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Functional and proteomic analyses reveal that *wxcB* is involved in virulence, motility, detergent tolerance, and biofilm formation in *Xanthomonas campestris* pv. *vesicatoria*

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ABSTRACT

The bacterial envelope possesses diverse functions, including protection against environmental stress and virulence factors for host infection. Here, we report the function of *wxcB* in *Xanthomonas campestris* pv. *vesicatoria* (Xcv), a causal agent of bacterial leaf spot disease in tomato and pepper. To characterize roles of *wxcB*, we generated a knockout mutant (XcvΔ*wxcB*) and found that the virulence of the mutant was weaker than that of the wild type in tomato plants. To predict the mechanism affected by *wxcB*, we compared protein expressions between the wild type and the mutant. Expression of 152 proteins showed a greater than 2-fold difference. Proteins involved in motility and cell wall/membrane were the most abundant. Through phenotypic assays, we further demonstrated that the mutant displayed reduced motility and tolerance to treatment, but it showed increased biofilm formation. Interestingly, the LPS profile was unchanged. These results lead to new insights into the functions of *wxcB* that is associated with cell wall/membrane functions, which contributes to pathogen virulence.

1. Introduction

The genus *Xanthomonas* is Gram-negative, motile, yellow-pigmented, plant-pathogenic bacteria with a single polar flagellum [1]. These bacteria can cause diseases in nearly 400 species, including economically important crops such as rice, tomato, soybean, pepper, citrus, and banana [2]. *Xanthomonas campestris* pv. *vesicatoria* (Xcv) is the causal agent of a bacterial leaf spot disease, which is one of the most serious bacterial diseases affecting pepper and tomato; the disease also reduces the yield and quality of these crops throughout the world [3].

Bacterial cell walls contain several factors necessary for virulence, such as lipopolysaccharides (LPSs) and pili [4]. The pili are essential for bacterial motility and are also involved in biofilm formation, a self-protective stage of growth that allows bacterial cells to survive harsh conditions [5,6]. In addition, LPS, a major component of the outer membrane in Gram-negative bacteria, has been shown to be required for protection against antimicrobial peptides and for interaction with the eukaryotic host [7]. These studies have revealed that bacterial cell wall components are essential for survival and virulence in Gram-negative bacteria.

The genome of Xcv strain 85-10 has been sequenced, and it is known to contain a gene cluster for LPS biosynthesis [3]. The LPS biosynthesis cluster in the genome contains genes homologous to those involved in the ABC transporter-dependent pathway, genes for the LPS core biosynthesis, and genes encoding proteins for O-antigen synthesis. These genes have been extensively characterized in *Xanthomonas* spp. and other Gram-negative bacteria [8–11]. However, functions of *wxcB* (Locus tag, XCV3720; Accession No., YP_365451) and its homologs encoding a putative kinase are poorly understood in Xcv and other bacteria. Although the pathway for LPS biosynthesis has been well studied and it is known that the *wxcB* gene is located within the LPS biosynthesis cluster, it is unclear whether *wxcB* is involved in virulence, LPS synthesis, or other functions of bacterial cell wall/membrane.

Many approaches are used for characterizing the function of genes at the RNA level; however, gene expression does not always correlate with the protein level due to post-transcriptional and translational regulation and processing [12]. Instead, proteomic analysis has become very important in order to elucidate the function of genes and proteins, especially for organisms where the genome sequence is established. Therefore, proteomics combined with genomic information allows the direct and efficient characterization of gene function at the protein level [13].

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In this study, to characterize the function of *wxcB*, we carried out shotgun proteomic and categorized proteins whose expression was affected by *wxcB* using cluster of orthologous group (COG) analysis. Based on the COG analysis, we further carried out various phenotypic assays related to cell wall/membrane function. This study reports that the function of *wxcB* in the LPS biosynthesis cluster is related to the regulation of virulence, motility, biofilm formation, and SDS tolerance, but not LPS synthesis.

2. Materials and methods

2.1. Bacterial strains and growth conditions

X. campestris pv. *vesicatoria* (Xcv) strain 85-10 was used as the wild type in this study [3]. *Xanthomonas* strains were grown at 28 °C in peptone sucrose (PS) medium (peptone: 10 g/L, sucrose: 10 g/L, L-glutamic acids: 1 g/L) and in tryptic soy (TS) medium (Tryptic Soy Broth Soybean-Casein Digested: 30 g/L). *Escherichia coli* DH5α was used as the host for the preparation of all vectors. *E. coli* was cultured in Luria Bertani (LB) medium at 37 °C. Antibiotics were added when required for selection: kanamycin (50 μg/mL), gentamicin (10 μg/mL), and ampicillin (100 μg/mL).

2.2. Construction of wxcB knockout and complemented strains in Xcv

Standard procedures described by Sambrook et al. [14] were used for all molecular techniques. To generate the *wxcB* gene-knockout mutant (Xcv*ΔwxcB*) a 1.3-kb fragment of *wxcB* was amplified using gene specific primers (F: 5′-atttctccctcaacctggcg-3′, R: 5′-tcgctgttcagcttgtcgat-3′) and ligated into the pGem T-easy vector (Promega). The kanamycin-resistance cassette was inserted into the plasmid digested with *Nru*I. The plasmid was introduced into the wild type strain by electroporation at 2.5 kV (Bio-Rad Micropulser™). Xcv*ΔwxcB* was selected by culturing on PSA media containing kanamycin (50 μg/mL). To construct a plasmid for complementation, *wxcB* ORF was amplified with the following primers: F, 5′-ctcgagatgacgcattccgtttcccc-3′; R, 5′-aagcttagtggtggtggtggtggt-gagcagactccccattgcta-3′. The product was cloned, confirmed by nucleotide sequencing, and then subcloned into pBBR1-MCS5 [15]. The plasmid was electroporated into Xcv*ΔwxcB* and cultured on selective TSA media containing kanamycin (50 μg/mL) and gentamicin (10 μg/mL).

2.3. Protein extraction and peptide preparation

All reagents were purchased from Sigma–Aldrich unless otherwise specified. Bacterial cells were harvested at an OD_{600} of 0.6, and 0.5 g of cells was resuspended in 1 mL of 50 mM Tris–HCl (pH 7.8) for washing. After removing the supernatant, the pellet was resuspended in 1 mL of lysis buffer (6 M Guanidine-HCl, 10 mM DTT, 50 mM Tris–HCl, pH 7.8). Resuspended cells were disrupted with an Ultrasonic Processor (Cole Parmer). After collecting the supernatant by centrifugation, the protein concentration was quantified by the BCA protein assay kit (ThermoFisher). Sample were alkylated by treatment with 100 mM iodoacetamide, and then incubated again with 20 mM DDT. For protein precipitation, 0.3 volumes of trichloroacetic acid was added to the sample, and incubated at 4 °C for 12 h. After centrifugation, the pellet was washed with acetone. The pellet was dried and dissolved in 50 mM ammonium bicarbonate (pH 7.8). For protein digestion, 5 μg of trypsin (Promega) was used for 200 μg of extracted protein. Trypsin-treated samples were acidified with 0.4% trifluoroacetic acid, and loaded into the Sep-Pak Vac 1-cc tC18 cartridges (Waters) for sample cleaning. The sample eluted was dried by Speed Vac concentrator (Vision), dissolved in 0.4% acetic acid, and quantified again using the BCA protein assay kit (ThermoFisher).

2.4. Mass spectrometry analysis

Tryptic peptide mixtures from each sample (2 μg) were analyzed by split free nano-liquid chromatography (LC, EASY-nLC II, ThermoFisher) connected to the LTQ Velos Pro instrument (ThermoFisher). Digested samples were separated by a column packed in-house with 7.5 cm of MAGIG C18AQ 200A (5 μm) material (Michrom). Peptides were eluted over a 420-min gradient at a flow rate of 300 nL/min by a water/ACN gradient (Solvent A: water with 0.1% formic acid; Solvent B: 100% acetonitrile with 0.1% formic acid). Full mass spectrometry (MS) spectra were acquired in six data-dependent MS/MS scans. Dynamic exclusion was allowed with a repeat count of 1, repeat duration of 0.5 min, and exclusion duration of 3.0 min, with charge state selection enabled to preferentially select 2+ and 3+ ions. The six most intense ions in each full MS scan were collected for fragmentation and examined in centroid mode within the linear ion trap part of the instrument. Two biological replicates were performed.

2.5. Protein/peptide identification and quantification

Thermo Proteome Discoverer 1.3 (ver. 1.3.0.399) with SEQUEST was used for interpretation of acquired MS/MS spectra. Spectra were searched against the Xcv strain 85-10 database. All accepted peptides had a false discovery rate (FDR) of 0.01 with reversed database searches, and precursor mass accuracies of 100 ppm. In addition, the probability score for all peptides was >20. Proteins matched by at least two unique peptides were considered as present in the sample. For comparative analyses, peptide spectra matches (PSM) was used [16]. PSMs of each protein were normalized against the total PSMs for all proteins in a sample. Proteins present in two biological replicates were used for comparison. The average of normalized PSMs was calculated per protein and used as a comparison value to identify differently expressed proteins between the wild type and Xcv*ΔwxcB*.

2.6. Virulence assay

We monitored Xcv growth in the plant *Solanum lycopersicum* cv. VF36. Bacterial suspensions (10^5 cfu/mL) in 10 mM $MgCl_2$ were infiltrated into tomato leaves with needleless syringes. Inoculated plants were grown in a growth chamber in 16-h light/8-h dark cycle at 25 °C with 70% humidity. Two leaf discs (0.4 cm^2) per treatment, at each time point, were ground, diluted and dotted onto PSA plates in triplicate to determine the bacterial population. Three biological replicates were used.

2.7. Biofilm assay

The polyvinyl chloride (PVC) microplate method was used as described previously [17]. Average values from 16 biological replicates were calculated. This experiment was repeated at least three times.

2.8. Motility assay

The Bacterial motility was examined using semi-solid plates as described previously [18]. The mean values were calculated from four biological replicates. This experiment was repeated at least four times.

2.9. SDS tolerance assay

The sensitivity of Xcv strains to SDS were assessed by growing the bacteria in PSB medium containing 0.005% SDS. Water was used as a negative control. The beginning concentration of bacterial cells was 10^5 cells/mL and cells were incubated overnight with shaking at 28 °C. Bacterial cells were serially diluted and dotted onto PSA plates for counting. The numbers were measured 2 days after dotting. This experiment was repeated at least three times.

2.10. LPS analysis

Xcv strains were incubated overnight at 28 °C in a shaking incubator. When the O.D value was about 0.8, 5 mL of bacterial cultures were harvested. The LPS samples were then extracted using the LPS extraction kit (Intron), separated by tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then visualized using the silver staining kit (Pierce® Silver Stain Kit). LPS from *E. coli* was used as a control. Evidence of protein contamination was checked by staining with Coomassie blue R-250 (Sigma). This experiment was independently carried out at least five times.

2.11. Statistical analysis

Statistical analyses were carried out by performing *t*-test or least significant difference using SPSS v.21 (IBM).

3. Results and discussion

To examine the impact of *wxcB* on virulence, leaves of the VF36 tomato [19] were injected by a 10^5 CFU/mL suspension of the wild type and Xcv*ΔwxcB* and the growth pattern of both strains was compared for 9 days (Fig. 1). The number of Xcv*ΔwxcB* in the infected plant leaf was significantly lower than that of the wild type at 3, 6, and 9 days after inoculation (DAI), demonstrating that the mutant is less virulent as compared to the wild type. These data indicate that *wxcB* is required for full virulence and/or maximal growth of Xcv in tomato. However, it is still unclear which mechanisms are related with the gene. Therefore, to predict the function of *wxcB*, we compared protein expression in the wild type and Xcv*ΔwxcB* using the shotgun proteomic approach. We observed a total of 837 and 807 proteins, detected from the wild type and Xcv*ΔwxcB*, respectively (Table S1). Among the detected proteins, 721 and 748 proteins were identified in two biological replicates in the wild type and Xcv*ΔwxcB*, respectively (Table S1), and were used for comparison. Thirty-one proteins were uniquely found in the wild type and 35 proteins were only present in Xcv*ΔwxcB*. In addition, 55 and 31 proteins were differentially (over 2-fold) enriched in the wild type and Xcv*ΔwxcB*, respectively (Fig. 2A). These data suggest that approximately 20% of the identified proteins were influenced either directly or indirectly by *wxcB* in Xcv.

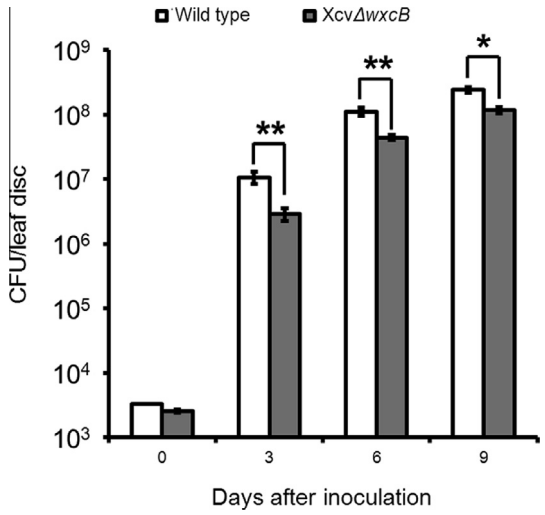

Fig. 1. *WxcB* is essential for full virulence of Xcv in tomato plant. Leaves of the VF36 tomato plant were inoculated with a 10^5 cfu/mL suspension of the wild type (white bars) and Xcv*ΔwxcB* (gray bars). Error bars represent standard error of the mean. The asterisk indicates a statistically significant difference (*t*-test, $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$).

Next, we predicted the function of the 152 altered proteins (86 and 66 in the wild type and Xcv*ΔwxcB* strains, respectively) and classified the proteins using COG analysis, which is widely used as a tool for categorization of proteins and prediction of their function [20]. Interestingly, the COG classifications of the unique or highly expressed proteins in the wild type were generally opposite to those of the proteins in Xcv*ΔwxcB* (Fig. 2B and C). Specifically, proteins belonging to cell motility, including 12 proteins predicted to be related to pili/fimbriae, were most abundant in the wild type (Fig. 2B, and Table S2), but they were not found in Xcv*ΔwxcB* (Fig. 2C and Table S3). Instead, proteins related to cell wall/membrane/envelop biogenesis were the most abundant in Xcv*ΔwxcB* (Fig. 2C). Bacterial pili/fimbriae are one of the major virulence factors for pathogens as well as a requirement for motility [21]. These data suggest that the roles of *wxcB* are mainly related to bacterial movement and cell envelope functions that contribute to virulence in Xcv.

Proteomic analyses indicated the possibility that the *wxcB* gene is positively associated with proteins involved in bacterial motility. Therefore, we investigated the influence of *wxcB* in a motility assay. We examined the phenotypes of wild type and Xcv*ΔwxcB* strains using a motility plate assay. In addition, we generated a complemented strain, Xcv*ΔwxcB*(WxcB), which carries a full-length *wxcB* gene driven by a *lacZ* promoter in Xcv*ΔwxcB*. We found that the motility of the wild type was 2-fold higher than that of Xcv*ΔwxcB* (Fig. 3A). Furthermore, the complemented strain could restore motility to a level comparable to the wild type (Fig. 3A). These results indicate that Xcv motility is affected by *wxcB*. It is well known that bacterial motility is a contributing factor for virulence [22]. Moreover, Liao et al. also reported that a *galU* mutant of *X. campestris* pv. *campestris* showed a reduction of both virulence and motility [23]. Possibly, the decrease in the virulence of Xcv*ΔwxcB* might be due to its motility defect.

In addition to virulence, bacterial motility is thought to be involved in biofilm formation [9]. To assess if *wxcB* is dispensable for biofilm formation, we examined its function in the wild type, Xcv*ΔwxcB*, and Xcv*ΔwxcB*(WxcB). Interestingly, Xcv*ΔwxcB* formed a significantly greater number (1.5-fold) of biofilms as compared with the wild type (Fig. 3B). The complemented strain could form biofilms similar to those formed by the wild type. Although Xcv*ΔwxcB* showed inverse phenotypes with regard to motility and biofilm formation, it is clear that *wxcB* is also involved in biofilm formation. Recently, Pehl et al. reported that the *shkS* gene, which encodes a histidine kinase in *Variovorax paradoxus*, showed inverse regulatory mechanisms of motility and biofilm formation [24]. Similar to our observation, the *shkS* mutant strain showed significantly delayed motility, but an increased ability to form biofilms. In the later stages of biofilm development, the mature biofilms must be dissociated in order to move to a new niche; bacterial motility is required for dissemination [25]. However, under conditions that inhibit motility, a biofilm will be less dissociated and remain intact. Therefore, we believe that the function of *wxcB* in motility and biofilm formation is related to this mechanism. In addition, several virulence-related genes, including *gdh*, *cps2*, and

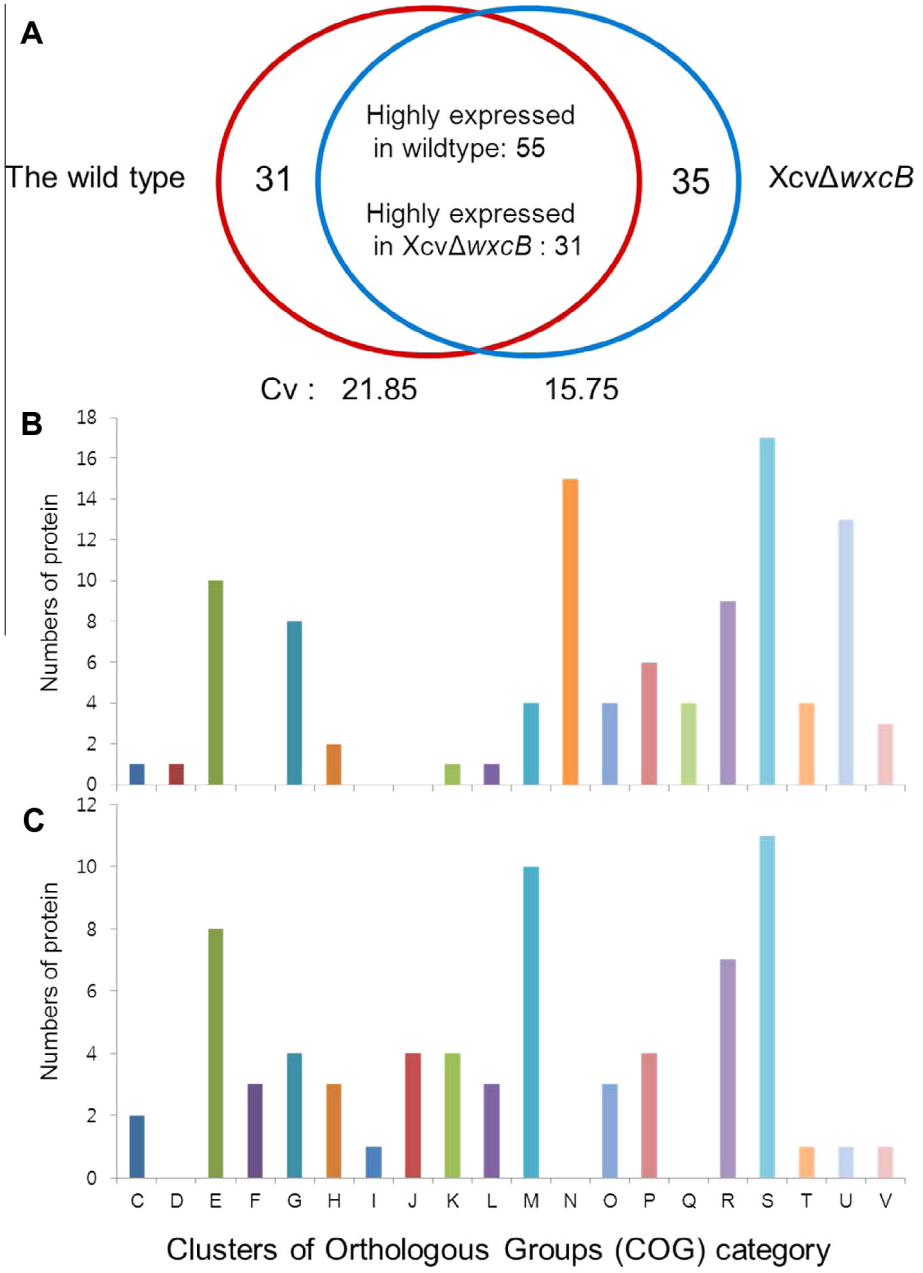

Fig. 2. Comparison and Clusters of Orthologous Groups analysis of proteins regulated by *WxcB*. (A) Venn diagrams present numbers of differentially enriched proteins from the set of commonly expressed proteins found in wild type (red circle) and Xcv*ΔwxcB* (blue circle) from two biological replicates. There are 31 proteins were present only in the wild type; 55 proteins were highly expressed in the wild type (expression was 2-fold higher than in Xcv*ΔwxcB*); 35 were present only in Xcv*ΔwxcB*; and 31 proteins were highly expressed in Xcv*ΔwxcB* (expression was 2-fold higher than in the wild type). CV, Coefficient of Variation. COG analysis of (B) proteins that were highly expressed in or unique to the wild type, and (C) proteins that were highly expressed in or unique to the Xcv*ΔwxcB*. C, Energy production and conversion; D, Cell cycle control and mitosis; E, amino acid metabolism and transport; F, nucleotide metabolism and transport; G, carbohydrate metabolism and transport; H, coenzyme metabolism; I, lipid metabolism; J, translation; K, transcription; L, replication and repair; M, cell wall/membrane/envelop biogenesis; N, cell motility; O, post-translational modification, protein turnover, chaperone functions; P, inorganic ion transport and metabolism; Q, secondary structure; R, general functional prediction only; S, function unknown; T, signal transduction; U, intracellular trafficking and secretion; V, defense mechanisms. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mrp, are down-regulated in the biofilm cells of *Streptococcus suis* [26], suggesting that virulence is reduced by forming a biofilm. This report supports our observation that Xcv*ΔwxcB* displays increased biofilm formation but reduced virulence.

Our proteomic data also revealed that proteins involved in cell wall/membrane biogenesis were differentially expressed in the wild type and Xcv*ΔwxcB*. In addition, four putative outer membrane receptors (XCV0772, XCV1963, XCV3301, and XCV3299) were highly expressed in the wild type as compared to the mutant (Table S1). Bacteria need to sense extracellular conditions in order to protect themselves against harsh environments [27]. Because the cell wall and membrane plays a significant role in protecting bacteria against various stresses [28], Xcv*ΔwxcB* was further investigated for tolerance against sodium dodecyl sulfate (SDS). Xcv strains were cultured in TSB medium at 10^5 CFU/mL in the presence of 0.005% SDS. The cell viability of tested strains was compared by counting colonies on agar plates. All tested strains showed similar viability in water control (Fig. 4). However, when subjected to treatment with 0.005% SDS, the viability of Xcv*ΔwxcB* was significantly lower (1000-fold) than that of the wild type

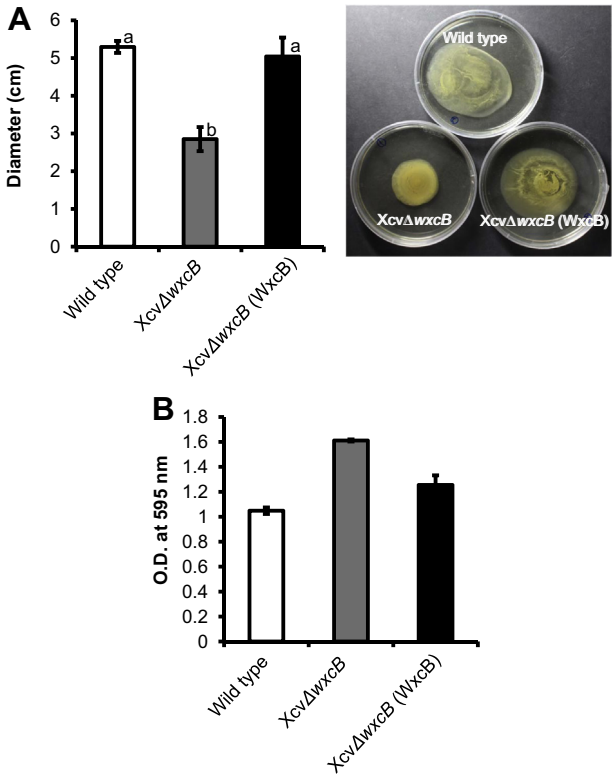

Fig. 3. Xcv*ΔwxcB* shows lower motility but enhanced biofilm formation. (A) Motility of the wild type (white bar), Xcv*ΔwxcB* (gray bar), and Xcv*ΔwxcB*(WxcB) (black bar) strains was analyzed by measuring the diameter of bacterial expansion in semi-solid media. Error bars represent standard error of the mean. A photograph was taken 7 days after incubation. (B) Biofilm formation of the wild type, Xcv*ΔwxcB*, and Xcv*ΔwxcB*(WxcB) strains was quantified by measuring the absorbance at A595 nm using the crystal violet staining assay in 96-well plates. Error bars represent standard error of the mean. Different letters on the bars represent significant differences. Different letters on the bars represent significant differences (the least significant difference test, $P \leqslant 0.05$ for A; $P \leqslant 0.01$ for B).

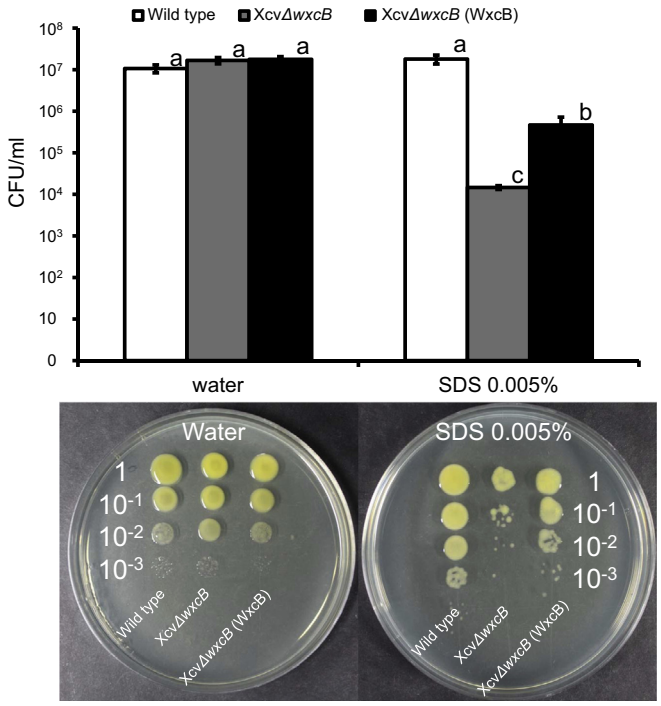

Fig. 4. WxcB is essential for tolerance to SDS. The wild type, Xcv*ΔwxcB*, and Xcv*ΔwxcB*(WxcB) strains were incubated in the presence of 0.005% SDS or water. After incubation, bacterial cells were serially diluted and quantified by a colony-dotting method. Bars represent the mean of three biological replicates with standard deviations. Different letters on the bars represent significant differences (the least significant difference test, $P \leqslant 0.05$).

(Fig. 4). Complementation of the mutant could restore viability towards to the wild type level. Together with the proteomic data, these results demonstrate that *wxcB* indeed has an effect on cell envelope function. As one of their defense mechanisms against pathogen infection, plants can secrete and produce antimicrobial molecules [29]. The wxcB mutant may have less tolerance against these molecules, resulting in slower growth compared to wild type (Fig. 1).

Because *wxcB* is located in the LPS biosynthesis gene cluster, we compared the LPS profile of the wild type, Xcv*ΔwxcB*, and Xcv*ΔwxcB*(WxcB). Surprisingly, all three strains showed similar LPS profiles, indicating that the gene is not essential for LPS synthesis in the tested condition (Fig. S1). Sequence analyses of *wxcB* revealed that this gene possesses a putative protein kinase domain, indicating that the substrates of the enzyme encoded by this gene are peptides/proteins. In agreement with the result of our observation, the results of a previous study have indicated that a protein kinase is not required for LPS biosynthesis or structural modification in *E. coli* [30]. Currently, further investigations are underway to elucidate the biochemical function of WxcB protein and the LPS structures in Xcv strains.

In this report, we used diverse phenotypic assays and a shotgun proteomic analysis to elucidate the function of a previously uncharacterized gene, *wxcB*, in Xcv. Based on the location of the gene in the Xcv genome, we hypothesized that this gene either is a component of the bacterial cell wall or is involved in the cell-wall related functions of the bacterium. In an agreement with our hypothesis, the proteomic analyses showed that mutation of *wxcB* had a considerable effect on the bacterial proteome, affecting proteins related to cell motility and cell wall/membrane biosynthesis. In addition, phenotypic assays also demonstrated that *wxcB* is involved in virulence as well as other functions related to the cell wall/membrane. Our study reveals that alterations in the functions of the bacterial cell wall/membrane and its related components can affect the virulence of the bacterium, because the cell wall and its components are responsible for bacterial protection and pathogenicity in plant hosts. Our findings show that the results of the proteomic analysis were well correlated to the observed phenotypic changes, suggesting that a proteomic approach combined with the use of diverse assays is an effective technique for characterizing virulence mechanisms and elucidating the role of genes in plant-pathogenic bacteria.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.076.

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