A Defect in the Twin-Arginine Translocation Pathway Decreases the Tolerance of *Xanthomonas campestris* pv. *campestris* to Phenazines

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Accepted for publication 11 July 2020.

ABSTRACT

Phenazine-1-carboxylic acid (PCA), a member of phenazines secreted by microorganisms, inhibits the growth of many bacteria and fungi. *Xanthomonas campestris* pv. *campestris* is the causal agent of black rot, the most important disease of cruciferous crops worldwide, and is more tolerant to PCA than other *Xanthomonas* species. Previous studies reported that reactive oxygen species (ROS) scavenging ability is involved in regulating the PCA tolerance of *Xanthomonas* species. Additionally, the cytochrome *c* maturation (CCM) system has been found to play a more important role in tolerance to phenazines than the ROS scavenging system. In this study, a highly PCA-sensitive insertion mutant of *X. campestris* pv. *campestris*, X-5, was identified and studied. The insertion site of X-5 was found to be in *tatB* gene (*XC_4183*), which encodes a subunit of the twin-arginine translocation (TAT) complex. Disruption of the three genes of TAT pathway resulted in decreased

biological fitness and reduced tolerance to phenazines in comparison with the wild-type strain 8004. These results imply that the tolerance mechanism of the TAT pathway to phenazines is related to the CCM system, but not due to the ROS scavenging system. Furthermore, respiration-related characteristic tests and peptide analysis suggested that disruption of the TAT complex causes a defect in the cytochrome *bcl* complex, which may be involved in the tolerance to phenazines. In summary, this study sheds new light on the critical role of the TAT pathway in influencing the fitness and phenazines tolerance of *Xanthomonas* species.

Keywords: genetics and resistance, phenazines, respiration, tolerance, twin-arginine translocation pathway, *Xanthomonas campestris* pv. *campestris*

Xanthomonas species cause most bacterial plant diseases, resulting in severe yield and quality loss in many crops worldwide (Hayward 1993). *X. campestris* pv. *campestris*, the causal agent of black rot, can infect many important cruciferous crops, including broccoli, cabbage, radish, cauliflower, and oilseed rape (Qian et al. 2005; Roberts et al. 2007). Citrus bacterial canker, which is caused by *X. citri* pv. *citri*, is a serious disease that significantly impacts citrus production (Jalan et al. 2011). Rice bacterial leaf blight (BLB), caused by *X. oryzae* pv. *oryzae*, and rice bacterial leaf streak (BLS), caused by *X. oryzae* pv. *oryzicola*, have recently been spreading across Asia (Lu et al. 2014; Zhang and Wang 2013). Strategies to control bacterial diseases have had limited success and mainly depend on cultivating resistant varieties, planting bacteria-free seeds and avoiding contamination (Cerutti et al. 2017; Taylor et al. 2002; Vicente and Holub 2013). Bactericides, such as streptomycin, have shown significant effectiveness against bacterial pathogens (Förster et al. 2015). However, the use of antibiotics on agricultural products is being restricted due to the concerns about the development of antibiotic resistance (Sundin and Wang 2018). Therefore, it is necessary to develop novel bactericides for controlling *Xanthomonas* species.

Phenazines are a class of antibiotics extracted from microorganisms (Baron and Rowe 1981; Morales et al. 2010), and are well-known for their potent antimicrobial activities against many organisms, including parasites, nematodes, fungi and bacteria (Laursen and Nielsen 2004; Morales et al. 2010; Xu et al. 2015; Zhou et al. 2016). In previous studies, phenazines were reported to impact a variety of processes in organisms, such as the intracellular reactive oxygen species (ROS) balance, DNA intercalation, respiration and biofilm formation (Gwee Kyo et al. 2011). In addition to their ability to inhibit the growth of specific microorganisms, phenazines have also been shown to accumulate around the rhizosphere, and have a relatively short half-life (3 to 4 days) in soil (Mavrodi et al. 2013). Historically, the low yield and difficult extraction of phenazines limited their use. However, with the development of genetic engineering and fermentation technology in recent years, the use of phenazines has become possible (Du et al. 2015). So far, phenazine-1-carboxylic acid (PCA), the biosynthetic precursor of the other phenazines, has been used for controlling rice sheath blight caused by *Rhizoctonia solani*, cucumber downy mildew caused by *Pseudoperonospora cubensis*, Fusarium head blight caused by *Fusarium graminearum*, and many other plant diseases in China (<http://www.icama.org.cn>).

In our previous study, PCA exhibited a strong inhibitory effect against *X. oryzae* pv. *oryzae* and a weak inhibitory effect against *X. oryzae* pv. *oryzicola*, *X. campestris* pv. *campestris*, and *X. citri* pv. *citri* (Xu et al. 2015). In addition, Pan et al. (2017, 2018) reported that defects in the transcriptional regulation factor of the ROS scavenging system, *oxyR*, and the catalase, *CatB*, decreased the PCA tolerance in both *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*. However, complementation of the *oxyR* deletion mutant of *X. oryzae* pv. *oryzae* with the *oxyR* fragment of *X. oryzae* pv. *oryzicola* increased the ROS scavenging ability of *X. oryzae* pv. *oryzae* but did not affect the PCA tolerance, indicating that some

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Funding: This research was supported by the National Natural Science Foundation of China (31272065), the Natural Science Foundation of Anhui Province (2008085QC125), and the Research Projects of Anhui Academy of Agricultural Sciences (2020YL075).

The author(s) declare no conflict of interest.

other mechanisms are involved in PCA tolerance (Pan et al. 2017, 2018). A study utilizing a transposon insertion mutant library of *X. campestris* pv. *campestris* demonstrated that cytochrome *c* proteins play a more important role in regulating tolerance to phenazines than the ROS scavenging system, and that the tolerance mechanism of cytochrome *c* proteins differs from that of the ROS scavenging system (Pan et al. 2018; Wu et al. 2019).

In this study, one PCA highly sensitive insertion mutant, X-5, was screened from the *X. campestris* pv. *campestris* insertion mutant library. By employing the plasmid rescue method, a gene of the twin-arginine translocation (TAT) pathway was identified as being disrupted by a transposon in X-5. The TAT pathway, also called the sec-independent preprotein translocation system, is an essential protein transport system that works in parallel with the sec dependent translocation system (Costa et al. 2015; Fröbel et al. 2012; Goosens and van Dijl 2017). The TAT complex generally consists of a hexahelical TatC-type protein and one or two TatA-like proteins (Fröbel et al. 2012). Multiple TatA-like proteins, which are termed TatA, TatB, and TatE, have been found in different species (Berks 2015). However, in some species, TatB and TatE seem to be unnecessary (Lee et al. 2006). In *X. campestris* pv. *campestris*, the TAT complex consists of three subunits, named TatA, TatB, and TatC (Qian et al. 2005). The TAT pathway is mainly responsible for exporting folded proteins across the cytoplasmic membrane or helping proteins achieve correct conformation, including proteins that contain molybdopterin cofactors, nucleotide-based cofactors, iron-sulfur clusters, and some other structures. Proteins folded and transported by this pathway are involved in many important cellular activities (Berks 2015; Goosens and van Dijl 2017; Lee et al. 2006; Palmer and Berks 2012). However, there has not been any report focused on how the TAT pathway impacts tolerance to phenazines.

MATERIALS AND METHODS

Bacterial strains, media, and bactericides. The strains and plasmids used in this study are listed in Table 1. *X. campestris* pv. *campestris* strains were grown at 28°C in nutrient broth (NB) media or on nutrient agar (NA) media. The *Escherichia coli* strain was grown at 37°C in Luria-Bertani (LB) broth media or on LB agar media. NB media contained 3 g of beef extract, 5 g of polypeptone, 1 g of yeast extract, and 10 g of sucrose per liter of distilled water. NA media contained the same components as NB media, with the addition of 16 g of agar per liter of distilled water. LB agar media contained 5 g of yeast extract, 10 g of NaCl, 10 g of tryptone, and 16 g of agar per liter of distilled water. LB broth media contained the

same components as LA media but lacked agar. The NAN, NBN, and NAS media were used in the transformation assays (Yu et al. 2015). NBN media contained the same components as NB media but lacked sucrose. NAN media contained the same components as NBN media, with the addition of 16 g of agar per liter of distilled water. NAS media contained the same components as NB media, with an additional 100 g of sucrose per liter of distilled water. XCM media was used for oxidant accumulation assays and contained 10.5 g of K₂HPO₄, 4.5 g of KH₂PO₄, 1 g of (NH₄)₂SO₄, 0.12 g of MgSO₄, 2.36 g of succinic acid, and 1.5 g of casein acid hydrolysate per liter of distilled water (Xu et al. 2015). The bactericide PCA (98%) was purchased from Shanghai Nongle Biological Products Co. Ltd. (China). Kanamycin was purchased from Sigma-Aldrich Co. Ltd. (China). Captan was purchased from Guanlong Agrochemical Co. Ltd. (China). Phenazine and 1-OH-phenazine were purchased from Tokyo Chemical Industry Co. Ltd. (Japan). All bactericides were dissolved in acetone, with the exception of kanamycin, which was dissolved in distilled water.

PCA sensitivity. PCA sensitivity, as indicated by EC₅₀ value, was determined in NB media with a turbidity indicator according to a previous study (Xu et al. 2015). The cell cultures were grown to an optical density (OD)₆₀₀ of approximately 1.0 (about 10⁹ CFU/ml) in a shaker (175 rpm) and diluted to 10⁷ CFU/ml. A 100 µl volume of diluted cultures was added to the conical flasks containing 25 ml of NB media amended with different concentrations of PCA. After the cultures were shaken (175 rpm) for 24 h at 28°C, the turbidity was measured. The data were analyzed with DPS software (Duan et al. 2013). Each combination of strain and PCA concentration was represented by three replicates, and the experiment was performed four times.

Plasmid rescue. The insertion site of the PCA-sensitive mutant X-5 was identified via the plasmid rescue method (Fig. 1B), which utilized the self-contained *ori* promoter and a kanamycin resistance-selective gene in the transposon (Grant et al. 1990). Genomic DNA was extracted with a DNA Prep Pure Cell/Bacteria Kit (Tiangen Co. Ltd., Beijing, China) (Yu et al. 2015). The genomic DNA of X-5 was digested with the restriction endonuclease *Eco*RI, and the enzyme-digested products were then treated with DNA ligase. The ligation products were transformed into *E. coli* S17-1λpir, and the transformants were then screened on plates containing kanamycin at 20 mg/liter. The plasmids present in the positive colonies were sequenced, and BLAST was employed to search the sequencing results against the National Center for Biotechnology Information database (NCBI) (<https://www.ncbi.nlm.nih.gov>). The sequencing primers, RP-F and RP-R, are listed in Table 2.

TABLE 1. Strains and plasmids used in this study

Strains and plasmids	Characteristics	Source
Strains		
<i>Escherichia coli</i> DH5α	φ80lacZpM15, p(lacZYA-argF)U169, <i>recA1</i>	Invitrogen, Shanghai, China
<i>Escherichia coli</i> S17-1λpir	<i>Tp^R</i> , <i>Sm^R</i> , <i>recA</i> , <i>Thi</i> , <i>pro</i> , <i>hsdR⁻</i> , <i>M⁺</i> , RP4: 2- <i>Tc::Mu</i> : <i>Km Tn7</i> , λpir	Shanghai Jiao Tong University
8004	<i>Rif^R</i> , wild-type strain of <i>X. campestris</i> pv. <i>campestris</i>	Guangxi University
X-5	<i>Km^R</i> , a PCA-sensitive Tn5 insertion mutant of 8004	This study
8004Δ4182	<i>Rif^R</i> , deletion of <i>tatC</i> in wild-type strain 8004	This study
8004Δ4183	<i>Rif^R</i> , deletion of <i>tatB</i> in wild-type strain 8004	This study
8004Δ4184	<i>Rif^R</i> , deletion of <i>tatA</i> in wild-type strain 8004	This study
8004Δ4182R	<i>Rif^R</i> , <i>tatC</i> complemented mutant of 8004Δ4182	This study
8004Δ4183R	<i>Rif^R</i> , <i>tatB</i> complemented mutant of 8004Δ4183	This study
8004Δ4184R	<i>Rif^R</i> , <i>tatA</i> complemented mutant of 8004Δ4184	This study
Plasmids		
pK18 <i>mobsacB</i> II	<i>Km^R</i> , allelic exchange suicide vector, <i>sacB</i> , <i>oriT</i> (RP4)	This study
pK18 <i>tatA</i>	<i>Km^R</i> , a fusion fragment of upstream and downstream of <i>tatA</i> gene ligated in pK18 <i>mobsacB</i>	This study
pK18 <i>tatB</i>	<i>Km^R</i> , a fusion fragment of upstream and downstream of <i>tatB</i> gene ligated in pK18 <i>mobsacB</i>	This study
pK18 <i>tatC</i>	<i>Km^R</i> , a fusion fragment of upstream and downstream of <i>tatC</i> gene ligated in pK18 <i>mobsacB</i>	This study
pK18 <i>RtatABC</i>	<i>Km^R</i> , whole TAT pathway gene cluster ligated in pK18 <i>mobsacB</i>	This study

Promoter prediction. Promoter analysis was conducted on the Softberry website (<http://www.softberry.com>) (Solov'yev et al. 2010). Sequence fragments from 300-bp nucleotides upstream of *tatA* to the end of *tatC* were entered into the program. The results contained the -10 box and -30 box RNA polymerase recognition sites of the promoter.

Construction of the deletion mutants and quantification of the transcripts of the TAT pathway genes. Deletion vectors were constructed and used to transform *X. campestris* pv. *campestris* cells by the homologous recombination method, as previously described, with minor modifications (Yu et al. 2015). The homologous recombination deletion vectors are shown in Figure 2B. The primers used for amplifying fragments and the fragment information are both listed in Table 2. The positive colonies on NAS plates were subsequently verified by PCR and qRT-PCR. The PCR products were amplified with primer TatF and TatR and then analyzed on 1% agarose gels. The complemented mutants were constructed with the same method as the genomic sequence of the wild-type (WT) strain. The complemented mutants were verified by the PCR and qRT-PCR (Fig. 2C and D).

For gene expression analysis, the deletion mutants and WT were cultured to an OD₆₀₀ of around 1.0 (about 10⁹ CFU/ml) in a shaker (175 rpm), and total RNA was extracted with the RNA Prep Pure Cell/Bacteria Kit (Tiangen Co. Ltd., Beijing, China) (Pan et al. 2018). The cDNA was generated with a HiScript II Reverse transcription kit (Vazyme Biotech Co. Ltd.). The cDNA was then diluted to 50 ng/μl and used as the template for qRT-PCR. The qRT-PCR reaction was conducted using a ChamQ SYBR qPCR Master Mix kit (Vazyme Biotech Co. Ltd.) with the CFX Connect Real-Time System (Bio-Rad). The primers used for qRT-PCR are listed in Table 2. Each mutant or WT was represented by three replicates, and the experiment was performed in triplicate.

Measurement of sensitivities to specific bactericides. The sensitivities of the WT or mutants to kanamycin, captan, phenazine, and 1-OH-phenazine, as indicated by survival rate, were determined via a turbidity indicator (Xu et al. 2015). The cultures were grown to an OD₆₀₀ of approximately 1.0 (about 10⁹ CFU/ml) in a shaker (175 rpm) and were then diluted to 1 × 10⁷ CFU/ml. A 100-μl volume of each diluted culture was added to flasks containing 25 ml of NB media amended with different concentrations of each bactericide. After the cultures were shaken (175 rpm) for 24 h at 28°C, the turbidity was measured. Each combination of mutants and bactericides was represented by three replicates, and the experiment was performed in triplicate.

Colony morphology and growth assay. For assessment of colony morphology, the cultures were grown to an OD₆₀₀ of approximately 1.0 (about 10⁹ CFU/ml) in a shaker (175 rpm) and then diluted to 1 × 10⁷ CFU/ml. A 2-μl volume of diluted culture was spotted onto NA plates and the colonies were photographed after their diameters increased to 1 cm.

Growth rate was determined in NB media (Pan et al. 2017). After the cultures were grown to an OD₆₀₀ of approximately 1.0 (about 10⁹ CFU/ml) in a Shaker (175 rpm), 100 μl of each culture was added to flasks containing 25 ml of NB media. The flasks were shaken (175 rpm) at 28°C and the OD₆₀₀ values of the cultures were measured at 3 h intervals. Each mutant or WT was represented by three replicate flasks, and the experiment was performed in triplicate.

Virulence test. The virulence of the mutants or WT was tested on Chinese radish (*Raphanus sativus* L. var. *radiculus* Pers.), which was grown in a greenhouse at 28°C with a 12-h day/night cycle. Seedlings with four expanded leaves were tested with different cultures. After the cultures were grown to an OD₆₀₀ of approximately 1.0 (about 10⁹ CFU/ml), two leaves per seedling

Fig. 1. Identification of the insertion site of the transposon in the phenazine-1-carboxylic acid (PCA)-sensitive mutant X-5. **A**, The mutant X-5 was screened on nutrient agar plates containing PCA at 0 or 32 mg/liter. The sensitivity to PCA was much greater for X-5 than for 8004. **B**, The plasmid rescue method utilizes the self-contained replication origin and kanamycin-resistance gene of the transposon. Genomic DNA was used to generate cyclic DNA via digestion and sequential ligation. The plasmids containing the completed transposon could replicate in the *Escherichia coli*, which expresses the Pir protein, and the positive clones then grew on the plates containing kanamycin. The plasmids were subsequently sequenced and analyzed. **C**, The insertion site of the transposon of X-5 is located in the *tatB* (XC_4183) gene. The graph shows the insertion site and transcript orientation of the transposon.

were inoculated with the leaf-clipping method (Dow et al. 2003). The lesion length was measured after 10 days. Thirty seedlings were inoculated for each mutants, and the experiment was performed in triplicate.

Oxidant accumulation assay. The accumulation of oxidants was assessed with an intracellular oxidants assay kit (Beyotime Co. Ltd., China), which monitored intracellular oxidants with a DCFH-DA probe (Pan et al. 2017). The DCFH-DA probe reacts with intracellular oxidants, like ROS and Fe³⁺, causing fluorescence (Wang and Newman 2008). A 1-ml volume of culture (OD₆₀₀ ≈ 1.0) was centrifuged at 3,500 × g for 5 min, and the pellets were incubated in XCM media containing a DCFH-DA probe for 20 min. The cells were then washed three times with fresh XCM media and added to the wells of a microplate. Each mutant or WT was then treated with PCA at 32 mg/liter in 0.4% (vol/vol) acetone or with 0.4% (vol/vol) acetone alone. The microplate was kept at 28°C, and the fluorescence was measured with the Spectra Max M5 fluorescence microplate reader (Molecular Devices Co.) for 4 h with an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Each treatment was represented by four replicates, and the experiment was performed in triplicate.

H₂O₂ tolerance and oxygen consumption rate. For assessment of the H₂O₂ tolerance, 2 µl of culture (OD₆₀₀ ≈ 1.0) was spotted onto the NA plates containing 0, 0.1, 0.2, 0.25, or 0.3 mM of H₂O₂ (Pan et al. 2018). When the colony diameter on the non-H₂O₂ plate reached 1 cm, all colonies were photographed. Each combination of strains and H₂O₂ treatment was represented by nine replicates, and the experiment was performed in triplicate.

The oxygen consumption rate was determined with a Clark electrode, which can monitor the change of oxygen concentration in an intact cell (Barrientos et al. 2009). A 1-ml volume of culture (OD₆₀₀ ≈ 1.0) was centrifuged at 3,500 × g for 5 min. The pellets were weighed and then resuspended in 1 ml of phosphate-buffered saline buffer (50 mM of phosphate, pH 7.0). The suspensions were added to the cells, which were then monitored for 3 min. The oxygen consumption rate was expressed as mM of oxygen consumed per gram of pellet per minute. Each mutant or WT was represented by four replicates, and the experiment was performed three times.

RESULTS

Insertion site and PCA sensitivity of X-5. The mutant X-5 was much more sensitive to PCA than 8004 (Fig. 1A). As determined in NB media, the EC₅₀ values of X-5 and WT under PCA treatment were 0.09 ± 0.08 mg/liter and >32 mg/liter, respectively. An accurate EC₅₀ value could not be calculated when the value was more than 32 mg/liter because of the limited solubility of PCA in broth media. By sequencing the rescued plasmid and employing BLAST against the NCBI database (Fig. 1B), we matched the insertion site with the gene *tatB* (XC_4183), which encodes a subunit of the TAT complex. Information about the transposon in the genomic DNA is shown in Figure 1C.

Construction of the TAT pathway deletion mutants. The promoter analysis results suggested that the TAT pathway genes share one promoter and are transcribed in the following order: *tatA* (XC_4184), *tatB* (XC_4183), and *tatC* (XC_4182) (Fig. 2A). XC_4184, XC_4183, and XC_4182 are the respective gene numbers of *tatA*, *tatB*, and *tatC* in a genomic database (Kyoto Encyclopedia of Genes and Genomes, KEGG). To investigate the role of different subunits of the TAT complex in tolerating PCA, we separately deleted the three genes in three mutants. The deletion mutants of *tatA*, *tatB*, and *tatC* were named 8004Δ4184, 8004Δ4183, and 8004Δ4182, respectively. The EC₅₀ values of 8004Δ4184, 8004Δ4183, and 8004Δ4182 to PCA were then determined to be 0.112 ± 0.032, 0.098 ± 0.026, and 0.13 ± 0.01 mg/liter, respectively. These values were substantially less than that of the WT (>32 mg/liter). 8004Δ4182R, 8004Δ4183R, and 8004Δ4184R were the complementation mutants of 8004Δ4182, 8004Δ4183m, and 8004Δ4184, respectively. The PCA sensitivities of the complemented mutants (EC₅₀ values to PCA > 32 mg/liter) were similar to that of the WT. The deletion mutants were verified by PCR and qRT-PCR. The PCR products of the WT, 8004Δ4182, 8004Δ4183, and 8004Δ4184 contained 3,331, 2,445, 2,887, and 3,140 bp on the gels, respectively (Fig. 2C). The expression levels of *tatA* and *tatB* in 8004Δ4182 (the deletion mutant of *tatC*) were 1.53-fold and 1.87-fold greater, respectively, than in the WT. The expression levels of *tatA* and *tatC* in 8004Δ4183 (the deletion mutant of *tatB*) were 2.43-fold and 2.20-fold greater than in the WT. The expression levels of *tatB* and *tatC* in 8004Δ4184 (the deletion mutant of *tatA*) were

TABLE 2. Primers used in this study

Primers	Sequence	Purpose
RP-F	ACCTACAACAAAGCTCTCATCAACC	Sequence the rescued plasmid
RP-R	CTACCCTGTGGAACACCTACATCT	
Up <i>tatA</i> F	CGGGATCCCAAGGCCGAAATCTGGTCCTGG	Amplify a 505-bp upstream fragment of <i>tatA</i> gene in <i>X. campestris</i> pv. <i>campestris</i> 8004
Up <i>tatA</i> R	GGGGTACCCAGTGCCAAATGCTGAAACTGCCAT	
Down <i>tatA</i> F	GGGGTACCCGCGACCGCGACGCGCGCTG	Amplify a 928-bp downstream fragment of <i>tatA</i> gene in <i>X. campestris</i> pv. <i>campestris</i> 8004
Down <i>tatA</i> R	GCGTCGACCGCGCTGGTACAGGCCCGGCGC	
Up <i>tatB</i> F	GCTCTAGAGCTGTCATCCCCCTG	Amplify a 301-bp upstream fragment of <i>tatC</i> gene in <i>X. campestris</i> pv. <i>campestris</i> 8004
Up <i>tatB</i> R	GGGGTACCCGACGCTGTTCGG	
Down <i>tatB</i> F	GGGGTACCCCCAGGCACAACC	Amplify a 614-bp downstream fragment of <i>tatC</i> gene in <i>X. campestris</i> pv. <i>campestris</i> 8004
Down <i>tatB</i> R	CGGGATCCGCCAGCACGAAGT	
Up <i>tatC</i> F	CGGGATCCGTGTTTCGATATTGGGTTT	Amplify a 590-bp upstream fragment of <i>tatB</i> gene in <i>X. campestris</i> pv. <i>campestris</i> 8004
Up <i>tatC</i> R	GGGGTACCCACGGCGTCTTCTCTCTGG	
Down <i>tatC</i> F	GGGGTACCGATCTGGTTCTAGGGC	Amplify a 642-bp downstream fragment of <i>tatB</i> gene in <i>X. campestris</i> pv. <i>campestris</i> 8004
Down <i>tatC</i> R	GCTCTAGACTGCTGATGGTGGCGA	
Tat F	CGGGATCCGCGGGTGTCTCAACGAGAT	Amplify a 3,331-bp fragment of <i>tat</i> cluster in <i>X. campestris</i> pv. <i>campestris</i> 8004 for constructing complementary mutants
Tat R	GCGTCGACGATGATCGGCCGAGCGGG	
Probe <i>tat</i> F	GATCTGGTTCTAGGGC	Amplify a 629-bp probe fragment for Southern blot of deletion mutants
Probe <i>tat</i> R	CTGCTGATGGTGGCGA	
Rt- <i>tatC</i> F	TTTCGTGGCGGTGTCTT	Test the gene expression level of <i>tatC</i>
Rt- <i>tatC</i> R	GGGAAGGCCAGCTTCTTT	
Rt- <i>tatB</i> F	GATTCGGTCAAGCAGGAACTC	Test the gene expression level of <i>tatB</i>
Rt- <i>tatB</i> R	CGACCTGTTGCTGCGTATT	
Rt- <i>tatA</i> F	TCGTGCTGCTGGTGT	Test the gene expression level of <i>tatA</i>
Rt- <i>tatA</i> R	CATGCCCTTCTGAATTCCTTG	
Rt-16S F	GCCTAACACATGCAAGTCGAACGGC	Test the gene expression level of 16S sequence
Rt-16S R	AATATCCCCACTGCTGCCTCCCC	

2.04-fold and 1.78-fold greater than in the WT (Fig. 2D). These results suggested that the other two genes are expressed more highly in each deletion mutant, indicating that the three TAT pathway genes are all vital for the TAT complex and PCA tolerance in *X. campestris* pv. *campestris*.

Sensitivities of the TAT pathway deletion mutants to some specific bactericides. Sensitivities of the mutants to the following bactericides were assessed: kanamycin, captan, phenazine, and 1-OH-phenazine. It has previously been shown that antibacterial mechanisms of both kanamycin and captan differ from

Fig. 2. Promoter prediction of the twin-arginine translocation (TAT) gene cluster and construction of the TAT mutants. **A**, Promoter analysis showed that the three TAT pathway genes share the same promoter. The graph shows the -10 box and -35 box RNA polymerase recognition sites of the predicted promoter. **B**, Schematic representation of the deletion strategy via homologous recombination. pK18*tatA*, pK18*tatB*, and pK18*tatC* were the deletion vectors. The gray parts in the vector represent the homologous arms of the deleted genes, and the gray parts on the genome represent the deleted fragments in the deletion mutants. The fragment information is listed in Table 2. **C**, PCR detection of the deletion mutant of *tatA* (XC_4184), *tatB* (XC_4183), and *tatC* (XC_4182). Strain 8004 is the wild-type strain. 8004Δ4182, 8004Δ4183, and 8004Δ4184 are the deletion mutants of *tatC*, *tatB*, and *tatA*, respectively. 8004Δ4182R, 8004Δ4183R, and 8004Δ4184R are the complementation mutants of 8004Δ4182, 8004Δ4183, and 8004Δ4184, respectively. The wild type (WT) and complementation mutants were both 3,331 bp in size. 8004Δ4182, 8004Δ4183, and 8004Δ4184 were 2,445, 2,887, and 3,140 bp, respectively. **D**, Expression levels of the TAT pathway genes in 8004Δ4182, 8004Δ4183, and 8004Δ4184. The deleted gene was not expressed in each corresponding mutant, and the other two genes were expressed at higher levels than in the WT. The columns indicate the expression level, in which the expression level of genes in WT was defined to be 1 unit, and error bars depict standard errors of the column. Each mutant or WT was represented by three replicates, and the experiment was performed in triplicate. The expression levels were compared using Fisher's least significant difference test. *, $P < 0.05$.

PCA (Alangaden et al. 1998; Barak and Edgington 1984; Jugheli et al. 2009). Phenazine and 1-OH-phenazine are both homologs of PCA (Mavrodi et al. 2010). The sensitivities of the mutants to kanamycin and captan were similar to that of WT (Fig. 3A and B). However, their sensitivities to phenazine and 1-OH-phenazine were significantly increased in comparison with 8004 (Fig. 3C and D).

Biological characteristics. The colony morphologies of the deletion mutants were similar to that of 8004 on the NA plates. The colony shapes were regular, and the surfaces were smooth (Fig. 4A). However, the deletion mutants grew more slowly than 8004 in the NB media (Fig. 4B). The virulence of 8004Δ4182, 8004Δ4183, and 8004Δ4184 was also substantially decreased compared with the WT (Fig. 4C). The lesion lengths of 8004Δ4182, 8004Δ4183, and 8004Δ4184 were 1.220 ± 0.084 , 1.060 ± 0.114 , and 1.160 ± 0.089 cm, respectively. The lesion lengths of 8004, 8004Δ4182R, 8004Δ4183R, and 8004Δ4184R were 1.920 ± 0.130 , 2.020 ± 0.277 , 1.880 ± 0.130 , and 2.080 ± 0.342 cm, respectively. The decreased growth rate and virulence suggested that the TAT pathway deletion mutants exhibit a fitness defect.

Intracellular oxidant accumulation of the TAT mutants and WT with PCA treatment. In a previous study, the ROS scavenging system played an important role in the PCA tolerance of *Xanthomonas* species (Pan et al. 2017, 2018). The accumulation of

intracellular oxidants in the deletion mutants and 8004 was measured after treatment with control or PCA at 32 mg/liter for 4 h. Considering that the growth rates differed between the deletion mutants and 8004, their OD₆₀₀ values were monitored to evaluate cell density. After the cells were kept in the reader for 4 h, the OD₆₀₀ values were similar for the deletion mutants and WT, possibly because the microplate is static in the reader, such that multiplication of the cells was restricted. The fluorescence results showed that the intracellular oxidants, indicated as relative fluorescence unit, of the deletion mutants and 8004 all increased with treatment of control, acetone or PCA. Additionally, the deletion mutants and the WT 8004 exhibited a similar accumulation of the intracellular oxidants in response to equal PCA treatment, suggesting that the oxidant scavenging system operated well in the TAT mutants (Fig. 5).

Respiration-related characteristic tests of the TAT mutants and 8004. The characteristics of the TAT mutants observed in the current study were similar to those of the CCM mutants of *X. campestris* pv. *campestris*, including decreased growth rate and reduced tolerance to phenazines (Wu et al. 2019). These findings motivated us to consider whether the TAT pathway and the CCM system influence the tolerance of *X. campestris* pv. *campestris* to phenazines through some related metabolic

Fig. 3. Sensitivities of the twin-arginine translocation (TAT) mutants to kanamycin, captan, phenazine, and 1-OH-phenazine. Sensitivities to **A**, kanamycin and **B**, captan were similar between the deletion mutants and wild type (WT) (8004), but sensitivities to **C**, phenazine and **D**, 1-OH-phenazine significantly increased for the deletion mutants in comparison with WT, which was similar to their phenazine-1-carboxylic acid (PCA) sensitivities. Strain 8004 is the wild-type strain. 8004Δ4182, 8004Δ4183, and 8004Δ4184 are the deletion mutants of *tatC*, *tatB*, and *tatA*, respectively. 8004Δ4182R, 8004Δ4183R, and 8004Δ4184R are the complementation mutants of 8004Δ4182, 8004Δ4183, and 8004Δ4184, respectively. Each combination of mutants and bactericides was represented by three replicates, and the experiment was performed in triplicate. The error bars depict standard errors of the replicates.

processes. The cytochrome bc1 complex is one obvious possibility because the TAT pathway and CCM complex are involved in its formation. Signal peptide analysis revealed that the iron-sulfur subunit of the cytochrome bc1 complex of *X. campestris* pv. *campestris* can be recognized by the TAT complex (Fig. 6A) (Fröbel et al. 2012; Qian et al. 2005).

The cytochrome bc1 complex, a key component of the respiratory chain, is responsible for transferring electrons from ubiquinone to some electron receptors. Cytochrome c552 degrades H_2O_2 and cytochrome c oxidases consume oxygen, both of which require the electrons supplied by the cytochrome bc1 complex (Husband et al. 2016; Millett and Durham 2004; Ow et al. 2008). The H_2O_2 tolerances and oxygen consumption rates of the deletion mutants were therefore tested to assess whether the cytochrome bc1 complex was functioning in TAT mutants. All three TAT pathway deletion mutants were more sensitive to H_2O_2 than the WT 8004 (Fig. 6B). Isolate 8004 and the complementation mutants could tolerate 0.2 mM of H_2O_2 on the NA plates, while the deletion mutants could not. The respiratory rates, as indicated by the oxygen consumption rate, were lower for the deletion mutants than for the complemented mutants and 8004 (Fig. 6C). The oxygen consumption rates of 8004, 8004 Δ 4182, 8004 Δ 4183, and 8004 Δ 4184 were 2.692 ± 0.125 nmol/(min mg), 1.246 ± 0.181 nmol/(min mg), 1.485 ± 0.275 nmol/(min mg), and 1.467 ± 0.252 nmol/(min mg), respectively. The oxygen consumption rates of 8004 Δ 4182R, 8004 Δ 4183R, and 8004 Δ 4184R were 2.543 ± 0.283 nmol/(min mg), 2.797 ± 0.096 nmol/(min mg), and 2.677 ± 0.327 nmol/(min mg), respectively. The results of H_2O_2 tolerance and oxygen consumption rate assays suggested that the cytochrome bc1 complex is defective in the TAT pathway deletion mutants.

DISCUSSION

To control bacterial plant diseases, farmers use crop rotation, plant bacteria-free seed, and employ resistant varieties, but these methods have had only limited effect (Roberts et al. 2007; Vicente and Holub 2013; Zhang and Wang 2013). Some bactericides have shown significant effectiveness against bacterial pathogens; however, the use of these bactericides are being restricted because of the antibiotic resistance (Förster et al. 2015; Nuñez et al. 2018; Sundin and Wang 2018). Phenazines, which are extracted from microorganisms, have been studied for a long time for their inhibitory effect on various organisms (Baron and Rowe 1981; Morales et al. 2010; Zhou et al. 2016). In previous studies, Pan et al. (2017, 2018) reported that ROS scavenging ability impacts PCA tolerance in both *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, and that there are other metabolic processes involved in tolerating PCA more directly in *Xanthomonas* species. In addition, a Tn5 transposon library of *X. campestris* pv. *campestris* was constructed to explore which genes are involved in the PCA tolerance of *Xanthomonas* species (Wu et al. 2019). That study revealed that the CCM system has a more dramatic impact on PCA tolerance than the ROS scavenging system, and the regulation mechanism involved with the CCM system is associated with the function of cytochrome c proteins, which require the CCM system and Dsb (transmembrane thioredoxin protein) pathway for proper maturation (Pan et al. 2017; Wu et al. 2019). However, the insertion site of a highly PCA-sensitive mutant, X-5 (Fig. 1A), could not be identified with the method of TAIL-PCR, which was used to detect the CCM system insertion mutants. We utilized the plasmid rescue strategy to determine that the insertion site of X-5 is located in the *tatB* (*XC_4183*) gene, which encodes a subunit of the TAT complex (Fig. 1C).

Fig. 4. Colony morphology, growth rate, and virulence of the twin-arginine translocation (TAT) mutants. Strain 8004 is the wild-type strain. 8004 Δ 4182, 8004 Δ 4183, and 8004 Δ 4184 are the deletion mutants of *tatC*, *tatB*, and *tatA*, respectively. 8004 Δ 4182R, 8004 Δ 4183R, and 8004 Δ 4184R are their respective complementation mutants. **A**, Colony morphology on nutrient agar media. The colonies were photographed after their diameters reached 1 cm. **B**, Growth rate in the nutrient broth media. The growth rates of the deletion mutants were significantly decreased compared with wild type (WT). Each mutant or WT was represented by three replicate flasks, and the experiment was performed in triplicate. Error bars indicate the standard deviation of three experiments. **C**, Virulence was tested on Chinese radish with the leaf-clipping method. The virulence of the deletion mutants was substantially decreased compared with that of the WT. Each mutant or WT was inoculated on 60 leaves, and the experiment was performed in triplicate.

The TAT pathway consists of a hexahelical TatC-type protein and one or two TatA-like proteins (Fröbel et al. 2012). The TAT complex of *Xanthomonas* species is encoded with conserved nucleotide sequence (Fig. 7) and contains two TatA-like proteins (TatA and TatB) and one TatC protein (Fig. 1C). *XC_4184*, *XC_4183*, and *XC_4182* are the respective gene names of *tatA*, *tatB*, and *tatC* in the KEGG genomic database. Promoter analysis showed that *tatA*, *tatB*, and *tatC* of *X. campestris* pv. *campestris* share the same promoter and that the three genes are transcribed in the order of *tatA*, *tatB*, and *tatC* (Fig. 2A), indicating that the inserted transposon might block the expression of *tatC* in X-5. Considering that TatB is not essential for some species, the decreased PCA tolerance of X-5 might be caused by the defect of TatC. To reveal the role of the three genes in the TAT complex, *tatA*, *tatB*, and *tatC* were separately knocked out in three mutants, which were named 8004Δ4184, 8004Δ4183, and 8004Δ4182 (Fig. 2C). The expression levels of the three genes in the deletion mutants indicated that the gene cluster of the TAT pathway is driven by one promoter in *X. campestris* pv. *campestris* and the downstream genes can still be express when an upstream gene is deleted (Fig. 2D). Recently, the different roles of TatA and TatB in *E. coli* have been reported (Hou et al. 2018), but the exact mechanism of this translocation complex still requires more investigation. However, the results of tolerance to phenazines and other characteristics indicated that the two TatA-like proteins and one TatC protein of *X. campestris* pv. *campestris* are all essential for the function of the TAT complex in *X. campestris* pv. *campestris*.

The EC₅₀ values of the TAT pathway deletion mutants to PCA decreased almost 250 folds compared with the WT 8004. In addition to PCA tolerance changes, defects of the TAT complex also impacted tolerance of *X. campestris* pv. *campestris* to other phenazines but not other types of bactericides. Kanamycin, which is a commonly used antibiotic in labs, interferes with protein

synthesis, while captan, which is typically used in the field, can interfere with the synthesis of glutathione (Alangaden et al. 1998; Barak and Edgington 1984; Jugheli et al. 2009). Like PCA, phenazine and 1-OH-phenazine are two members of phenazines (Mavrodi et al. 2010). The sensitivities of the three deletion mutants to kanamycin and captan were similar to that of 8004 (Fig. 3A and B). The mutants, however, were more sensitive to phenazine and 1-OH-phenazine than the WT 8004 (Fig. 3C and D), indicating that the functions of the TAT complex are specifically involved in the tolerance of *X. campestris* pv. *campestris* to phenazines. Our previous study also reported that the defective mutants of the CCM system of *X. campestris* pv. *campestris* were more sensitive to PCA and its phenazine homologs, but not to other types of bactericides (Wu et al. 2019). In addition to the decreased tolerance to phenazines, the deletion mutants of the TAT complex also exhibited reduced fitness-related characteristics. The growth rate and virulence of 8004Δ4184, 8004Δ4183, and 8004Δ4182 notably decreased compared with 8004 (Fig. 4B and C), suggesting a critical role of the TAT pathway in the growth and infection capability of *X. campestris* pv. *campestris*. The decreased growth and infection abilities of the TAT mutants might be caused by the essential role of the TAT pathway for many metabolic processes in organisms (Fröbel et al. 2012; Goosens and van Dijk 2017).

Phenazines are well-known for their various negative impacts to organisms, and their ability to cause intracellular ROS accumulation has been studied extensively (Baron and Rowe 1981). In our previous study, we found that PCA treatment can induce the accumulation of intracellular oxidants of *Xanthomonas* species, and the ROS scavenging system has a weak influence on PCA tolerance in *Xanthomonas* species (Pan et al. 2018; Xu et al. 2015). However, the tolerance mechanism of the CCM system to phenazines was found to be different from that of the ROS scavenging system. In this study, oxidants were also significantly accumulated in TAT mutants and WT after treatment with PCA (Fig. 5). Furthermore, the accumulated oxidants in response to equal PCA treatment were similar between the TAT mutants and WT, indicating that the oxidants induced by PCA could be metabolized in the TAT mutants and had no impact on the decreased tolerance of the TAT mutants to PCA. Pan et al. (2018) also observed that defects in the ROS scavenging system had limited impact on the PCA tolerance of *Xanthomonas* species. Mutants of the TAT pathway exhibit dramatic reductions in PCA tolerance compared with the reduction seen in ROS scavenging system mutants, which is similar to that of the CCM system. The EC₅₀ values of the *oxyR* or the *CatB* deletion mutants to PCA decreased within two to threefold in both *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, while the EC₅₀ values of the deletion mutants of the TAT pathway and CCM system to PCA decreased more than 100-fold. These studies indicated a more critical role of the TAT complex and CCM system in regulating the tolerance of *Xanthomonas* species to PCA (Pan et al. 2017, 2018; Wu et al. 2019).

The characteristics of the TAT mutants presented in the current study were similar to the CCM mutants. Like the TAT mutants, the CCM mutants exhibited reduced fitness, decreased tolerance to phenazines but unchanged tolerance to other bactericides, and similar accumulation of oxidants to the WT after treatment with equal PCA. Based on the similarities between the mutants of the TAT pathway and CCM system, we suspected that the two metabolic processes impact the tolerance of *X. campestris* pv. *campestris* to phenazines via related processes. Our previous study revealed that the CCM system can significantly impact the phenazine tolerance of *X. campestris* pv. *campestris* by impacting the maturation process of cytochrome *c* proteins (Wu et al. 2019). Given the functions of the two metabolic processes, we hypothesized that the cytochrome bc₁ complex was involved (Fig. 8). The cytochrome bc₁ complex is the electron transferring center of the respiratory chain, which is the main energy source of organisms (Ow et al. 2008), and consists of three proteins: cytochrome *b*

Fig. 5. Oxidant accumulation of the twin-arginine translocation (TAT) mutants after treatment with phenazine-1-carboxylic acid (PCA). The oxidant level is indicated as relative fluorescence unit (RFU); A.U. stands for arbitrary unit. Strain 8004 is the wild-type strain. 8004Δ4182, 8004Δ4183, and 8004Δ4184 are the deletion mutants of *tatC*, *tatB*, and *tatA*, respectively. R indicates the complementation mutant. The levels of intracellular oxidants of the TAT mutants and wild type (WT) were significantly increased when treated with PCA, while the oxidant accumulation between the TAT pathway deletion mutants and WT was similar with equal PCA treatment. The results of oxidant accumulation indicated that the oxidants induced by PCA can be metabolized in the TAT mutants and are not involved in the decreased tolerance of the TAT mutants to PCA. Each treatment was represented by four replicates, and the experiment was performed in triplicate. Error bars indicate the standard deviation of three replicates, and columns with same letter represent significant differences ($P < 0.05$) according to Fisher's least significant difference test.

subunit, iron-sulfur subunit, and cytochrome C1 subunit (Zara et al. 2009). Maturation of the cytochrome C1 protein requires the CCM system (Stevens et al. 2011; Verissimo and Daldal 2014), and it was reported that defects of the TAT pathway of *Streptomyces coelicolor* cause an incorrect assembly of the iron-sulfur subunit of the cytochrome bc1 complex (Hopkins et al. 2014). The results of peptide analysis suggested that the signal peptide of the iron-sulfur subunit of *X. campestris* pv. *campestris* is recognized by the TAT

complex (Fig. 6A) (Fröbel et al. 2012; Qian et al. 2005). The TAT system recognizes the substrates via a consensus amino acid sequence. In Proteobacteria, the recognizable sequence is generally defined as “S/T-R-R-x-F-L-K” (x is any polar amino acid) where the arginine residues are almost invariant and the other amino acids are found at a frequency exceeding 50% (Lee et al. 2006). For example, the signal sequences of three most common TAT substrates in *E. coli*, Sufl, TorA, and CueO, are S-R-R-Q-F-I-Q, S-R-R-R-F-L-A,

Fig. 6. The signal peptide of the cytochrome bc1 iron-sulfur subunit and the respiration-related characteristic assays of the twin-arginine translocation (TAT) mutants. **A**, The signal peptide of the cytochrome bc1 iron-sulfur subunit is G-R-R-R-F-L-T in *Xanthomonas campestris* pv. *campestris*, which is generally consistent with the TAT pathway recognition signal peptide. The arginine residues are almost invariant, and the other amino acids are found at a frequency exceeding 50%. The x in the graph denotes polar amino acids. **B**, H₂O₂ tolerances of the TAT mutants and wild type (WT) (8004). The H₂O₂ tolerances were decreased for the deletion mutants as compared with 8004. Strain 8004 could tolerate 0.2 mM of H₂O₂ on the plates, while the deletion mutants could not. Each combination of strains and H₂O₂ was represented by nine replicates, and the experiment was performed in triplicate. **C**, Oxygen consumption rates of the TAT pathway mutants and WT (8004). The oxygen consumption rates were lower for the deletion mutants than for the WT 8004. Each mutant or WT was represented by four replicates, and the experiment was performed in triplicate. Error bars indicate the standard deviation of three replicates, and the columns with same letter are not significantly different ($P < 0.05$) according to Fisher's least significant difference test.

Fig. 7. Homology of the twin-arginine translocation (TAT) pathway of *Xanthomonas* species. The sequences of the TAT gene cluster of different *Xanthomonas* species were acquired from the Kyoto Encyclopedia of Genes and Genomes database (KEGG) (<http://www.genome.jp/kegg>). The cladogram was built using MEGA 5.0 software with 10,000 bootstrap replications performed. The graph suggests the conserved relationship of the TAT pathway genes between *Xanthomonas* species.

and Q-R-R-D-F-L-K, respectively (Fröbel et al. 2012). The signal peptide of the cytochrome bc1 iron-sulfur subunit of *X. campestris* pv. *campestris* is G-R-R-R-F-L-T, which contains conserved arginine residues. Moreover, the results of the H₂O₂ sensitivities and oxygen consumption rates indicated that respiration is defective in the TAT mutants (Fig. 6B and C). Cytochrome c552 protein degrades H₂O₂ and the cytochrome c oxidases consume oxygen, both of which require electrons supplied by the cytochrome bc1 complex (Berry et al. 2000; Husband et al. 2016). These results suggested that the cytochrome bc1 may be involved in PCA tolerance, although further study is required to elucidate the mechanism of this interaction.

In this study, we utilized a PCA-sensitive insertion mutant and confirmed that the three subunits of the TAT complex, TatA, TatB, and TatC, are all essential for the functions of the TAT complex in *X. campestris* pv. *campestris*. The characteristics of the TAT mutants suggested that disruption of the TAT pathway can significantly decrease fitness and phenazines' tolerance in *X. campestris* pv. *campestris*. Moreover, the similar characteristics (the decreased fitness and tolerance to phenazines) between the TAT and CCM mutants, and the defective respiratory characteristics (decreased oxygen consumption rate and H₂O₂ tolerance) of the TAT mutants indicated that the cytochrome bc1 complex is associated with the tolerance mechanism of *X. campestris* pv. *campestris* to phenazines. Overall, this study sheds new light on the tolerance mechanism to phenazines in *Xanthomonas* species and suggests new areas of further investigating the mode of action of phenazines and their utility in controlling plant diseases caused by *Xanthomonas* species.

Fig. 8. Diagram of the relationship between twin-arginine translocation (TAT) pathway, cytochrome *c* maturation (CCM) system, and cytochrome bc1 complex in *Xanthomonas campestris* pv. *campestris*. The cytochrome bc1 complex consists of the cytochrome *b* subunit, the iron-sulfur subunit (ISP), and the cytochrome *C1* subunit. The cytochrome *C1* subunit and the iron-sulfur subunit require the CCM system and TAT pathway for proper maturation. The characteristics of the TAT pathway deletion mutants indicated that the cytochrome bc1 complex is associated with the tolerance mechanism to phenazines in *X. campestris* pv. *campestris*. **A**, The cytochrome bc1 complex can function well and increases the tolerance of *X. campestris* pv. *campestris* to phenazine-1-carboxylic acid (PCA) in wild type (WT). **B**, The cytochrome bc1 complex cannot be correctly assembled in the deletion mutants of the CCM system and TAT pathway, causing a significant decrease in PCA tolerance.

ACKNOWLEDGMENTS

We thank TopEdit LLC for the linguistic editing and proofreading during the preparation of this manuscript.

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