves of 5- to 6-week-old rice plants were inoculated with the tested strains (optical density at 600 nm = 0.5) using the leaf-clipping method. Lesion lengths were recorded 16 days postinoculation. Values are the means $\pm$ standard deviation from three independent experiments. Asterisks above error bars indicate significant differences compared with the wild type (*t* test, ** indicates *P* < 0.01). **B**, Disease symptoms caused by *X. oryzae* pv. *oryzae* strains on IR24 rice leaves. Representative photographs were taken 16 days postinoculation. In A and B, deletion in four genes, including *PXO_02203*, *PXO_02523*, *PXO_03477*, and *PXO_02951*, attenuated the virulence of *X. oryzae* pv. *oryzae*. Genetic complementation (*CΔPXO_02203*, *CΔPXO_02523*, *CΔPXO_03477*, and *CΔPXO_02951*) could fully or partially rescue the disease symptoms and lesion lengths of the respective mutants.

Fig. 4. Determination of the roles of the twin-arginine translocation (Tat) pathway and 15 Tat-dependent translocation proteins (TDTP) in multiple stress tolerance using plate-based assays. The *Xanthomonas oryzae* pv. *oryzae* strains were precultured to the exponential phase of the indicated value (optical density at 600 nm = 1.0, initial culture) in nutrient broth (NB) medium, and the initial culture (1×) was diluted 5-fold (5×) and 25-fold (25×). The initial culture and diluted cultures for each strain (3 μ l each) were spotted onto NB agar (NA) plates and NA plates containing multiple stressors at the indicated concentrations. **A**, No growth defects were observed among PXO99^Δ, Δ tatC, and 15 TDTP-coding gene mutants on NA plates (experimental control). **B**, Mutations in *tatC*, *PXO_02951*, and *PXO_02523* resulted in reduced tolerance of *X. oryzae* pv. *oryzae* against oxidative stress. **C**, Mutations in *tatC* and *PXO_02523* resulted in reduced tolerance of *X. oryzae* pv. *oryzae* against sodium dodecyl sulfate stress. **D**, Mutations in *tatC* and *PXO_03132* resulted in reduced tolerance of *X. oryzae* pv. *oryzae* against copper stress. **E**, Mutations in *PXO_02523* and *PXO_03841* resulted in reduced tolerance of *X. oryzae* pv. *oryzae* against sodium stress. In A, B, C, D, and E, genetic complementation could fully or partially rescued the stress tolerance of the mentioned mutants to wild-type levels. All plates were incubated at 28°C, and representative colony morphologies were photographed 3 to 5 days after spotting on agar plates. The experiment was repeated at least three times, with similar results.

DISCUSSION

In this study, we have, for the first time, identified 15 potential TDTP via comparative proteomics and detailed bioinformatics analyses, and dissected their roles in virulence and multiple stress adaption in the economically significant phytopathogenic bacterium *X. oryzae* pv. *oryzae*. The findings of this work led us to discover three previously uncharacterized virulence determinants that are required for full virulence in *X. oryzae* pv. *oryzae*, including PXO_02203, PXO_03477, and PXO_02523, all of which are conservatively distributed in the genus of *Xanthomonas*. We also provided the first evidence to show that different TDTP play differential roles in multiple stress tolerance (PXO_02523, PXO_02951, PXO_03132, and PXO_03841), EPS production (PXO_02671), and swarming motility (PXO_02523 and PXO_02671). These phenotypes are considered to be associated with *X. oryzae* pv. *oryzae* infection and environmental adaption. Therefore, these results, along with our earlier findings, raise the possibility that the *X. oryzae* pv. *oryzae* Tat pathway controls virulence and stress adaption, probably via its dependent translocation proteins, which provides new insights into the working mechanism of the Tat pathway in *X. oryzae* pv. *oryzae*.

In the present study, we identified 10 potential TDTP in *X. oryzae* pv. *oryzae* via comparative proteomics. Surprisingly, excluding PXO_02671 (XadA), which was predicted to have a Tat signal peptide, the remaining eight proteins were found to contain Sec but not the Tat signal peptide (Table 1). This situation has also been observed in other bacterial pathogens, where the majority of proteins present in significantly reduced amounts or absent in the exoproteome of $\Delta tatC$ were not predicted to contain Tat signal peptides as well (Ball et al. 2016; Joshi et al. 2010; Widdick et al. 2006). These findings collectively raise an interesting question: why are an unexpectedly high number of proteins containing Sec

but not Tat signal peptides easy to detect in the Tat-dependent exoproteome? The possible explanations for this query could be summarized as follows. (i) Technical limitation. It is likely that the significance threshold (>fivefold) used for filtering ambiguous protein spots and the limited ability of 2-DE to detect proteins with a low abundance prevented us from discovering “true” TDTP that contain Tat signal peptides. (ii) Cultural condition. Several laboratories have demonstrated that the number and types of extracellular TDTP identified by proteomics under different culture conditions can vary greatly (Ball et al. 2016; Bronstein et al. 2004; Joshi et al. 2010), suggesting that some true TDTP are most likely poorly expressed or secreted using the test nutritional condition in the present study. (iii) ‘Hitchhiker’ mechanism. The hitchhiker mechanism of the Tat pathway has been demonstrated to translocate proteins lacking Tat signal peptides across the cytoplasmic membrane by forming heteromeric protein complexes with specific Tat substrates (Tullman-Ercek et al. 2007). Therefore, the Tat pathway may contribute directly or indirectly to the translocation of specific Sec substrate proteins (Bronstein et al. 2004). Moreover, inactivation of the Tat pathway has also been demonstrated to have pleiotropic effects on a variety of cellular processes, including alterations of the Sec pathway (Bronstein et al. 2004; Joshi et al. 2010), which may subsequently result in reduced secretion of Sec-dependent proteins in $\Delta tatC$. Remarkably, of the 10 identified TDTP, 2 (PXO_02951 and PXO_01001) of their homologs, LipA and flagellin (FliC), were demonstrated to be secreted via the Tat pathway (Kouwen et al. 2009) or have been identified in the Tat-dependent exoproteome (Ball et al. 2016; Pradel et al. 2003) in other bacterial system under specific conditions, thus supporting our abovementioned hypothesis.

One of the important finding of the present study is to discover three previously uncharacterized virulence determinants in *X. oryzae* pv. *oryzae*. The first one is PXO_02203, which is annotated as a

Fig. 5. Effects of mutations in 15 twin-arginine translocation-dependent translocation protein-coding genes on extracellular polysaccharide (EPS) production and swarming motility of *Xanthomonas oryzae* pv. *oryzae*. **A**, EPS production of *X. oryzae* pv. *oryzae* strains in nutrient broth (NB) liquid medium after 5 days of growth with shaking at 220 rpm at 28°C. EPS production of mutant $\Delta tatC$ was significantly impaired but the mutation in PXO_02671 resulted in an unexpected increase in EPS production. **B**, Swarming motility of *X. oryzae* pv. *oryzae* strains on NB agar plates supplemented with 0.6% agar. The halo diameter of the motility zone of each strain was measured after 5 days of incubation at 28°C. Mutations in *tatC*, PXO_02523, and PXO_02671 led to impaired swarming motility of *X. oryzae* pv. *oryzae*. In A and B, genetic complementation could fully or partially rescued the EPS production and swarming motility of the mentioned mutants to wild-type levels. Values presented are the means $\pm$ standard deviation from three independent experiments. Significant differences compared with wild-type are indicated by ** (*t* test, *P* < 0.01).

putative hydrolase with similarity to the *Pseudoalteromonas carrageenovora* arylsulfatase (AtsA) or the *E. coli* ElaC domain-containing protein. In *E. coli*, the enzymatic analysis confirmed that this protein does not possess AtsA activity but, rather, has zinc-dependent phosphodiesterase activity that participates in tRNA 3'-processing (Vogel et al. 2002). To our knowledge, the AtsA or ElaC domain-containing proteins were rarely linked to virulence; thus, addressing the precise mechanism of PXO_02203 in virulence deserves future study. The second one, PXO_03477, is annotated as a tetratricopeptide repeat (TPR) domain protein. The TPR motif usually serves as a protein-protein interaction module and as a multiprotein complex mediator (Cervený et al. 2013). Notably, several TPR domain-containing proteins in bacterial pathogens have been reported to be associated with virulence-related processes (Cervený et al. 2013), which can be regarded as supporting evidence for our results. The third one is PXO_02523, which is predicted to be an OmpA family outer membrane protein. The OmpA-like proteins in bacteria mainly function in the maintenance of membrane structure and stability, and homologs of OmpA have been demonstrated to be required for pathogenicity in several plant and animal pathogens (Confer and Ayalew 2013; Rott et al. 2009). However, we demonstrated herein, for the first time, that the OmpA homolog (PXO_02523) in *X. oryzae* pv. *oryzae* is required for bacterial fitness and colonization in infected rice leaves, and it has a critical role in multiple types of stress tolerance and motility. Furthermore, in contrast, 2 of the 15 TDTP, flagellin (PXO_01001) and T6SS Hcp family protein (PXO_02047), which have homologs that are confirmed virulence factors in the bacterial pathogens *E. coli* O157: H7 (Pradel et al. 2003) and *Pseudomonas aeruginosa* (Mougous et al. 2006), did not involve pathogenicity in *X. oryzae* pv. *oryzae* under the test conditions, thus suggesting that the involvement of certain TDTP in virulence could vary in different bacterial pathogens.

The second important finding of this work is to show that multiple TDTP contribute differentially to *X. oryzae* pv. *oryzae* adaption under various environmental stresses, which provides evidence to connect the differential involvement of Tat pathways in these functions. In particular, we demonstrated that only the mutants $\Delta tatC$ and ΔPXO_03132 had impaired copper tolerance but PXO_03132 (CopA) was not required for *X. oryzae* pv. *oryzae* virulence under laboratory conditions. In *P. aeruginosa* and *P. syringae* pv. *tomato*, both homologs of TatC were also reported to be involved in copper tolerance. These studies also speculated that the increased copper sensitivity of $\Delta tatC$ presumably resulted from the incorrect translocation of the Tat substrate CopA (Bronstein et al. 2005; Ochsner et al. 2002). Testing such a hypothesis in *X. oryzae* pv. *oryzae* will facilitate addressing the detailed relationship between the Tat pathway and PXO_03132 in adaption to copper stress. Moreover, we also found that $\Delta tatC$ and ΔPXO_02951 ($\Delta lipA$) were associated with pathogenicity and H₂O₂ tolerance in *X. oryzae* pv. *oryzae*. In fact, the LipA homologs have been reported to be secreted lipolytic enzymes that are expressed in planta and contribute to pathogenesis during infection by several pathogens (Aparna et al. 2009; Tamir-Ariel et al. 2012). In this study, based on the above findings and data obtained for PXO_02951 (LipA) in the proteomics study, we hypothesized that the reduced secretion of PXO_02951 (LipA) in $\Delta tatC$ was most likely one of the reasons explaining the impairment of virulence and H₂O₂ tolerance of $\Delta tatC$ in *X. oryzae* pv. *oryzae*. For the impaired SDS tolerance observed in $\Delta tatC$, some studies in *E. coli* (Ize et al. 2003) and *Ralstonia solanacearum* (González et al. 2007) indicate that these phenotypes (SDS hypersensitivity) arise as a result of defects in the integrity of the cell envelope in the Tat mutant. Coincidentally, we found that mutation in PXO_02523, which is implicated in maintaining the integrity of the outer membrane, exhibited impaired virulence and increased sensitivity to H₂O₂ and SDS, similar to the phenotypes in $\Delta tatC$ in *X. oryzae* pv. *oryzae*, thus indicating that the reduced amount of PXO_02523 observed in the exoproteome of $\Delta tatC$ was likely a contributor for the Tat pathway

to withstand H₂O₂ stress and infection. Finally, it is also worth mentioning that PXO_02523 and PXO_03841 (lipid metabolism-associated) were required for sodium tolerance but $\Delta tatC$ was not defective in this function, thus suggesting that a Tat-independent mechanism may be used by PXO_02523 and PXO_03841 in adaption to sodium stress in *X. oryzae* pv. *oryzae*.

Adhesins are known to play a crucial role in the attachment of plant-pathogenic bacteria to specific host surfaces (Büttner and Bonas 2010). Particularly, the *Xanthomonas* adhesin-like protein A (XadA) has been demonstrated to be required for early attachment of BXO43 to rice leaves (Das et al. 2009). Extracellular levels of XadA (PXO_02671) are shown to be regulated by the Tat pathway in strain PXO99A; thus, along with earlier reports described above, it is possible that the Tat pathway may contribute to *X. oryzae* pv. *oryzae* attachment on the rice leaf surface via XadA (PXO_02671). Indeed, our results supported this hypothesis, because we found that, compared with the WT, both $\Delta tatC$ and ΔPXO_02671 exhibited a significant deficiency in bacterial adhesion on rice leaves via confocal laser-scanning microscopy and crystal violet staining analyses (Supplementary Fig. S6). These results suggested that the Tat pathway may affect *X. oryzae* pv. *oryzae* adhesion on rice leaves by modulating the biological behavior of PXO_02671. We also noticed that ΔPXO_02671 exhibited a fitness defect on the rice leaf surface, suggesting an important role of bacterial adhesion in the epiphytic fitness of *X. oryzae* pv. *oryzae*. Furthermore, bacterial adhesion has also been reported to be implicated in the epiphytic fitness of *X. fuscans* subsp. *fuscans* (Darsonval et al. 2009), which could be regarded as a supporting evidence for our result. Notably, in *X. oryzae* pv. *oryzae* BXO43, the *xadA* mutant is deficient in the ability to cause disease via surface inoculation (not by wound inoculation) (Das et al. 2009), indicating that bacterial adhesion and epiphytic fitness were likely contributors to the development of bacterial leaf blight disease.

EPS and swarming motility are important virulence determinants in *Xanthomonas* spp. (Büttner and Bonas 2010; González et al. 2013; Zheng et al. 2016). In this study, the mutation in PXO_02671 resulted in an increased level of EPS production. No significant reduction of EPS production was observed in the remaining 14 TDTP-coding gene mutants, indicating that additional factors rather than these 15 TDTP or cooperation between tested TDTP probably account for the reduced EPS production related to $\Delta tatC$. In addition, ΔPXO_02523 exhibited impaired swarming motility, which may be one of the reasons for the attenuation of virulence of this mutant on host rice. We also observed that the absence of PXO_02671 induced a significant decrease in swarming motility. However, the swimming motility of ΔPXO_02671 was not impaired compared with the WT (data not shown). Such an observation for one certain protein playing different roles in swarming motility and swimming motility has also been reported in *E. coli* (Inoue et al. 2007). The mechanism underlying this phenomenon regarding PXO_02671 is worthy of future study. Remarkably, of 15 TDTP, we had an initial expectation that the flagellin protein FlhC (PXO_01001), the homolog of which in *E. coli* is involved in a strongly repressed-swarming phenotype (Inoue et al. 2007), could explain the impaired virulence and decreased swarming motility of $\Delta tatC$; however, we did not observe any significant difference in virulence and swarming motility between ΔPXO_01001 and the WT. This result suggests that inactivation of a single flagellin protein in PXO99A may not be enough to cause visible phenotype changes (i.e., swarming motility). Such a hypothesis has been highlighted in related studies, wherein the authors show that the single flagellin gene mutant $\Delta fliC$ was still sufficient for forming swarming motility in *Salmonella typhimurium* (Harshey and Matsuyama 1994) and also exhibited WT virulence in *X. campestris* (Lee et al. 2003).

In summary, the present study shows that 15 TDTP have differential roles in virulence, colonization, attachment, stress tolerance, EPS production, and swarming motility in *X. oryzae* pv. *oryzae*. Interestingly, similar defects in varying degrees in these

phenotypes were also found in *ΔtatC*, indicating that the *X. oryzae* pv. *oryzae* Tat pathway most likely performs its multiple virulence-associated functions by directly or indirectly coordinating the biological behaviors of one or more of the above-characterized TDTP. This is helpful to understand how the Tat pathway controls pathogenicity in *X. oryzae* pv. *oryzae*.

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