Plant Pathol. J. 32(3) : 266-272 (2016)
http://dx.doi.org/10.5423/PPJ.NT.12.2015.0273
pISSN 1598-2254 eISSN 2093-9280

The Plant Pathology Journal

Note Open Access

Deciphering the Role of Tyrosine Sulfation in *Xanthomonas oryzae* pv. *oryzae* Using Shotgun Proteomic Analysis

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(Received on December 23, 2015; Revised on February 23, 2016; Accepted on February 23, 2016)

A bacterial tyrosine sulfotransferase, RaxST, is required for activation of rice XA21-mediated immunity, and it catalyzes sulfation of tyrosine residues of Omp1X and RaxX in *Xanthomonas oryzae* pv. *oryzae*, a causal agent of bacterial blight in rice. Although RaxST is biochemically well-characterized, biological functions of tyrosine sulfation have not been fully elucidated. We compared protein expression patterns between the wildtype and a raxST knockout mutant using shotgun proteomic analysis. Forty nine proteins displayed a more than 1.5-fold difference in their expression between the wildtype and the mutant strains. Clusters of orthologous groups analysis revealed that proteins involved in cell motility were most abundant, and phenotypic observation also showed that the twitching motility of the mutant was dramatically changed. These results indicate that tyrosine sulfation by RaxST is essential for *Xoo* movement, and they provide new insights into the biological roles of RaxST in cellular processes.

Keywords **:** proteomics, tyrosine sulfation, *Xanthomonas oryzae* pv. *oryzae*

Xanthomonas oryzae pv. *oryzae* (*Xoo*), a Gram-negative bacterium, motile by means of a single polar flagellum, produces polysaccharide xanthan gum, with its characteristic yellow pigments, and also causes bacterial blight disease in rice plants (Mew, 1987). Blight disease is one of the most destructive diseases plaguing rice in Africa and Asia, and the losses caused by this disease account for those of up to half of rice production worldwide, resulting in tremendous economic and social impact (Gnanamanickam et al., 1999). In addition to its importance, the bacterium is a model organism for studying the function of genes and proteins because its full genome has been sequenced and it has been studied with diverse well-established analytical techniques (Han et al., 2008; Park et al., 2014b; Salzberg et al., 2008).

Tyrosine sulfation is one of the most common post-translational modifications in eukaryotic organisms, and up to 1% of tyrosine residues on total proteins can be sulfated in eukaryotic organisms (Monigatti et al., 2006). Tyrosine sulfation is mediated by tyrosine sulfotransferases, which transfer a sulfate group (SO_3^-) from the universal sulfate donor, 3′-phosphoadenosine-5′-phosphosulfate, to specific substrates, and it occurs as an enzymatic modification of peptides and proteins. In contrast to serine/threonine phosphorylation, which is often involved in signal transduction pathways, tyrosine sulfation modulates protein-protein/peptide or ligand-receptor interactions with high affinity (Stone et al., 2009). A notable example in plants is phytosulfokine-α (PSK), which possesses sulfate tyrosine residues and serves as a self-recognition factor for the proliferation of plant cells (Matsubayashi et al., 2002). PSK is recognized by the phytosulfokine receptor kinase present in the plant plasma membrane, and tyrosine sulfation of PSK is required for ligand-receptor recognition. In animals, sulfation of tyrosine residues on the CC-chemokine receptor 5 is critical for both the binding of glycoprotein 120 from human immunodeficiency virus (HIV) and for

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the entry of HIV into human cells (Farzan et al., 1999). However, tyrosine sulfotransferases in prokaryotic organisms have not been well described compared to their counterparts in eukaryotes.

RaxST protein from *Xoo* is required for the triggering of XA21-mediated immunity and it shows homology with sulfotransferases, including human tyrosylprotein sulfotransferase 1 (TPST1) and TPST2, as well as *Sinorhizobium meliloti* nodulation protein H (da Silva et al., 2004). In the central region of the protein, RaxST possesses the 5′-phosphosulfate-binding and 3′-phosphate-binding motifs that are hallmarks of sulfotransferases and critical for the binding of 3′-phosphoadenosine-5′-phosphosulfate. Recently, the tyrosine sulfotransferase activity of RaxST was demonstrated using a novel sulfotransferase activity assay and ultraviolet photodissociation mass spectrometry (Han et al., 2012). RaxST specifically catalyzes the sulfation of tyrosine residues on both Omp1X (outer membrane protein 1 in *Xoo*) and a synthetic N-terminal peptide of CC-chemokine receptor 5. Omp1X is involved in *Xoo* motility and biofilm formation (Park et al., 2014b). Comparative proteomic analysis with an Omp1X knockout strain (*XooΔomp1X*) showed that the expression of 106 proteins was significantly changed. Clusters of orthologous groups (COG) analysis revealed that proteins related to cell motility and signal transduction were most abundant. Phenotypic observation also displayed that pili- and flagella-dependent motility of *XooΔomp1X* was significantly impaired. In addition, RaxST transfers sulfuryl groups to RaxX protein that is an activator of XA21-mediated immunity (Pruitt et al., 2015).

Although RaxST is the first biochemically characterized prokaryotic tyrosine sulfotransferase, it is still unclear which biological processes are associated with RaxST in *Xoo*. In this study, we carried out a label-free comparative shotgun proteomic analysis and a COG analysis in order to understand the biological processes regulated by RaxST in *Xoo*. Our results indicate that RaxST is involved in *Xoo* motility. Phenotypic observation also confirmed that RaxST is indispensable for *Xoo* motility. These results allow us to elucidate protein functions via a combination of proteomic analysis and phenotypic changes in *Xoo*.

We used the wildtype *Xoo* strain Philippines race 6, PXO99Az, and the *raxST* knockout mutant (*XooΔraxST*) that have been employed in previous studies (da Silva et al., 2004; Hopkins et al., 1992). To generate a complemented strain *XooΔraxST* (*RaxST*), the raxST gene was amplified using the forward primer 5′-CTCGAGATGCACCACCACCACCACCACGCTTGGCTGGCGATGCGTCCCGCCG-3′ and the reverse primer 5′-CTTCGCATGGAGCGCTGCTGGTATGAAGCTT-3′. The amplified DNA fragment was cloned into pTOP blunt V2 vector (Invitrogen, Carlsbad, CA, USA) and the cloned DNA, cut out with *Xho*I and *Hind*III, was inserted into a broad host range vector, pBBR1-MCS5, digested with same restriction enzymes (Kovach et al., 1995). The construct was introduced into *XooΔraxST* by electroporation. The complimented strain *XooΔraxST* (*RaxST*) successfully triggered XA21-mediated immunity in the rice cultivar Kitaake expressing the XA21 protein, but not in *XooΔraxST* (Fig. S1). These results indicate that the interruption of the *raxST* gene does not have any polar activity, which is consistent with a previous report (da Silva et al., 2004).

To understand the biological processes associated with RaxST, we compared protein expression between the wildtype and *XooΔraxST* using a label-free shotgun proteomic approach following previously established protocols (Park et al., 2014a). Briefly, *Xoo* strains were grown in peptone sucrose broth to 0.6 OD at 600 nm, and harvested. Bacterial cells were dissolved in 1 ml lysis buffer (6 M guanidine-HCl, 50 mM Tris-HCl, and 10 mM DTT; pH 7.8), and then were disturbed by sonication using an Ultrasonic Processor (Cole Parmer, Niles, IL, USA). Extracted proteins (1 mg) were precipitated using trichloroacetic acid and then digested with trypsin (Promega, Madison, WI, USA). The trypsin-digested protein (2 μg) was examined with an LTQ Velos Pro dual-pressure linear ion trap mass spectrometer combined with a split-free nano liquid chromatograph (EASY-nLC II; Thermo Fisher Scientific, Waltham, MA, USA) in nanospray ionization mode. The samples were separated in a column composed of MAGIG C18AQ 200A (5

Table 1. Detected numbers of proteins and PSM in three biological replicates from the label-free shotgun proteomic experiments

Strain	1st		2nd		3rd		Shared proteins in three biological replicates
	Protein	PSM	Protein	PSM	Protein	PSM	
Wildtype	753	57,274	771	53,045	779	60,401	661
XooΔraxST	768	56,642	782	57,027	781	60,071	662

PSM, peptide-spectrum matches.

μm; Michrom Bioresources, Auburn, CA, USA) and the peptides were eluted. Full MS spectra were gained with six data-dependent MS/MS scans (m/z 300–2,000 mass range). Three biological replicates from each strain were carried out.

To interpret the MS/MS spectra, we have used previously reported methods (Park et al., 2014a, 2014b), combination of a Thermo Proteome Discoverer 1.3 (ver. 1.3.0.399; Thermo Fisher Scientific) and the SEQUEST search algorithm was employed. The spectra were analyzed using the *Xoo* PXO99Az reference sequence database containing 4,375 proteins, from National Center for Biotechnology Information. Trypsin was situated as a cleavage enzyme, and up to two missed cleavages were allowable. All peptides had a 0.01 false discovery rate with forwarded and reversed database searches. One hundred ppm of precursor mass accuracies were acceptable and scores of probability from all peptides were over 20. The methionine oxidation was reflected as a possible modification. Proteins that matched at least two unique peptides were appreciated to be present in all samples. For comparative analysis of protein expression between the wildtype and *XooΔraxST*, a peptide-spectrum match (PSM) was used (Choi et al., 2008). PSMs from individual proteins were normalized against the total number of PSMs from the total proteins in each sample. Proteins shared in three biological replicates were chosen and used for comparison. The coefficient of variation calculated with all conserved proteins in three biological replicates was used for quality assessment. The mean value of the normalized PSMs was calculated per each protein and employed as a comparison value between the wildtype and *XooΔraxST* to identify differently expressed proteins. Proteins whose expression has shown over 1.5-fold difference between the wildtype and *XooΔraxST* were selected as differently expressed proteins.

In the three biological replicates with the wildtype strain, 753, 771, and 779 proteins were detected from 57,274, 53,045, and 60,401 PSMs, respectively. In the *XooΔraxST* strain, 768, 782, and 781 proteins were identified from 56,642, 57,027, and 60,071 PSMs, respectively (Table 1). Among the identified proteins, 661 and 662 proteins commonly detected in all three biological replicates of the wildtype and *XooΔraxST* strains, respectively, were used for comparative analysis. Counts of PSMs from 661 and 662 proteins from the wildtype and *XooΔraxST* strains, respectively, were compared after normalization. The average of coefficient of variation from all shared proteins in the wildtype and *XooΔraxST* strains was 15.88 and 16.12, respectively, suggesting that the degree of variation from means of PSMs is relatively low and the comparative analysis shows high confidence. The expression of a total of 49 proteins was affected by the presence of functional RaxST (Fig. 1A). Among these differentially expressed proteins, 22 and 27 proteins were only detected or highly expressed (over 1.5 fold) in the wildtype and *XooΔraxST* strains, respectively. These results indicate that approximately 7% of the observed

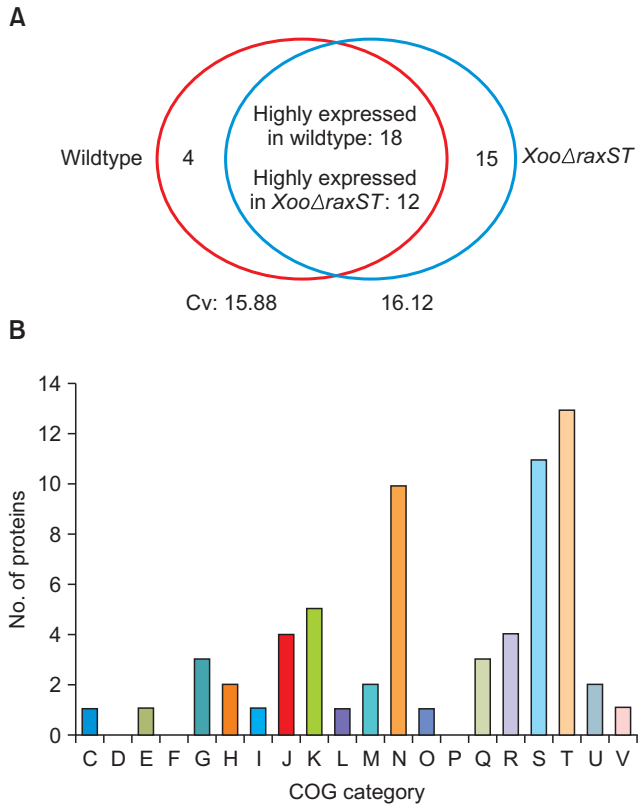

Fig. 1. Comparison and clusters of orthologous groups (COG) analysis of proteins whose expression is altered by RaxST. (A) Venn diagram showing the number of differently expressed proteins in wildtype (red circle) and *XooΔraxST* (blue circle) strains for the commonly detected proteins in three biological replicates. Four proteins were found only in the wild type; 18 were highly expressed in the wild type; 15 were only detected in *XooΔraxST*; and 12 were highly expressed in *XooΔraxST*. Cv, coefficient of variation. (B) COG analysis of proteins whose expression was affected by RaxST. C, energy production and conversion; D, cell cycle control and mitosis; E, amino acid metabolism and transport; F, nucleotide metabolism and transport; G, carbohydrate metabolism and transport; H, coenzyme metabolism; I, lipid metabolism; J, translation; K, transcription; L, replication and repair; M, cell wall/membrane/envelope biogenesis; N, cell motility; O, post-translational modification, protein turnover, chaperone functions; P, inorganic ion transport and metabolism; Q, secondary structure; R, general functional prediction only; S, function unknown; T, signal transduction mechanisms; U, intracellular trafficking and secretion; V, defense mechanisms.

proteins were specifically influenced by tyrosine sulfation by RaxST in *Xoo*. This is a relatively low number compared with phosphorylation by kinases on response regulators of bacterial two-component systems, leading to massive changes in gene expression (Mitrophanov and Groisman, 2008). Relatively low effects on gene expression by tyrosine sulfation in *Xoo* are consistent with a result observed in eukaryotic cells in which tyrosine sulfation is mostly involved in protein-protein/peptide or receptor-ligand interaction with high affinity, rather than in gene expression for cell signaling cascades via phosphorylation by kinases (Kehoe and Bertozzi, 2000).

Next, we carried out COG analysis (Tatusov et al., 2000) to classify 49 RaxST-associated proteins according to their predicted functions (Table 2, 3). The majority of proteins whose expression is affected by RaxST belong to the "cell motility" (N) and "signal transduction mechanisms" (T) categories (Fig. 1B). Eight of ten proteins belonging to "cell motility" are also categorized in "signal transduction mechanisms" (Table 3, included in other group T). Interestingly, eight chemotaxis-related proteins associated with twitching motility were identified as proteins altered by RaxST, and all of them (PXO_00057, PXO_00050, PXO_00047, PXO_00046, PXO_00032, and PXO_06212 from "cell motility"; PXO_06210 and PXO_00031 from "signal transduction mechanisms") were detected only in *XooΔraxST* but not in the wildtype strain under the given conditions (Table 2, 3). In agreement with these observations, a comparative proteomic analysis with an Omp1X-knockout mutant

Table 2. Classification of highly expressed (> 1.5 fold) proteins in the wildtype using COG

COG function	Accession	Locus tag	Gene function	Fold change (*ΔraxST/wild*)	Included in other group
C (energy production and conversion)	188578710	PXO_03114	F0F1 ATP synthase subunit epsilon	1.67	
E (amino acid transport and metabolism)	188577842	PXO_01920	Aminopeptidase N	1.63	
G (carbohydrate transport and metabolism)	188579071	PXO_02746	Polyvinylalcohol dehydrogenase	3.24	
	188574985	PXO_04104	Beta-glucosidase	2.37	
	188579118	PXO_03298	Endoglucanase	1.66	
H (coenzyme transport and metabolism)	188578413	PXO_02519	2-amino-3-ketobutyrate coenzyme A ligase	2.71	
J (translation, ribosomal structure and biogenesis)	188577053	PXO_01112	TrmH family RNA methyltransferase	*	
M (cell wall/membrane/ envelope biogenesis)	188575566	PXO_04698	OmpA family domain-containing protein	1.97	
	188577175	PXO_01117	UDP-N-acetylglucosamine acyltransferase	1.86	
O (post-translational modification, protein turnover, and chaperones)	188577468	PXO_01524	Protein GntY	1.55	
Q (secondary metabolites biosynthesis, transport, and catabolism)	188579085	PXO_03211	ABC transporter ATP-binding protein	*	
	188576165	PXO_00406	Acetoacetyl-CoA reductase	1.61	I, R
R (general function prediction only)	188576411	PXO_00684	Alkaline phosphatase	1.55	
S (function unknown)	188577991	PXO_01984	Hypothetical protein	*	
	188578598	PXO_02671	Adhesin-like protein A	3.16	
	188576643	PXO_00888	Hypothetical protein	2.31	
	188578763	PXO_03057	Hypothetical protein	2.08	
	188574531	PXO_03595	Hypothetical protein	2.05	
	188577492	PXO_01502	Hypothetical protein	1.76	
	188577147	PXO_01147	Hypothetical protein	1.75	
T (signal transduction mechanisms)	188578484	PXO_02599	Response regulator	1.58	
V (defense mechanisms)	188575698	PXO_04833	Beta-lactamase	*	

COG, clusters of orthologous groups; ATP, adenosine triphosphate; ABC, ATP-binding cassette.
*The protein is unique to the wild type.

Table 3. Classification of highly expressed (> 1.5 fold) proteins in the *XooΔraxST* strain using COG analysis

COG function	Accession	Locus tag	Gene function	Fold change (*wild/ΔraxST*)	Included in other group
J (translation, ribosomal structure and biogenesis)	188577392	PXO_01601	Glutathione synthetase	1.56	H
	188576286	PXO_00525	Queuine tRNA-ribosyltransferase	*	
	188578674	PXO_03147	ATP-dependent RNA helicase	1.75	L, K
K (transcription)	188578888	PXO_02929	Chromosome partitioning protein	1.63	
	188577000	PXO_06209	RNA polymerase sigma factor FliA	*	
N (cell motility)	188576956	PXO_06165	Flagellin	14.08	
	188577385	PXO_01608	Glutamate methyltransferase	1.53	T
	188577389	PXO_01604	Pilus biogenesis protein	1.59	T
	188576008	PXO_00057	Chemotaxis-specific methylesterase	*	T
	188576015	PXO_00050	Chemotaxis signal transduction protein	*	T
	188576018	PXO_00047	Chemotaxis protein	*	T
	188576019	PXO_00046	Chemotaxis protein	*	T
	188576031	PXO_00032	Chemotaxis protein CheA	*	T
	188577003	PXO_06212	Chemotaxis protein CheA	*	T
	188578248	PXO_02353	Fimbrial assembly membrane protein	2.38	U
Q (secondary metabolites biosynthesis, transport, and catabolism)	188579087	PXO_03209	Toluene tolerance protein	1.52	
R (general function prediction only)	188578113	PXO_02228	ABC transporter ATP-binding protein	1.66	
	188577094	PXO_01199	GNAT family acetyltransferase	*	
S (function unknown)	188578099	PXO_02242	Hypothetical protein	1.82	
	188574981	PXO_04100	GatB/Yqey	*	
	188576619	PXO_00915	Hypothetical protein	*	
	188576905	PXO_06111	Hypothetical protein	*	
T (signal transduction mechanisms)	188576100	PXO_00466	Two-component system regulatory protein with GGDEF domain	*	
	188577001	PXO_06210	Chemotaxis protein CheY	*	
	188576618	PXO_00916	Methyl-accepting chemotaxis protein	8.79	K
	188576032	PXO_00031	Chemotaxis response regulator	*	K
U (intracellular trafficking, secretion, and vesicular transport)	188579256	PXO_03487	Inner membrane protein translocase component YidC	1.57	

COG, clusters of orthologous groups; ATP, adenosine triphosphate; ABC, ATP-binding cassette.
*The protein is unique to the *XooΔraxST* strain.

(*XooΔomp1X*) also displayed differential expression of chemotaxis-related proteins (Park et al., 2014b). These results suggest that tyrosine sulfation by RaxST is most likely associated with bacterial movement. In addition to bacterial motility, the expression of 11 proteins classified as "function unknown" (S) was also altered (Table 2, 3), suggesting that RaxST might play a role in uncharacterized biological processes.

Bacterial chemotaxis is closely related to twitching motility in terms of pili-dependent motility (Miller et al., 2008). Overexpression and knockout mutations of genes involved in chemotaxis mechanisms resulted in impaired pili-dependent twitching motility (Kearns et al., 2001; Whitchurch et al., 2004). Because a proteomic analysis with *XooΔraxST* also implies that RaxST seems to have an effect on bacterial movement, the twitching motility of the wild type, *XooΔraxST*, and the complemented strain *XooΔraxST* (*RaxST*) was examined on potato sucrose agar (PSA) media containing 1.5% agar (Fig. 2). A twitching motility assay was carried out as described previously (Park et al., 2014b). Ten microliters of bacterial suspension (1.0×10^8 CFU/ml) was dropped into the center of a PSA plate, dried, and incubated at 28°C for 3 days. The marginal morphology of each dropped colony was observed under a light microscope. This experiment was repeated at least three times with

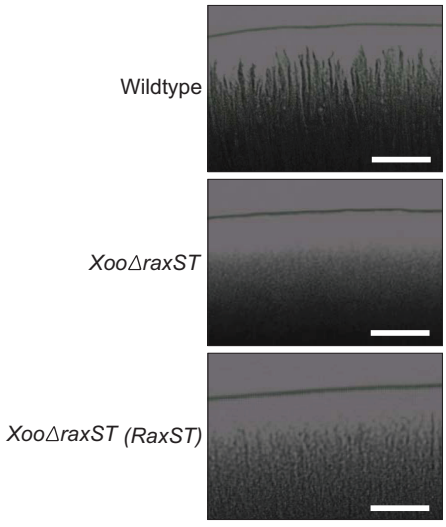

Fig. 2. Twitching motility of the wild type, *XooΔraxST*, and *XooΔraxST* (*RaxST*) strains. Ten microliters of bacterial suspension (1.0×10^8 CFU/ml) was dropped on potato sucrose agar containing 1.5% agar and incubated for 3 days. Twitching motility was evaluated by examination of the marginal shapes under a light microscope. Scale bars = 100 μm.

four biological replicates. The margins of the colonies from the wildtype strain were significantly irregular, but *XooΔraxST* colonies exhibited smooth edges, indicating that RaxST is essential to bacterial twitching motility. In the complementation experiment, *XooΔraxST* (*RaxST*) was restored toward to the phenotype of the wildtype strain. Similar to *XooΔraxST*, the twitching motility of the *XooΔomp1X* strain was significantly impaired (Park et al., 2014b), indicating that sulfation by RaxST is indispensable for normal twitching motility in *Xoo*. Flagella-dependent motility was not affected by RaxST (data not shown), although the expression of flagellin (PXO_06165) in the *XooΔraxST* strain was higher than in the wildtype strain (Table 3).

It is clear that RaxST is critical for full twitching motility in *Xoo* (Fig. 2). However, because many chemotaxis-related proteins are highly expressed in *XooΔraxST*, it remains to be investigated whether the impaired twitching motility was affected by the increased proteins directly, indirectly, or both. In addition, *XooΔraxST* may retain functional pili because PXO_0164 and PXO_02353, which are related to pili/fimbriae biosynthesis, are still expressed in the *XooΔraxST* strain (Table 3). Therefore, one possible explanation is that uncontrolled high expression of chemotaxis-related proteins in *XooΔraxST* may lead to abnormal twitching motility. In support of our hypothesis, overexpression of ChpA, a regulatory protein for chemosensory systems in *Pseudomonas aeruginosa*, has been shown to hamper the normal twitching motility of the wildtype strain (Whitchurch et al., 2004). To determine how tyrosine sulfation by RaxST affects the expression of proteins involved in bacterial motility, each molecular target of RaxST needs to be studied in depth.

In conclusion, this report elucidates the biological function of a tyrosine sulfotransferase, RaxST, using comparative shotgun proteomic analysis and phenotypic observation. The comparative proteomic analysis in this study found correlations between phenotypic changes and the biological functions of target proteins, indicating that a label-free shotgun proteomic technique is a very useful tool for postulating the biological roles of proteins. Our results also reveal that RaxST, the first biochemically characterized prokaryotic tyrosine sulfotransferase, has diverse functions in bacterial biology, including cell motility and signal transduction. However, the specific molecular mechanisms of tyrosine sulfation by RaxST, and its role in biological processes, remain to be characterized. Because Omp1X may function as a porin-like channel required for bacterial motility (Park et al., 2014b), it is possible that tyrosine sulfation by RaxST on Omp1X plays a role in discriminating or differentiating molecules being transported through the sulfated Omp1X on outer membranes of *Xoo*. The biological functions of RaxX, another substrate of RaxST and an activator of XA21-mediated immunity (Pruitt et al., 2015), are still not fully understood. Therefore, in addition to Omp1X and RaxX, it has to be carried out to identify other substrates of RaxST and to test phenotypes influenced by this protein in *Xoo*.

Acknowledgments

This work was supported by grants from the Next-Generation BioGreen 21 Program (No. PJ011033012016) of the Rural Development Administration (RDA), and by the Chung-Ang University Excellent Student Scholarship in 2016.

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Supplementary data

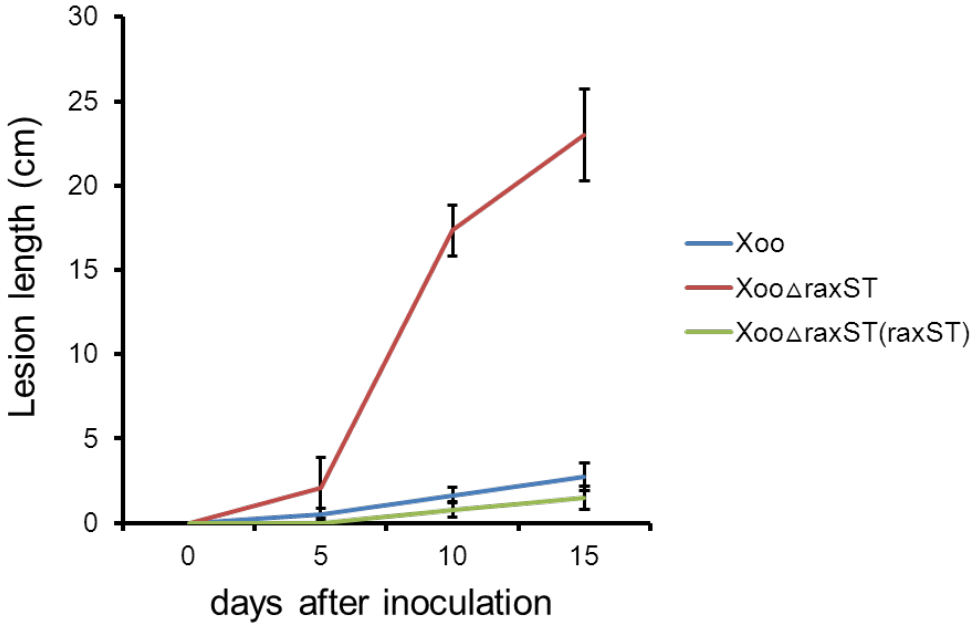

Figure S1. Pathogenicity test of *Xoo* strains. After culturing *Xoo*, *XooΔraxST*, and *XooΔraxST*(*RaxST*) strains, bacterial cells were diluted to about 1.0 × 10^8 CFU/ml, and inoculated onto rice leaves of Kitaake expressing XA21 using the scissor clipping method. Lesion lengths were measured at 0, 5, 10, and 15 days after inoculation. Data points indicate averages ± standard deviations of at least three biological replicates.